Handbook of Experimental Pharmacology

Volume 186

Editor-in-Chief
F. Hofmann, München

Editorial Board

J. Beavo, Seattle, WA
D. Ganten, Berlin
M. Michel, Amsterdam
C. Page, London
W. Rosenthal, Berlin

Enno Klussmann • John Scott
Editors

Protein–Protein Interactions as New Drug Targets

Contributors

E.M. Aandahl, A. Bauman, M. Berrera, J. Bode, M.D. Brown, G. Cesareni, N.L. Chudasama, W.M. Cleghorn, S. Costa, S.W. Deacon, K.L. Dodge-Kafka, G. Dodoni, C. Freund, A. Gluch, E.V. Gurevich, V.V. Gurevich, V. Haucke, M.D. Houslay, C. Hundsrucker, E. Huston, P.A. Insel, M.S. Kapiloff, S. Keller, E. Klussmann, D. Krappmann, M. Krause, H.-J. Kreienkamp, R. Kühne, F.-Q. Li, X. Li, S.O. Marx, A.C. McCahill, S. Monterisi, F. Murray, M. Ohmitsu, H.H. Patel, V. Pertegato, J.R. Peterson, G. Pula, C. Rodemer, W. Rosenthal, D.B. Sacks, H.-G. Schmalz, S.F. Steinberg, J. Sticht, H.M. Strauss, K.-I. Takemaru, K. Taskén, K.M. Torgersen, M. Vidakovic, J.W. Walker, E. Wegener, M. Zaccolo, I. Zamparo

Springer

Dr. Enno Klussmann
Leibniz-Institut für Molekulare
Pharmakologie
Robert-Rössle-Str. 10
13125 Berlin
Germany
klussmann@fmp-berlin.de

John Scott Ph.D.
The Vollum Institute
Oregon Health & Science University
Medical Research Bldg, Rm 322D
3181 SW Sam Jackson Park Road
Portland, OR 97239-3098
USA
scott@ohsu.edu

ISBN 978-3-540-72842-9 e-ISBN: 978-3-540-72843-6

DOI: 10.1007/978-3-540-72843-6

Handbook of Experimental Pharmacology ISSN 0171-2004

Library of Congress Control Number: 2008920053

© 2008 Springer-Verlag Berlin Heidelberg

This work is subject to copyright. All rights are reserved, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilm or in any other way, and storage in data banks. Duplication of this publication or parts thereof is permitted only under the provisions of the German Copyright Law of September 9, 1965, in its current version, and permission for use must always be obtained from Springer. Violations are liable to prosecution under the German Copyright Law.

The use of general descriptive names, registered names, trademarks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.

Product liability: The publisher cannot guarantee the accuracy of any information about dosage and application contained in this book. In every individual case the user must check such information by consulting the relevant literature.

Cover design: WMXDesign GmbH, Heidelberg

Printed on acid-free paper

9 8 7 6 5 4 3 2 1

springer.com

Preface

Biological processes are controlled by protein–protein interactions. The specificity of a protein–protein interaction permits highly selective pharmacological interference with a defined cellular process. Due to the vast diversity of protein–protein interactions they represent a large class of potential drug targets that offer great opportunities for therapeutic intervention. However, targeting of protein–protein interactions is a difficult task (Wells and Clendon, 2007, *Nature* **450**:1001).

Peptides mimicking binding domains disrupt protein–protein interactions with high selectivity by competitively binding to one of the interacting partners. Their use has provided insight into the function of a plethora of protein–protein interactions. However, therapeutic applications of peptides are limited as they need to be administered parentarally, possess a short half-life and may evoke immune responses. Such drawbacks may be overcome through the development of stabilized peptides and peptidomimetics (for review see: Yin and Hamilton, 2005, *Angew Chem Int Ed* **44**:2).

Interfaces for protein–protein interactions do not possess evolutionarily conserved hormone-, substrate-, or cofactor-binding pockets. On the contrary, protein–protein interactions often rely on extended, relatively flat interaction surfaces (750-1500 Å^2) which cannot easily be blocked by small molecules (Freund et al., this volume). However, the exchange of a few amino acids within a defined part of the contact area ('hot spot') may completely abolish a protein–protein interaction. For example, key residues contribute a large fraction of the free energy of binding between the growth hormone and its cognate receptor (Clackson and Wells, 1995, *Science* **267**:383). Thus, it is not necessary for a small molecule to cover the entire protein-binding surface for blocking a protein–protein interaction. The concept of 'hot spots' as areas crucial for the affinity of the interaction has been confirmed for numerous protein–protein interactions (for review see: Wells and Clendon, 2007, *Nature* **450**:1001; Arkin and Wells, 2004, *Nat Rev Drug Disc* **3**:301; Yin and Hamilton, 2005, *Angew Chem Int Ed* **44**:2). Besides targeting 'hot spots', small molecules may also bind to allosteric sites distant from the interface. The conformational change induced by the binding prevents a protein interaction. In addition, disrupting the homooligomeric assembly of an enzyme complex by the use of a small molecule can result in a highly isotype-specific allosteric inhibition, e.g. of inducible nitric oxide synthetase (iNOS; McMillan et al., 2000, *Proc Natl Acad Sci USA* **97**:1506).

Most if not all diseases are associated with or are caused by dysregulation of signal transduction processes. Generally, signalling proteins, including protein kinases, protein phosphatases and phosphodiesterase, are ubiquitously expressed. However, it is increasingly recognised that subsets of signalling proteins are encompassed in cell type-specific multi protein complexes that are tethered to defined cellular compartments in close proximity to their substrates (e.g. Torgersen et al., Kreienkamp, Freund et al., Costa and Cesarini, McCahill et al., all in this volume). This compartmentalization is mediated by scaffolding proteins including arrestins (Gurevich et al, this volume), A kinase anchoring proteins (AKAPs; Dodge-Kafka et al., and Hundsrucker et al., this volume), receptors of activated C kinases (RACKs; Walker, this volume) and caveolin (Patel et al., this volume). Scaffolding proteins temporally and spatially coordinate signal processing, facilitate the assorted flow of information through a cell and thereby participate in converting an exogenous stimulus into a specific cellular response. Displacing selected proteins from complexes assembled by scaffolding proteins is likely to cause subtle local changes in signal processing that affect defined cell functions.

Disruption of protein kinase A (PKA) anchoring by AKAPs has, for example, been achieved using 17-25 amino acid residues-long peptides derived from the PKA-binding domains of different AKAPs. The PKA anchoring disruptor peptides prevent the interaction by binding to the AKAP interaction sites on regulatory subunits of PKA (Hundsrucker et al., this volume). In cardiac myocytes AKAP18α tethers PKA to L-type Ca^{2+} channels by direct interaction of the AKAP with the channel. The PKA anchoring disruptor peptides abolish β-adrenoreceptor-induced, AKAP18α-dependent PKA phosphorylation of L-type Ca^{2+} channels and the consequent increase of channel open probability by displacing PKA from AKAP18α. This in turn prevents the enhanced entry of Ca^{2+} that increases contractility (Hulme et al., *Proc. Natl. Acad. Sci. USA* **100**, 13093, 2003; *Proc. Natl. Acad. Sci. USA* **103**, 16574, 2006). This effect resembles that of β-blockers (Dodge-Kafka et al. and Chudasama et al., this volume). Also similar to the effect of β-blockers is that of a peptide disrupting the direct interaction of AKAP18δ and phospholamban (PLN) in cardiac myocytes. This peptide, derived from the PLN interaction site for AKAP18δ, reduces the velocity of Ca^{2+} reuptake into the sarcoplasmic reticulum (Lygren et al., *EMBO Rep.* **8**, 1061, 2007; Hundsrucker et al., this volume).

This book discusses therapeutically relevant protein–protein interactions with a major focus on scaffolding proteins tethering signal transduction processes to defined cellular compartments by direct protein–protein interactions. Recent advances in the development of peptides and small molecules as pharmacological agents interfering specifically with defined protein–protein interactions are reviewed and potential therapeutic applications of the agents are highlighted.

Enno Klussmann and Walter Rosenthal

Contents

Part I Organization of Scaffolds

A-Kinase Anchoring Proteins as the Basis for cAMP Signaling 3
K.L. Dodge-Kafka, A. Bauman,
and M.S. Kapiloff

Arrestins as Multi-Functional Signaling Adaptors 15
V.V. Gurevich, E.V. Gurevich,
and W.M. Cleghorn

Role of Ena/VASP Proteins in Homeostasisand Disease 39
G. Pula and M. Krause

Scaffold/Matrix Attachment Regions (S/MARs):
Relevance for Disease and Therapy. 67
A. Gluch, M. Vidakovic, and J. Bode

Clathrin/AP-2-Dependent Endocytosis:
A Novel Playground for the Pharmacological Toolbox? 105
C. Rodemer and V. Haucke

Part II Scaffolding Proteins and Cellular Signalling

PDE4 Associates with Different Scaffolding
Proteins: Modulating Interactions as Treatment for Certain Diseases . . . 125
A.C. McCahill, E. Huston, X. Li,
and M.D. Houslay

G-Protein-Coupled Receptor-Signaling Components in Membrane
Raft and Caveolae Microdomains 167
H.H. Patel, F. Murray, and P.A. Insel

Protein Scaffolds, Lipid Domains and Substrate Recognition in Protein Kinase C Function. Implications for Rational Drug Design 185
J.W. Walker

Compartmentalised MAPK Pathways 205
M.D. Brown and D.B. Sacks

Dynamic Protein Complexes Regulate NF-κB Signaling 237
E. Wegener and D. Krappmann

An Oncogenic Hub: β-Catenin as a Molecular Target for Cancer Therapeutics 261
K.-I. Takemaru, M. Ohmitsu, and F.-Q. Li

A Toolkit for Real-Time Detection of cAMP: Insights into Compartmentalized Signaling 285
M. Berrera, G. Dodoni, S. Monterisi,
V. Pertegato, I. Zamparo, and M. Zaccolo

Part III Cell Type-Specific Anchoring

Scaffolding Proteins in Cardiac Myocytes 301
N.L. Chudasama, S.O. Marx, and S.F. Steinberg

Molecular Architecture of Signal Complexes Regulating Immune Cell Function 327
K.M. Torgersen, E.M. Aandahl, and K. Taskén

Scaffolding Proteins at the Postsynaptic Density:Shank as the Architectural Framework 365
H.J. Kreienkamp

Part IV Interference with Protein–Protein Interaction Sites as a New Pharmacological Concept

Domains Mediate Protein–Protein Interactions and Nucleate Protein Assemblies 383
S. Costa and G. Cesareni

Proline-Rich Sequence Recognition Domains (PRD): Ligands, Function and Inhibition 407
C. Freund, H.G. Schmalz, J. Sticht, and R. Kühne

Chemical Inhibition Through Conformational Stabilization of Rho GTPase Effectors 431
S.W. Deacon and J.R. Peterson

Pharmacological Interference with Protein–Protein Interactions Mediated by Coiled-Coil Motifs 461
H.M. Strauss and S. Keller

Direct AKAP-Mediated Protein–Protein Interactions as Potential Drug Targets 483
C. Hundsrucker and E. Klussmann

Index 505

Contributors

Einar Martin Aandahl
The Biotechnology Centre of Oslo, Gaustadalléen 21, PO Box 1125 Blindern, 0317 Oslo, Norway

Andrea Bauman
Department of Cell and Developmental Biology, Heart Research Center, Oregon Health & Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA

Marco Berrera
Dvn Biochemistry and Molecular Biology, IBLS, Wolfson Link Building, University Avenue, Glasgow G12 8QQ, Scotland, UK

Juergen Bode
Helmholtz-Zentrum für Infektionsforschung MBIO/Epigenetic Regulation, Inhoffenstraße 7, 38124 Braunschweig, Germany

Matthew D. Brown
Brigham and Women's Hospital and Harvard Medical School, Thorn 530, 75 Francis St, Boston, MA 02115, USA

Gianni Cesareni
University of Rome Tor Vergata, Via della Ricerca Scientifica, Rome, Italy

Neelesh L. Chudasama
Departments of Pharmacology and Medicine, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

Whitney M. Cleghorn
Department of Pharmacology, Vanderbilt University, Nashville, TN 37232, USA

Stefano Costa
University of Rome Tor Vergata, Via della Ricerca Scientifica, Rome, Italy

Sean W. Deacon
Division of Basic Science, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA

Kimberly L. Dodge-Kafka
Pat and Jim Calhoun Center for Cardiology, Department of Cell Biology, University of Connecticut Health Center, 263 Farmington Ave, Farminton, CT 06030, USA

Giuliano Dodoni
Dulbecco Telethon Institute, Venetian Institute of Molecular Medicine, via Orus 2, 35100 Padova, Italy

Christian Freund
Protein Engineering Group, FU and FMP Berlin, Robert-Rössle-Str. 10, 13125 Berlin, Germany

Angela Gluch
Helmholtz-Zentrum für Infektionsforschung MBIO/Epigenetic Regulation, Inhoffenstraße 7, 38124 Braunschweig, Germany

Eugenia V. Gurevich
Department of Pharmacology, Vanderbilt University, Nashville, TN 37232, USA

Vsevolod V. Gurevich
Department of Pharmacology, Vanderbilt University, Nashville, TN 37232, USA

Volker Haucke
Department of Membrane Biochemistry, Institute of Chemistry & Biochemistry, Freie Universität Berlin, Takustraße 6, 14195 Berlin, Germany

Miles D. Houslay
Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G128QQ, UK

Christian Hundsrucker
Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Str. 10, 13125 Berlin, Germany

Elaine Huston
Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G128QQ, UK

Paul A. Insel
Department of Pharmacology, University of California, San Diego, 9500 Gilman Drive, BSB 3076, La Jolla, CA 92093-0636, USA

Michael S. Kapiloff
Department of Cell and Developmental Biology, Heart Research Center, Oregon Health & Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA

Sandro Keller
Leibniz Institute of Molecular Pharmacology FMP, Robert-Rössle-Str. 10, 13125 Berlin, Germany

Enno Klussmann
Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Str. 10, 13125 Berlin, Germany

Daniel Krappmann
GSF – Research Center for Environment and Health, Institute of Toxicology, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

Matthias Krause
Randall Division of Cell and Molecular Biophysics, King's College London, New Hunt's House, Guy's Campus, London SE1 1UL, UK

Hans-Jürgen Kreienkamp
Institut für Humangenetik, Universitätsklinikum Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany

Ronald Kühne
Molecular Modeling Group, FU and FMP Berlin, Robert-Rössle-Str. 10, 13125 Berlin, Germany

F.-Q. Li
Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, NY 11794, USA

Xiang Li
Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G128QQ, UK

Steven O. Marx
Departments of Pharmacology and Medicine, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

Angela C. McCahill
Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G128QQ, UK

Stefania Monterisi
Dulbecco Telethon Institute, Venetian Institute of Molecular Medicine, via Orus 2, 35100 Padova, Italy

Fiona Murray
Pharmacology, University of California, San Diego, La Jolla, CA 92093, USA

M. Ohmitsu
Department of Pharmacological Sciences, State University of New York
at Stony Brook, Stony Brook, NY 11794, USA

Hemal H. Patel
Departments of Anesthesiology, University of California, San Diego, La Jolla,
CA 92093, USA

Vanessa Pertegato
Dulbecco Telethon Institute, Venetian Institute of Molecular Medicine,
via Orus 2, 35100 Padova, Italy

Jeffrey R. Peterson
Division of Basic Science, Fox Chase Cancer Center, 333 Cottman Avenue,
Philadelphia, PA 19111, USA

Giordano Pula
Randall Division of Cell and Molecular Biophysics, King's College London,
New Hunt's House, Guy's Campus, London SE1 1UL, UK

Claus Rodemer
Department of Membrane Biochemistry, Robert-Rossle-Str.10, 13125 Berlin,
Institute of Chemistry & Biochemistry, Freie Universität Berlin, Takustraße 6,
14195 Berlin, Germany

Walter Rosenthal
Leibniz-Institut für Molekulare Pharmakologie, Robert- Rössle-Str. 10, 13125
Berlin, Institut für Pharmakologie, Charité – Universitätsmedizin, Berlin,
Freie Universität Berlin, Germany

David B. Sacks
Brigham and Women's Hospital and Harvard Medical School, Thorn 530,
75 Francis St, Boston, MA 02115, USA

Hans-Günther Schmalz
Institute of Organic Chemistry, University Köln, Greinstr. 4, 50939 Köln,
Germany

Susan F. Steinberg
Departments of Pharmacology and Medicine, College of Physicians
and Surgeons, Columbia University, New York, NY 10032, USA

Jana Sticht
Protein Engineering Group, FU and FMP Berlin, Robert-Rössle-Str. 10,
13125 Berlin, Germany

Holger M. Strauss
Nanolytics, Gesellschaft für Kolloidanalytik mbH, Am Mühlenberg 11,
14476 Potsdam, Germany

K.-I. Takemaru
Department of Pharmacological Sciences, State University of New York
at Stony Brook, Stony Brook, NY 11794, USA

Kjetil Taskén
The Biotechnology Centre of Oslo, Gaustadalléen 21, PO Box 1125 Blindern,
0317 Oslo, Norway

Knut Martin Torgersen
The Biotechnology Centre of Oslo, Gaustadalléen 21, PO Box 1125 Blindern,
0317 Oslo, Norway

Melita Vidakovic
Molecular Biology Laboratory, Institute for Biological Research,
11060 Belgrade, Serbia

Jeffery W. Walker
Department of Physiology, University of Wisconsin, 1300 University Avenue,
Madison, WI 53706, USA

Elmar Wegener
GSF – Research Center for Environment and Health, Institute of Toxicology,
Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

Manuela Zaccolo
Dvn Biochemistry and Molecular Biology, IBLS, Wolfson Link Building,
University Avenue, Glasgow G12 8QQ, Scotland, UK

Ilaria Zamparo
Dulbecco Telethon Institute, Venetian Institute of Molecular Medicine,
via Orus 2, 35100 Padova, Italy

Arrestins as Multi-Functional Signaling Adaptors

V.V. Gurevich(✉), E.V. Gurevich, and W.M. Cleghorn

Contents

1 Introduction . . . 16
2 The Functional Cycle of Arrestin Proteins . . . 17
3 Arrestin-Binding Partners . . . 19
4 Arrestin Conformation Determines Its Binding and Signaling Capabilities . . . 20
5 Arrestin as a Target for Conventional Pharmacological Tools . . . 24
5.1 Arrestin–Receptor Interface . . . 25
5.2 Clathrin and AP2 Sites in the C-Tail . . . 26
5.3 Arrestin-Binding Sites for the Non-Receptor Partners . . . 26
6 Custom-Designed Proteins: An Alternative Approach to Harness Arrestin-Mediated Signaling for Therapeutic Purposes . . . 27
6.1 Modification of Receptor-Binding Elements . . . 27
6.2 Changing the Affinity for Non-Receptor Partners . . . 29
6.3 "Conformationally Biased" Mutants . . . 30
7 Looking Farther Ahead: The Combination of Conventional and Unconventional . . . 31
References . . . 33

Abstract Arrestins are versatile regulators of cellular signaling expressed in every cell in the body. Arrestins bind active phosphorylated forms of their cognate G-protein-coupled receptors, shutting down G-protein activation and linking receptors to alternative signaling pathways. Arrestins directly interact with more than 20 surprisingly diverse proteins, such as several Src family kinases, ubiquitin ligases, protein phosphatases, microtubules, etc., and serve as scaffolds facilitating signaling in two MAP kinase cascades, leading to the activation of ERK1/2 and JNK3. A number of arrestin-binding partners are key players in signaling pathways that regulate cell proliferation, survival, and apoptotic death, which make arrestin interactions with these proteins inviting targets for therapeutic intervention. For example, enhancement of pro-survival or pro-apoptotic arrestin-dependent signaling is a promising strategy in treating disorders such as neurodegenerative diseases or cancer, respectively. Recent studies show that in the cell arrestin exists in at

V.V. Gurevich
Department of Pharmacology, Vanderbilt University, Nashville, TN 37232, USA
vsevolod.gurevich@vanderbilt.edu

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

least three distinct conformations, free, receptor-bound, and microtubule-bound, with very different signaling capabilities. Precise identification of arrestin elements mediating its interactions with each partner and elucidation of conformational dependence of these interactions will pave the way to the development of molecular tools for targeted enhancement or attenuation of arrestin interactions with individual partners. This structural information is necessary to devise conventional drug-based approaches and to engineer specialized "designer" arrestins that can compensate for defects in receptor regulation associated with congenital disorders and/or redirect arrestin-mediated signaling to desired pathways. Arrestins are at the crossroads of crucial pathways that determine cell fate and behavior. Therefore, targeted manipulation of arrestin-dependent signaling has an enormous therapeutic potential.

1 Introduction

The first member of the arrestin family was identified in the visual system (Kuhn 1978). In rod photoreceptors, arrestin binds light-activated rhodopsin phosphorylated by rhodopsin kinase and stops its signaling via G protein (Kuhn et al. 1984). Within a few years, it was discovered that the phosphorylation of other G protein-coupled receptors (GPCRs) by G-protein-coupled receptor kinases (GRKs) facilitates desensitization, but does not fully explain their functional uncoupling from G proteins (Benovic et al. 1987). This pioneering work led to a search for the non-visual arrestin homologues. Indeed, soon after rod arrestin (Shinohara et al. 1987), two ubiquitously expressed non-visual counterparts were cloned (Lohse et al. 1990; Attramadal et al. 1992; Sterne-Marr et al. 1993). At the time, β2-adrenergic receptor (b2AR) was the only non-rhodopsin GPCR available in purified form in Dr. Lefkowitz's lab, where these pioneering studies were performed. The first cloned non-visual arrestin clearly preferred b2AR over rhodopsin, so it was named β-arrestin (Lohse et al. 1990, 1992). The cloning of the second non-visual arrestin prompted the change of this purely historic name to β-arrestin1, whereas the other was named β-arrestin2 (Attramadal et al. 1992). Because even at that time it was perfectly clear that non-visual arrestins are not specific for b2AR but interact with a variety of GPCRs, a systematic nomenclature was also proposed in which rod arrestin became arrestin1, the non-visual homologues became arrestins 2 and 3 in the order of their cloning, and "s" or "l" was added to their names to distinguish between their short and long splice variants (Sterne-Marr et al. 1993). In this chapter, we use the systematic names of mammalian arrestins.

Arrestin functions in the biology of the cell are surprisingly diverse. Arrestins bind and regulate the signaling of hundreds of different GPCRs (Carman and Benovic 1998; Gurevich and Gurevich 2006b). After direct arrestin interaction with clathrin was discovered 10 years ago (Goodman et al. 1996), numerous additional non-receptor binding partners were described. The list now includes protein kinases and phosphatases, ubiquitin ligases, small G proteins and their regulators, etc.

(recently reviewed in Lefkowitz and Shenoy 2005; Gurevich and Gurevich 2006a). Arrestin interactions with various receptors and non-receptor partners affect virtually every aspect of cell function. Here we describe arrestin interactions with receptors and other signaling proteins as potential targets for therapeutic intervention using conventional pharmacological tools, such as drugs, as well as engineered arrestin proteins with modified functional capabilities.

2 The Functional Cycle of Arrestin Proteins

Every cell in the body constantly receives a variety of signals, so an "unstimulated cell" is as imaginary as an "ideal gas" in physics, but it is a convenient reference point. In the unstimulated cell, arrestins are fairly evenly distributed in the cytoplasm, where they may be free or bound to soluble signaling proteins (Fig. 1) (Gao et al. 2004; Witherow et al. 2004; Song et al. 2006; Wu et al. 2006). A certain proportion of cytoplasmic arrestins is bound to the microtubules via a relatively low-affinity interaction with K_D of about 25 μM (Hanson et al. 2007b), which is comparable to the ~25–50 μM concentration of polymerized tubulin in most cells (Hiller and

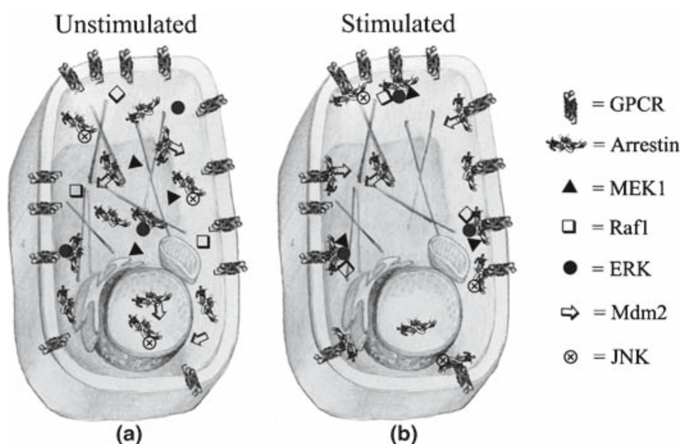

Fig. 1 The functional cycle of arrestin proteins. (**a**) In a cell where most GPCRs are silent, arrestin is distributed throughout the cytoplasm. Some of the cytoplasmic arrestin is bound to microtubules. The extent of nuclear localization depends on arrestin subtype (arrestin 2 >> arrestin 3) and cell type. Free cytoplasmic and nuclear arrestin interacts with several non-receptor binding partners: JNK3, Mdm2, and likely many others. Microtubule-bound arrestin mobilizes ERK1/2 and Mdm2 to the cytoskeleton. (**b**) Upon stimulation of one or more GPCR subtypes a significant proportion of arrestin is mobilized to the receptor(s), so that the abundance of free and microtubule-bound arrestin decreases. Receptor-bound arrestin serves as an organizer of signalosome, mobilizing numerous signaling proteins to the receptor and scaffolding c-Raf-1->MEK1->ERK1/2 and ASK1->MKK4->JNK3 MAP kinase cascades

Weber 1978; Ackmann et al. 2000). A certain amount of arrestin is also present in the nucleus (Scott et al. 2002; Wang et al. 2003; Song et al. 2006) or anchored to the plasma membrane via interactions with other proteins (Hunzicker-Dunn et al. 2002). The proportion of nuclear arrestin 2 varies depending on the cell type (Song et al. 2006). How much the stimulation of a particular GPCR affects the overall arrestin distribution in the cell depends on the relative expression of the receptor and arrestins. For example, arrestins 2 and 3 are expressed in the adult brain at ~11 and 0.5 ng mg^{-1} total protein, which is equivalent to 250 and 10 fmol mg^{-1} protein, respectively (Gurevich et al. 2002, 2004). Most GPCRs are expressed at 30–300 fmol mg^{-1}. Therefore, any individual GPCR in a neuron is likely to engage only a fraction of total arrestin. However, neuronal GPCRs are often concentrated in pre- or post-synaptic areas, enabling them to exhaust the immediately available “local” supply. GPCRs are expressed in other types of cells at comparable levels, whereas the expression of both non-visual arrestins is significantly lower. Thus, in many cell types activated receptors may bind most, if not all, available arrestin (Fig. 1).

Receptor stimulation induces arrestin translocation to the plasma membrane (where the receptors reside) within minutes (Barak et al. 1997), although one should keep in mind that these experiments were performed in cells over-expressing both receptors and arrestins. However, similar receptor activation-driven arrestin redistribution is apparent in rod photoreceptors (Nair et al. 2005), the only cell type expressing sufficient amounts of endogenous arrestin to make its movement easily observable by immunochemistry (Nair et al. 2005; Hanson et al. 2007a). Massive binding of free arrestin by active phosphoreceptors reduces its concentration in the cytoplasm, shifting the equilibrium towards its dissociation from microtubules (Gurevich et al. 2007). Binding sites for GPCRs and microtubules on the arrestin molecule largely overlap, meaning that arrestin cannot simultaneously bind the receptor and microtubules (Hanson et al. 2006a, 2007b). Nanomolar arrestin affinity for phosphorylated receptors (Gurevich et al. 1995) as compared to micromolar affinity for microtubules (Hanson et al. 2006a, 2007b) ensures that the receptor always wins in this competition. Consequently, soon after receptor stimulation virtually all arrestin ends up in a complex with the receptor. Arrestin may come to the receptor “empty-handed” or may bring one or more signaling proteins already bound to it (Fig. 1) (Song et al. 2006). Some arrestin-binding partners bind to both receptor-bound and free arrestin, whereas some prefer one form to the other (Gurevich and Gurevich 2003; Lefkowitz and Shenoy 2005). For proteins with higher affinity for free arrestin, “hitching a ride” with arrestin when it is mobilized to the receptor may be the only way to become a part of the receptor–arrestin complex. For example, the affinity of ubiquitin ligase Mdm2 for arrestin “frozen” in the basal state is higher than for the “pre-activated” arrestin mutant mimicking its receptor-bound conformation. Most likely, free arrestin in the basal conformation binds Mdm2, brings it to the receptor, and releases it upon receptor binding (Song et al. 2006). Nonetheless, receptor-bound arrestin may be a better substrate for Mdm2, for the level of arrestin ubiqutination by Mdm2 is proportional to the stability of the receptor–arrestin complex (Shenoy et al. 2001).

Binding to the receptor induces the release of the arrestin C-tail (Hanson et al. 2006b) carrying clathrin and AP2 interaction sites (Goodman et al. 1996; Laporte et al. 1999; Kim and Benovic 2002). This enhances arrestin affinity for these components of the trafficking machinery (Kim and Benovic 2002; Xiao et al. 2004), ensuring that free arrestin in the cytoplasm does not compete with receptor–arrestin complex for clathrin and AP2 (Gurevich and Gurevich 2003). By virtue of mobilizing clathrin and AP2 to the complex, bound arrestin promotes receptor internalization via coated pits, although arrestin binding per se does not necessarily mean that the receptor will follow this route of internalization (Pals-Rylaarsdam et al. 1997). Receptor-bound arrestin also mobilizes a number of signaling proteins, such as c-Src, PDE4, ARNO, Arf6, etc., and serves as a scaffold for MAP kinase cascades, facilitating the activation of JNK3 and ERK1/2 (reviewed in Lefkowitz and Shenoy 2005; Gurevich and Gurevich 2006a,b). Arrestin interaction with some GPCRs is transient, so that arrestin is likely released soon after bringing the receptor to the coated pit. With other GPCRs, arrestin forms fairly stable complexes that internalize without dissociation (Oakley et al. 2000). Eventually, low pH in the endosome promotes the release of the activating agonist, whereupon arrestin dissociates and the receptor is dephosphorylated and recycled. Alternatively, internalized receptor can be transported to the lysosome and destroyed. Several lines of evidence suggest that increased stability of the complex makes the latter outcome more likely, whereas the formation of transient complexes favors recycling (Shenoy et al. 2001; Pan et al. 2003). Apparently, the partners that preferentially interact with receptor-bound arrestin dissociate when arrestin is released from the receptor (Gurevich and Gurevich 2003), although direct experimental evidence proving this scenario is still lacking. In the end, the system returns to its original state, with the majority of arrestin localized in the cytoplasm, some of it bound to the microtubules and/or other partners (Fig. 1).

3 Arrestin-Binding Partners

Arrestins apparently bind the great majority of GPCRs, in most cases demonstrating a strong preference for the phosphorylated agonist-activated functional form of the receptor. Arrestin selectivity is ensured by a sequential multi-site binding mechanism (Gurevich and Benovic 1993). In a nutshell, this mechanism involves the “phosphate sensor” in arrestin that binds receptor-attached phosphates and an “activation sensor” that binds to parts of the receptor that change conformation upon activation. Each of these interactions is low affinity. However, when the active phosphoreceptor engages both sensors simultaneously, arrestin undergoes a transition into a high-affinity receptor-binding state involving global conformational rearrangement of the molecule that brings additional receptor-binding sites into action (reviewed in Gurevich and Gurevich 2004). Thus, free arrestin diffusing in the cytoplasm transiently binds via the “phosphate” and “activation” sensors to GPCRs it randomly encounters, “checking” their functional state. Because arrestin affinity for the other functional forms of the receptor is low enough to ensure rapid dissociation, only the encounter with the active phosphoreceptor turns arrestin on and results in tight binding.

For a long time the fact that an individual receptor molecule is activated by an agonist (or, in case of rhodopsin and other photopigments, by a photon of light) was believed to imply that a single arrestin molecule interacts with a single receptor (reviewed in Gurevich and Gurevich 2004, 2006b). However, the propensity of GPCRs to dimerize under certain circumstances inspired an alternative model, where a single arrestin molecule interacts with both receptors in a dimer (Liang et al. 2003). The controversy surrounding the mechanistically fundamental issue of the stoichiometry of the arrestin–receptor interaction was resolved only recently (Hanson et al. 2007a). It has been demonstrated that each rhodopsin molecule binds its own arrestin, so that the binding is saturated at an ~1:1 ratio both in vivo in mouse photoreceptors and in the in vitro experiments with purified and carefully quantified proteins. Considering an amazing conservation of the receptor-binding mechanisms within the arrestin family (Kovoor et al. 1999; Vishnivetskiy et al. 1999, 2004; Celver et al. 2002; Sutton et al. 2005), this finding strongly suggests that all arrestins bind individual molecules of their cognate receptors regardless of the receptor dimerization status.

Direct binding of arrestins to clathrin, the first non-GPCR partner discovered, was described 10 years ago (Goodman et al. 1996). The number of known non-receptor binding partners of arrestin proteins has been growing ever since; it exceeded 30 at the last count (Gurevich and Gurevich 2006a) and keeps growing (Nelson et al. 2007). The variety of the signaling proteins interacting with arrestin is even more astonishing than their number. The list includes key players in protein trafficking (clathrin, AP2, and *N*-ethylmaleimide-sensitive fusion protein, commonly known as NSF); small GTPases and their regulators (Arf6, ARNO, Ral-GDS, RhoA); tyrosine kinases c-Src, Hck, Yes, and Fgr; MAP kinases JNK3 and ERK1/2 and their respective upstream activators ASK1 and c-Raf-1; protein phosphatases PP2A and MKP7; protein kinase Akt, ubiquitin ligase Mdm2, NFκB inhibitor IκBα, cAMP phosphodiesterase PDE4, calmodulin, microtubules, several receptors that do not belong to the GPCR superfamily, and many other proteins (reviewed in Lefkowitz and Shenoy 2005; Gurevich and Gurevich 2006a,b).

4 Arrestin Conformation Determines Its Binding and Signaling Capabilities

The most important question from the biological standpoint is whether arrestin interaction with a particular partner is enhanced, reduced, or not affected at all by its binding to other proteins, particularly GPCRs. Receptor binding induces a major conformational change in arrestin (Fig. 2) (Schleicher et al. 1989; Gurevich and Benovic 1993; Vishnivetskiy et al. 2002; Hanson et al. 2006b; Hanson and Gurevich 2006, reviewed in Gurevich and Gurevich 2004, 2006a,b). The receptor can affect arrestin interactions with other partners via a simple mechanism: free and receptor-bound arrestin presents a different "face" to the world (Gurevich and Gurevich 2003). In other words, the conformation and/or accessibility of the elements in the arrestin molecules available to bind other partners is different in free and

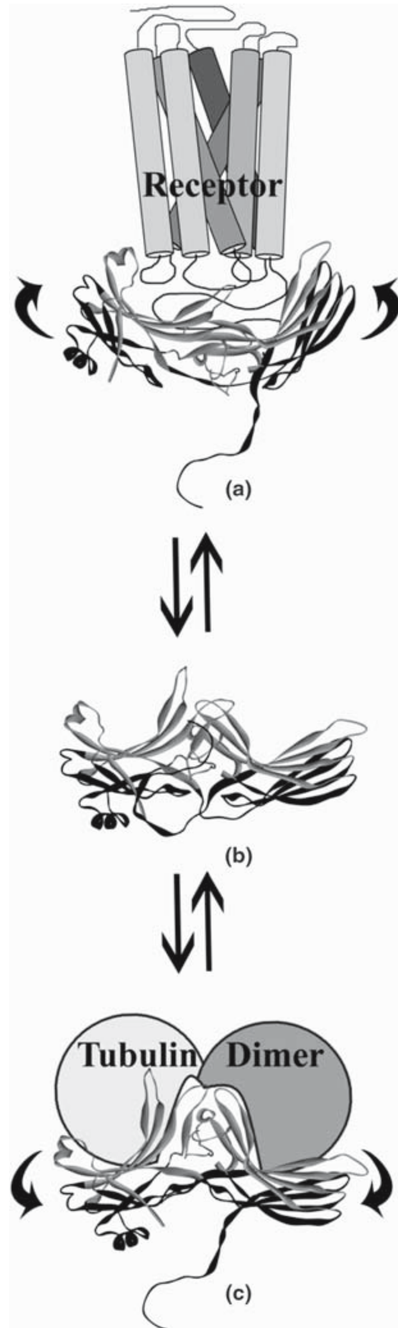

Fig. 2 Conformational states of arrestin. The basal conformation of free arrestin is stabilized by several interactions between the two domains and by the C-tail anchored to the body of the N-domain (**b**). The concave surfaces of both domains (*lighter shading*) are involved in arrestin binding to receptors and microtubules, whereas the other side of the molecule (*darker shading*) carries the binding sites for the partners that interact with receptor- and/or microtubule-bound arrestin. Arrestin binding to both receptors (**a**) and microtubules (represented by a tubulin dimer that contains most of the arrestin binding site; (**c**) induces the release of the arrestin C-tail. These interactions also change the relative orientation of the two domains, which apparently move "forward" (in the direction of the concave sides) in the receptor-bound arrestin and "backward" in the microtubule-bound state

Fig. 3 Conformational dependence of arrestin interactions with signaling proteins. Although the effect of arrestin conformation on the binding of the majority of arrestin partners remains to be elucidated, differential interactions of several proteins with free, receptor- and microtubule-bound arrestin have been demonstrated. Clathrin and AP2 preferentially bind receptor-associated arrestin via its released C-tail; it is not known whether microtubule binding-induced C-tail release has a similar effect. Calmodulin binding site includes the elements involved in receptor and microtubule interaction, so that it can only bind free arrestin. Ubiquitin ligase Mdm2 binds all forms of arrestin, but apparently has lower affinity for the receptor-bound state. JNK3 binds free and receptor-bound arrestin, but not the microtubule-bound form. ERK1/2 interacts with both receptor- and microtubule-bound arrestin, whereas upstream kinases c-Raf-1 and MEK1 prefer the receptor-bound form. Note that in real life receptor-bound arrestin cannot simultaneously interact with all the partners shown here

receptor-bound arrestins (Fig. 3). Interestingly, this obvious mechanism was actually proved only for arrestin interactions with clathrin and AP2. Arrestin C-tail is anchored to the body of the arrestin molecule via two sets of interactions, both of which are disrupted by the receptor-attached phosphates (Fig. 4) (Vishnivetskiy et al. 1999, 2000). The resulting release of the C-tail (Hanson et al. 2006b) makes clathrin and AP2 sites localized in this element more exposed, promoting their binding (Fig. 3). Indeed, mutations in arrestin (Kim and Benovic 2002) or phosphopeptides and other polyanions that facilitate the release of the C-tail (Palczewski et al. 1991; Xiao et al. 2004) enhance arrestin binding to these components of the trafficking machinery. It seems very likely that receptors similarly regulate arrestin interactions with other signaling proteins. However, experimental measurements of their affinity for free and receptor-bound arrestin, or even qualitative comparison of their binding to these two functional forms of arrestin, remain to be performed.

Nonetheless, several lines of indirect evidence suggest that the affinity of many partners and signaling consequences of their binding to arrestins depend on arrestin conformation. First, there are differences in signaling properties among arrestin subtypes. For example, free visual and both non-visual arrestins 2 and 3 bind JNK3 comparably (Song et al. 2006), but arrestin 3 promotes receptor activation-dependent JNK3 phosphorylation more effectively (McDonald et al. 2000). Free arrestin 2, but not arrestin 3, is released from the membrane via the ARNO/Arf6-dependent mechanism (Mukherjee et al. 2000; Hunzicker-Dunn et al. 2002), whereas both non-visual arrestins when bound to the receptor mediate ARNO/Arf6-dependent trafficking of β2-adrenergic receptor (Claing et al. 2001). Second, numerous arrestin-mediated signaling events are initiated by receptor activation (Lefkowitz and Shenoy 2005),

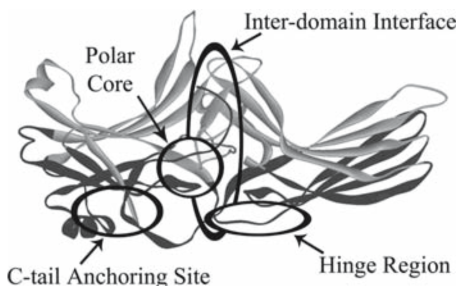

Fig. 4 Intra-molecular interactions in arrestin proteins. The basal arrestin conformation is stabilized by three interactions. The polar core in the center of the molecule includes the side chains of two positively charged residues (Arg169 and Arg393 in the β-strand X and the arrestin C-tail, respectively) and three negative charges (Asp26 in the N-domain and Asp290 and Asp 297 in the C-domain). It acts as the main phosphate sensor: receptor-attached phosphates neutralize the positive charge of Arg169, thereby disrupting the polar core and facilitating domain movement. The C-tail is anchored via hydrophobic interactions with the β-strand I and α-helix I to the body of the N-domain. This interaction is also destabilized by receptor-attached phosphates interacting with adjacent lysines on the β-strand I. It serves as an auxiliary phosphate sensor. An extensive hydrophobic interface between the bodies of the two domains is affected by arrestin binding to the non-phosphorylated receptor elements that change conformation upon activation. When the two domains move upon receptor or microtubule binding (Fig. 2), they "slide" along each other at this interface. Destabilization of all three interactions allows the movement of the arrestin domains relative to each other, which is limited by the length of the inter-domain hinge. It contains 12 residues in all arrestins. Hinge deletions impede the "forward" (in the direction of the concave sides of the two domains), but not the "reverse" domain movement (Fig. 2), thereby compromising receptor binding and enhancing microtubule interaction

indicating that they require arrestin in the receptor-bound conformation. Several recent studies show that the situation may be more complex. It turns out that even with the same receptor, arrestin can form more than one type of complex with functionally different consequences (Key et al. 2003; Kim et al. 2005; Ren et al. 2005; Vishnivetskiy et al. 2007). Thus, "receptor-bound conformation" may represent a whole family of distinct conformational states. Interestingly, in all cases the nature and functionality of the complex appears to be determined by differential receptor phosphorylation. For example, the localization of the phosphates in a particular serine-threonine cluster in the *N*-formyl-peptide receptor (Key et al. 2003), the incorporation of the phosphates into angiotensin II (Kim et al. 2005) or V2 vasopressin receptor (Ren et al. 2005) by a particular GRK (presumably in different receptor elements), or the sheer number of attached phosphates in rhodopsin (Vishnivetskiy et al. 2007) yields functionally different arrestin–receptor complexes.

It was recently shown that arrestins bind microtubules (Nair et al. 2004; Hanson et al. 2006a, 2007b) and that the conformation of microtubule-bound arrestin differs from that of both free and receptor-bound states (Hanson et al. 2006a, 2007b). Thus, arrestins in the cell exist in at least three distinct conformations. The deletions in the inter-domain hinge (Fig. 4) impede arrestin–receptor interaction (Vishnivetskiy et al. 2002), but promote its binding to microtubules (Hanson et al. 2006a, 2007b),

suggesting that these interactions require the movement of the two arrestin domains in opposite directions (Fig. 2). The microtubule-binding site on arrestin significantly overlaps with the receptor-binding site, leaving the "back" of the molecule (Fig. 2) free to interact with other proteins. Interestingly, some of them do just that: ERK1/2 and Mdm2 bind microtubule-associated arrestin, as evidenced by arrestin-dependent mobilization of these proteins to the cytoskeleton (Hanson et al. 2007b). However, in contrast to the receptor-bound state, microtubule-bound arrestin does not mobilize upstream activators of ERK1/2, c-Raf-1, and MEK1 (Hanson et al. 2007b). As a result, arrestin-dependent ERK1/2 mobilization to microtubules reduces the ERK1/2 phosphorylation level in the cell (Hanson et al. 2007b), whereas ERK1/2 mobilization to the receptor increases its activation (Luttrell et al. 2001). Mdm2 apparently prefers basal (Song et al. 2006) and microtubule-bound forms, so that its arrestin-dependent mobilization to the microtubules dramatically increases the ubiquitination of cytoskeleton-associated proteins (Hanson et al. 2007b). To summarize, even when a binding partner interacts with different conformational states of arrestin with comparable affinity, the physiological consequences of these interactions are determined by arrestin conformation.

The comparison of the known crystal structures of receptors (Palczewski et al. 2000; Li et al. 2004), arrestins (Hirsch et al. 1999; Han et al. 2001; Sutton et al. 2005), and arrestin-binding partners suggests that a single arrestin molecule or a unitary arrestin–receptor complex can accommodate no more than four to six interacting proteins (Gurevich and Gurevich 2006b). Thus, certain arrestin partners likely compete with each other. The competition between proteins interacting with overlapping or sterically close sites on arrestin has been demonstrated experimentally. GPCRs engage the concave sides of both arrestin domains (Fig. 2) (Pulvermuller et al. 2000; Vishnivetskiy et al. 2004; Hanson et al. 2006b; Hanson and Gurevich 2006). "Footprints" of microtubules (Fig. 2) (Hanson et al. 2006a, 2007b) and Ca^{2+}-liganded calmodulin (Wu et al. 2006) map to the same arrestin surface. Indeed, GPCRs compete for arrestin with microtubules and calmodulin (Nair et al. 2005; Hanson et al. 2006a; Wu et al. 2006), and receptors usually win due to much higher affinity (Gurevich et al. 1995). It is very likely that other arrestin-binding partners also compete for the overlapping sites, but this competition and its biological significance remain to be experimentally elucidated.

5 Arrestin as a Target for Conventional Pharmacological Tools

Arrestins are ubiquitous adaptors linking GPCRs to a variety of signaling pathways by virtue of assembling multi-protein complexes (Lefkowitz and Shenoy 2005), sometimes referred to as "signalosomes." It seems likely that the same arrestin–receptor complex can serve as the core of several types of signalosomes with different biological consequences (Gurevich and Gurevich 2003, 2006b). Free and microtubule-bound arrestins also interact with many of the same signaling proteins, often with different functional outcomes (Song et al. 2006; Hanson et al. 2007b). Manipulating the structure

and composition of signalosomes in order to modulate specific signaling pathways is likely to have enormous experimental and, ultimately, therapeutic potential. Generally speaking, every protein–protein interaction interface within signalosomes can be selectively targeted. Conventional thinking usually focuses on molecules that disrupt the interaction by binding to one of the interaction sites and thereby occluding it. However, a similar approach can be used to strengthen the interaction with appropriately designed small molecules that bind right next to the interaction interface in both proteins and create an additional "bridge" between them.

5.1 Arrestin–Receptor Interface

Receptor-binding elements in the arrestin molecule were identified by progressive deletions (Gurevich and Benovic 1992, 1993), differential chemical modifications and H/D exchange (Ohguro et al. 1994), element swapping between arrestin subtypes (Gurevich et al. 1995; Vishnivetskiy et al. 2004), site-directed mutagenesis (Gurevich and Benovic 1995, 1997; Kovoor et al. 1999; Vishnivetskiy et al. 1999, 2000; Celver et al. 2002; Hanson and Gurevich 2006), peptide competition (Pulvermuller et al. 2000), epitope insertion (Dinculescu et al. 2002), and site-directed spin labeling-EPR (Hanson et al. 2006b). These elements clearly fall into two categories: phosphate-binding residues (Gurevich and Benovic 1995, 1997; Vishnivetskiy et al. 1999, 2000; Sutton et al. 2005; Hanson and Gurevich 2006) and elements that bind non-phosphorylated parts of the receptor (Vishnivetskiy et al. 2004; Hanson et al. 2006b; Hanson and Gurevich 2006), presumably those that either change conformation or become exposed upon receptor activation. Both kinds invariably map to the concave sides of the two arrestin domains (Gurevich and Gurevich 2004, 2006b). Receptor-attached phosphates are the common feature of different arrestin-binding GPCRs. Every cell expresses several different GPCRs, but the signaling of just one receptor subtype usually has to be manipulated for therapeutic purposes. To achieve the desired selectivity of therapeutic intervention, the interaction of non-phosphate-binding arrestin elements with subtype-specific non-phosphorylated parts of the receptor must be targeted. Considering that fewer than 25 non-conserved arrestin residues localized in two elements on the concave sides of the two domains determine receptor preference of arrestins (Vishnivetskiy et al. 2004), small-molecule mimics of different versions of these arrestin elements can be designed to disrupt arrestin interactions with selected receptors, while minimally affecting its binding to others. Further improvement of our understanding of the structural basis of arrestin preference for particular GPCRs is needed to make this approach feasible. However, receptor mimics that bind arrestin appear less promising. These molecules would bind to endogenous arrestin and thus indiscriminately prevent its interaction with a variety of GPCRs.

Selective reduction of arrestin interactions with a particular GPCR would slow down its desensitization and internalization, thereby enhancing and prolonging G protein-mediated signaling. This kind of intervention has therapeutic potential in conditions usually treated with GPCR agonists (e.g., asthma) and agonist precursors (e.g., Parkinson's disease) because it counteracts the very process that severely

limits the beneficial effects of the agonists, i.e., receptor desensitization. Currently, loss of responsiveness resulting from repeated agonist administration necessitates the increase of doses, which leads to even more severe desensitization and exacerbates side effects. Excessive desensitization of β2-adrenergic receptor and compensatory over-stimulation apparently underlies congestive heart failure (Rockman et al. 2002). It seems likely that selective blockade of arrestin binding to β2-adrenoreceptor has a potential to interrupt this vicious cycle in the heart.

5.2 Clathrin and AP2 Sites in the C-Tail

Clathrin and AP2 binding sites in the arrestin C-tail were localized with high precision (Kim and Benovic 2002). Over-expressed arrestin C-tail binds clathrin and AP2, effectively functioning as a competitive suppressor of arrestin-dependent GPCR internalization (Orsini and Benovic 1998), suggesting that small molecule mimics of these two sites can be quite effective. However, pharmacological disruption of these interactions would unavoidably affect the internalization of all GPCRs in the cell, which reduces their appeal as possible therapeutic targets.

5.3 Arrestin-Binding Sites for the Non-Receptor Partners

Selective targeting of arrestin interactions with individual non-receptor partners has a tremendous therapeutic potential. Arrestin functions affect a huge variety of signaling mechanisms (Lefkowitz and Shenoy 2005; Gurevich and Gurevich 2006a), some of which may underlie the pathology of multiple diseases. For example, increases in arrestin expression in the brain of MPTP-treated monkeys with Parkinsonian symptoms (Bezard et al. 2005) and human patients with Parkinson's disease-dementia combination (Bychkov et al. 2007) were recently reported. Many arrestin-binding proteins (c-Src, ASK1, JNK3, c-Raf-1, ERK1/2, Mdm2, IκBα, etc.) are key players in pro-survival and pro-apoptotic pathways, suggesting that arrestin-mediated signaling participates in "life-or-death" decisions in the cell. Thus, enhancing pro-survival signaling at the arrestin level has a potential to prevent the excessive cell death characteristic for neurodegenerative diseases, such as Parkinson's, Alzheimer's, and retinitis pigmentosa, whereas enhancing arrestin-dependent pro-apoptotic signaling may counteract the excessive proliferation characteristic for every form of cancer. Pro-survival or pro-apoptotic "branches" of arrestin-mediated signaling could be modulated by enhancing or inhibiting the binding of pro- or antiapoptotic proteins, which can be achieved using properly designed small molecules. In the cell, interference with any one of the signaling pathways would also influence alternative arrestin-dependent signaling mechanisms. In a simplistic example, selective blockade of ERK1/2 binding would shift the balance toward the activation of JNK3, attenuating proliferation or even inducing apoptosis. Similarly, the disruption

of arrestin-JNK3 interaction would re-direct the signaling to ERK1/2 and promote cell survival.

However, to realize the full potential of selective channeling of arrestin-mediated signaling, the binding sites for various partners must be mapped on the arrestin molecule with high precision. These studies are urgently needed: at the moment only arrestin elements involved in the binding to receptors (Vishnivetskiy et al. 2004; Hanson et al. 2006b), clathrin and AP2 (Kim and Benovic 2002), microtubules (Hanson et al. 2006a, 2007b), and calmodulin (Wu et al. 2006) have been properly mapped. The interaction sites for the remaining >20 binding partners are identified very imprecisely or not at all. Another issue that urgently requires thorough investigation is the competition and/or possible cooperation between different partners. For example, receptor-bound arrestin is believed to scaffold at least two MAP kinase cascades, c-Raf-1->MEK1->ERK1/2 and ASK1->MKK4->JNK3. A unitary arrestin–receptor complex is too small to accommodate all these proteins simultaneously (Gurevich and Gurevich 2006b), which suggests that it scaffolds either one of these cascades or the other. If arrestin interactions with MAPKKK (c-Raf-1 or ASK1) and MAPK (ERK1/2 or JNK3) were independent, half of the complexes would contain wrong combinations of kinases and therefore be unproductive. It stands to reason that the binding of MAPKKKs and MAPKs is coordinated, so that the complexes preferentially contain combinations of ERK1/2 with c-Raf-1 and JNK3 with ASK1. Direct experiments with purified proteins are needed to test which partners compete for the limited "parking space" on receptor-bound arrestin and which partners cooperate to end up in the same complex. Although dimerization of receptors (Angers et al. 2002), each recruiting its own arrestin molecule (Hanson et al. 2007a), may relieve the "overcrowding" of signaling proteins, it is unlikely to solve this particular problem, as members of the same MAP kinase cascade must be properly positioned relative to each other for the scaffold to work.

6 Custom-Designed Proteins: An Alternative Approach to Harness Arrestin-Mediated Signaling for Therapeutic Purposes

6.1 Modification of Receptor-Binding Elements

For therapeutic purposes it is usually necessary to modify the signaling via just one subtype of GPCR out of many expressed by the same cell type. For example, naturally occurring constitutively active receptor mutants were implicated in Jansen-type metaphyseal chondrodysplasia (Schipani et al. 1995), autosomal dominant non-autoimmune hyperthyroidism and toxic thyroid nodules (Paschke 1996; Khoo et al. 1999), certain forms of cancer (Allen et al. 1991; Parnot et al. 2002), and familial nephrogenic diabetes insipidus (Barak et al. 2001). Existing

arrestin mutants with enhanced ability to quench signaling (Gray-Keller et al. 1997; Gurevich et al. 1997; Kovoor et al. 1999; Celver et al. 2002) appear to be promising tools for gene therapy in these cases. Unfortunately, both non-visual arrestins demonstrate very broad receptor specificity (Gurevich and Gurevich 2006b), so tinkering with either would affect many GPCR subtypes, likely doing more harm than good. However, rod arrestin is fairly selective for its natural target, rhodopsin (Gurevich et al. 1995; Kovoor et al. 1999; Celver et al. 2002), demonstrating that it is possible to construct an arrestin that preferentially binds just one type of receptor. Using a series of rod-arrestin 2 chimeras, the elements that determine receptor specificity were narrowed down to two relatively small pieces on the concave sides of the two arrestin domains. Swapping these two elements, which contain only 22 non-conservative substitutions between rod and arrestin 2, completely reverses their receptor preference (Vishnivetskiy et al. 2004). Thus, it is likely that by manipulating these residues one can construct arrestins with narrow receptor specificity, even with preference for just one GPCR subtype. Existing "super-arrestins" with enhanced desensitizing ability (Gray-Keller et al. 1997; Kovoor et al. 1999; Celver et al. 2002) appropriately modified to enhance selectivity for a mutant receptor subtype are promising tools for gene therapy in diseases associated with constitutive activity of GPCRs. The combination of high receptor specificity with other special characteristics, such as the ability to bind an active unphosphorylated form of the cognate receptor (Kovoor et al. 1999; Celver et al. 2002), or preferential interaction with pro-survival or pro-apoptotic signaling molecules (Sect. 6.2 below), can further extend the therapeutic potential of these "custom-designed" arrestins.

However, the construction of receptor subtype-specific non-visual arrestins is not a trivial task. The main difficulty is in numbers: even if only 10–15 amino acids determine receptor specificity of arrestin proteins (Vishnivetskiy et al. 2004), the number of possible combinations is enormous ($>10^{20}$). Obviously, screening so many mutants to identify receptor subtype-specific arrestins is impractical. Thus, relatively few residues that play the most important role must be identified first. Our recent data indicate that we can narrow down the list of "prime suspects" to four or five (Vishnivetskiy S.A. and Gurevich V.V., unpublished observations). Next, "intelligently designed" combination mutants that contain a residue of one of the four main types (positively charged, negatively charged, hydrophilic neutral, or hydrophobic) in each of these four to five positions should be constructed. This strategy brings the number of arrestin mutants that have to be tested to a manageable range (from 4^4 to 5^4, i.e., $<1,000$). Still, relatively high-throughput interaction assay (e.g., yeast-based) must be developed to screen even a few hundred mutants with the first batch of the 20–40 most common GPCR subtypes. The first round is unlikely to yield perfect receptor-specific arrestins, but it will generate useful variants that can be further fine-tuned. Importantly, these data will greatly improve our understanding of the structural basis of receptor specificity of arrestin proteins, paving the way to engineering true receptor subtype-specific arrestins.

6.2 Changing the Affinity for Non-Receptor Partners

This task is simpler and much more straightforward than the design of receptor-specific arrestins. However, it is currently hampered by inadequate identification of the arrestin sites mediating its interactions with the great majority of partners. This situation can be easily remedied by systematic mutagenesis of the non-receptor-binding side of arrestins (Figs. 2 and 3). Established interaction assays with purified (Hanson et al. 2007b) or radiolabeled translated proteins in vitro (Hanson and Gurevich 2006), site-directed spin-labeling (Hanson et al. 2006b, 2007b; Wu et al. 2006), as well as cell-based methods, such as "nuclear exclusion assay" (Song et al. 2006, 2007), can be used as readouts. For example, we have recently found that all four vertebrate arrestins comparably bind JNK3 and Mdm2, which identifies a relatively small number of highly conserved residues on the non-receptor-binding side of the molecule as key players in these interactions (Song et al. 2007). The very process of mapping the "footprint" of a particular partner (e.g., by alanine-scanning mutagenesis) generates a number of mutants with reduced binding. These can be further fine-tuned to completely obliterate the interaction by combining selected mutations. Enhancing protein–protein interactions is a bit harder than destroying them, but it is feasible. Most binding sites have an area of 2,000–4,000 $Å^2$, where fewer than ten residues in each partner are in direct contact with the other. These residues are easily identified in the process of site mapping. Usually 10–20 additional side chains within the interface do not contact the partner. These residues can be replaced with those that have bigger side chains to bring them in contact with the partner. The first round is likely to yield a few arrestin mutants with increased affinity for the partner of interest, as well as a number of new mutants with reduced affinity. An additional H-bond increases free energy of interaction by 2–5 kcal mol^{-1}, a salt bridge adds ~5 kcal mol^{-1}, and a hydrophobic interaction up to 3 kcal mol^{-1}. The relationship between the free energy of association and affinity, $\Delta G^o = -RT\ln K_A$ (where R is gas constant = 1.99 cal mol^{-1} K^{-1}; T is temperature in Kelvin, and K_A is the equilibrium association constant) suggests that a combination of mutations that adds two to four direct contacts between side chains of the two proteins can increase the affinity of the interaction 10–100-fold.

Mutant proteins with enhanced ability to bind particular partners are excellent tools for gene therapy, since gain of function can be achieved with a relatively low level of expression of these proteins. Loss of function, however, would require disrupting interactions of endogenous arrestins with specific proteins, which could be achieved using small mimics of arrestin elements that mediate its binding to individual partners. Alternatively, mutant proteins of a dominant negative type could induce loss of function. For example, if a mutant arrestin tightly binds one member of a signaling cascade but not the other(s) required for signaling, it would effectively act as a scavenger of the protein it binds, thereby reducing the activity of the whole pathway. Curiously, microtubule-bound arrestin acts in such a manner with respect to the MAPK pathway: it binds ERK1/2, but not c-Raf-1 or MEK1, causing reduction in cellular ERK1/2 activity (Hanson et al. 2007b). The existence of a native arrestin conformation with

such "dominant negative" properties further supports the feasibility of constructing arrestin proteins with desired signaling behavior. Additionally, as discussed above, often the same effect can be obtained either by reducing the interaction with one partner (e.g., pro-apoptotic kinases JNK3 or ASK1) or enhancing the binding of another with opposing biological activity (e.g., pro-survival kinases ERK1/2, c-Raf-1, or c-Src). Thus, the identification of arrestin elements mediating its interactions with non-receptor partners will open almost limitless possibilities of modulating arrestin-mediated signaling in different ways. From the standpoint of therapeutic potential, some arrestin partners appear more promising than others. Arrestin interactions with proteins that enhance cell survival (protein kinases c-Src, c-Raf-1, ERK1/2, Akt; ubiquitin ligase Mdm2) or promote cell death (protein kinases ASK1, JNK3; NFκB inhibitor IκBα) are of particular interest in this regard, as discussed in Sect. 5.3.

6.3 "Conformationally Biased" Mutants

Arrestin conformation is the key determinant of its functional capabilities (Gurevich and Gurevich 2003). Thus, mutations that affect the flexibility of the molecule and/or limit the conformational space it can "explore" would enhance or reduce its ability to interact with some partners without affecting the binding to others, thereby dramatically shifting arrestin-mediated signaling. Crystal structure of arrestins (Hirsch et al. 1999; Han et al. 2001; Sutton et al. 2005) and functional studies (Vishnivetskiy et al. 2002; Hanson et al. 2006a,b) identify two significant conformational rearrangements in the molecule: receptor- and microtubule-binding induced the release of arrestin C-tail and the movement of the two arrestin domains relative to each other (Fig. 2). Destabilization of intramolecular interactions that hold arrestin in the basal conformation, the polar core and the one that anchors the C-tail to the body of the N-domain (Fig. 4), yield mutants that are more flexible than the wild type (Carter et al. 2005). These "pre-activated" arrestins demonstrate dramatically enhanced binding to active unphosphorylated receptor (Gurevich 1998), shut off the signaling even without receptor phosphorylation (Gray-Keller et al. 1997; Kovoor et al. 1999; Celver et al. 2002), and change the pattern of receptor trafficking, greatly reducing its down-regulation and facilitating recycling (Pan et al. 2003). Interestingly, in cell-based assays these mutant forms of all arrestin subtypes bind JNK3 normally, but show reduced affinity for Mdm2 (Song et al. 2006, 2007), demonstrating that mutations designed to change arrestin conformation differentially affect its interactions with receptors and non-receptor partners. Extensive deletions in the inter-domain hinge region (Fig. 4) restrict domain mobility, limiting their ability to move "forward," in the direction of receptor-binding concave sides (Fig. 2). These mutations severely reduce receptor binding (Vishnivetskiy et al. 2002), considerably enhance the binding to microtubules (Hanson et al. 2007b) and ubiquitin ligase Mdm2, but do not appreciably affect JNK3 interaction (Song et al. 2006, 2007). Mutations that drastically change arrestin conformation and therefore simultaneously affect its interactions with multiple partners demonstrate the feasibility

of constructing arrestins with “biased” signaling capabilities, but are not likely to yield proteins with therapeutic potential. Mutations that change arrestin flexibility in more subtle ways and affect its interactions with just one or very few partners appear more promising in this regard. Arrestin structure identifies the part of the molecule that must be targeted to achieve this goal: the extensive hydrophobic inter-domain surface along which the domains “slide” relative to each other (Fig. 4) (Sutton et al. 2005). The introduction of appropriately positioned opposite charges in the two domains can “fix” their relative orientation, “freezing” the arrestin molecule in any conformation between the two extremes, receptor-bound-like and microtubule-bound-like (Fig. 2). Recent findings suggest that a similar strategy is actually used by cells, where arrestin bound to differentially phosphorylated receptor demonstrates a distinct signaling bias: it does or does not stabilize agonist-receptor interaction (Key et al. 2003), and does or does not promote ERK1/2 activation (Kim et al. 2005; Ren et al. 2005), while enhancing receptor internalization in both cases.

Obviously, binding partners that interact with both arrestin domains, such as receptors (Vishnivetskiy et al. 2004), microtubules (Hanson et al. 2007b), calmodulin (Wu et al. 2006), JNK3, Mdm2 (Song et al. 2007), and probably a number of others, are sensitive to these conformational manipulations, in contrast to the partners with the binding site localized on a single domain. Subtle manipulation of the conformational state of arrestin has a potential to change its signaling capabilities to a great extent. Moreover, the mutations affecting interactions with individual partners (described in Sect. 6.2) will likely produce a more dramatic effect in the context of conformationally restricted arrestins. Thus, the combination of both approaches has a better chance of yielding “designer arrestins” with precisely fine-tuned signaling bias specifically crafted for particular therapeutic purposes. Further development of vectors targeting individual tissues and cell types is needed to enable the delivery of these molecular tools to their intended targets.

7 Looking Farther Ahead: The Combination of Conventional and Unconventional

The main drawback of custom-designed arrestins described above is that each mutant is going to be essentially a “one-trick pony”: it will maintain the same change in the pattern of arrestin-mediated signaling as long as it is expressed. No doubt, there are many situations where tools of this type would be highly beneficial. For example, excessive signaling of mutant GPCRs is responsible for metaphyseal chondrodysplasia (Schipani et al. 1995), autosomal dominant hyperthyroidism (Paschke 1996; Khoo et al. 1999), cancer (Allen et al. 1991; Parnot et al. 2002), and familial diabetes insipidus (Barak et al. 2001). Successful gene therapy of these disorders may well be accomplished by the expression of phosphorylation-independent “super-arrestins” fine-tuned for high selectivity for the receptor that causes the disease. Enhanced desensitizing potential of these arrestins has a good chance to bring

the signaling of the mutant receptor back to normal levels. Similarly, arrestins with anti-proliferative or simply pro-apoptotic signaling bias selectively expressed in cancerous cells by means of appropriate targeting vectors would likely solve the existing problem without creating new ones: after all, the ideal therapeutic outcome is the death of every cancer cell in the body. In contrast, it will be necessary to control the effectiveness of arrestin mutants with pro-survival signaling bias, the expression of which in affected neurons may be beneficial in neurodegenerative disorders, such as Alzheimer's and Parkinson's. Excessive signaling shift in that direction may lead to cell dedifferentiation, which would be just as harmful as the disease itself.

This problem can be solved by the use of "adjustable" arrestin mutants that can be constructed in different ways. For example, the binding site for an interaction partner can be mutated to make arrestin affinity for that particular partner dependent on the presence of a small molecule (that should be deliverable as a drug and should have no unwanted biological activity), e.g., is low in its absence and higher in its presence. Then the administration of different doses of this drug would gradually change arrestin-mediated signaling, allowing for dynamic control of mutant's effect. It is worth noting that small molecules that change wild-type arrestin affinity for individual partners also can be designed (Sect. 5.3 above). However, drugs affecting endogenous protein would change arrestin-mediated signaling everywhere. This may not be a fatal flaw: after all, the great majority of clinically used drugs reaches their targets via systemic circulation, thus producing a rather indiscriminate effect on signaling everywhere, but still yields beneficial effects. However, the combination of protein engineering with pharmacological agents can do better: target specially designed arrestin mutants expressed in a particular tissue or cell types to achieve selective local control.

An alternative approach to designing arrestin mutants amenable to the regulation by exogenously administered drugs involves conformational control. It would require mutations in the inter-domain interface (Fig. 4) that create a binding site for the small molecule. If the occupancy of this site changes preferred arrestin conformation to that favorable (or unfavorable) for the interaction with a particular partner, the drug would enhance (or attenuate) arrestin-dependent activation of the pathway involving this interaction. Similar molecules targeting wild-type arrestin would have global effects, whereas tissue-specific control would require expression of a specially designed mutant in the tissue or cell type of interest. Because endogenous wild-type arrestins expressed in these cells will keep doing their job, adjustable gain-of-function mutants will be required to effectively change the balance of arrestin-mediated signaling.

These two approaches can be used simultaneously and combined with additional mutations in the binding sites for other arrestin partners to produce a virtually unlimited variety of "designer" arrestins with any combination of functional characteristics. Many of these mutants will have therapeutic potential that can be realized with rapid development of cell type and tissue-specific expression vectors. To make these exciting prospects a reality, fine molecular mechanisms of arrestin interactions with various signaling proteins must be elucidated. This includes precise identification of their binding sites on arrestin and determination of the conformational dependence of arrestin interactions with each binding partner.

Acknowledgements The authors are grateful to numerous colleagues in collaboration with whom many of the studies referenced here were performed. This work was supported in part by NIH grants EY11500 (VVG) and NS45117 (EVG). WMC is a recipient of pre-doctoral NIH training grant GM07628.

References

Ackmann M, Wiech H, Mandelkow E (2000) Nonsaturable binding indicates clustering of tau on the microtubule surface in a paired helical filament-like conformation. J Biol Chem 275:30335–30343

Allen LF, Lefkowitz RJ, Caron MG, Cotecchia S (1991) G-protein-coupled receptor genes as protooncogenes: constitutively activating mutation of the alpha 1B-adrenergic receptor enhances mitogenesis and tumniorigenicity. Proc Natl Acad Sci USA 88:11354–11358

Angers S, Salahpour A, Bouvier M (2002) Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. Annu Rev Pharmacol Toxicol 42:409–435

Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, Snyder SH, Caron MG, Lefkowitz RJ (1992) Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. J Biol Chem 267:17882–17890

Barak LS, Ferguson SS, Zhang J, Caron MG (1997) A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. J Biol Chem 272:27497–27500

Barak LS, Oakley RH, Laporte SA, Caron MG (2001) Constitutive arrestin-mediated desensitization of a human vasopressin receptor mutant associated with nephrogenic diabetes insipidus. Proc Natl Acad Sci USA 98:93–98

Benovic JL, Kuhn H, Weyand I, Codina J, Caron MG, Lefkowitz RJ (1987) Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). Proc Nat Acad Sci USA 84:8879–8882

Bezard E, Gross CE, Qin L, Gurevich VV, Benovic JL, Gurevich EV (2005) L-DOPA reverses the MPTP-induced elevation of the arrestin2 and GRK6 expression and enhanced ERK activation in monkey brain. Neurobiol Dis 18:323–335

Bychkov ER, Gurevich VV, Joyce JN, Benovic JL, Gurevich EV (2008) Arrestins and two receptor kinases are upregulated in Parkinson's disease with dementia. Neurobiol Aging 29:379–396.

Carman CV, Benovic JL (1998) G-protein-coupled receptors: turn-ons and turn-offs. Curr Opin Neurobiol 8:335–344

Carter JM, Gurevich VV, Prossnitz ER, Engen JR (2005) Conformational differences between arrestin2 and pre-activated mutants as revealed by hydrogen exchange mass spectrometry. J Mol Biol 351:865–878

Celver J, Vishnivetskiy SA, Chavkin C, Gurevich VV (2002) Conservation of the phosphate-sensitive elements in the arrestin family of proteins. J Biol Chem 277:9043–9048

Claing A, Chen W, Miller WE, Vitale N, Moss J, Premont RT, Lefkowitz RJ (2001) Beta-arrestin-mediated ADP-ribosylation factor 6 activation and beta 2-adrenergic receptor endocytosis. J Biol Chem 276:42509–42513

Dinculescu A, McDowell JH, Amici SA, Dugger DR, Richards N, Hargrave PA, Smith WC (2002) Insertional mutagenesis and immunochemical analysis of visual arrestin interaction with rhodopsin. J Biol Chem 277:11703–11708

Gao H, Sun Y, Wu Y, Luan B, Wang Y, Qu B, Pei G (2004) Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways. Mol Cell 14:303–317

Goodman OB, Jr., Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL (1996) Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. Nature 383:447–450

Gray-Keller MP, Detwiler PB, Benovic JL, Gurevich VV (1997) Arrestin with a single amino acid sustitution quenches light-activated rhodopsin in a phosphorylation independent fashion. Biochemistry 36:7058–7063

Gurevich VV (1998) The selectivity of visual arrestin for light-activated phosphorhodopsin is controlled by multiple nonredundant mechanisms. J Biol Chem 273:15501–15506

Gurevich VV, Benovic JL (1992) Cell-free expression of visual arrestin. Truncation mutagenesis identifies multiple domains involved in rhodopsin interaction. J Biol Chem 267:21919–21923

Gurevich VV, Benovic JL (1993) Visual arrestin interaction with rhodopsin: Sequential multisite binding ensures strict selectivity towards light-activated phosphorylated rhodopsin. J Biol Chem 268:11628–11638

Gurevich VV, Benovic JL (1995) Visual arrestin binding to rhodopsin: diverse functional roles of positively charged residues within the phosphorylation-recignition region of arrestin. J Biol Chem 270:6010–6016

Gurevich VV, Benovic JL (1997) Mechanism of phosphorylation-recognition by visual arrestin and the transition of arrestin into a high affinity binding state. Mol Pharmacol 51:161–169

Gurevich VV, Gurevich EV (2003) The new face of active receptor bound arrestin attracts new partners. Structure 11:1037–1042

Gurevich VV, Gurevich EV (2004) The molecular acrobatics of arrestin activation. TIPS 25:59–112

Gurevich EV, Gurevich VV (2006a) Arrestins are ubiquitous regulators of cellular signaling pathways. Genome Biol 7:236

Gurevich VV, Gurevich EV (2006b) The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. Pharmacol Ther 110:465–502

Gurevich VV, Dion SB, Onorato JJ, Ptasienski J, Kim CM, Sterne-Marr R, Hosey MM, Benovic JL (1995) Arrestin interaction with G protein-coupled receptors. Direct binding studies of wild type and mutant arrestins with rhodopsin, b2-adrenergic, and m2 muscarinic cholinergic receptors. J Biol Chem 270:720–731

Gurevich VV, Pals-Rylaarsdam R, Benovic JL, Hosey MM, Onorato JJ (1997) Agonist-receptor-arrestin, an alternative ternary complex with high agonist affinity. J Biol Chem 272:28849–28852

Gurevich EV, Benovic JL, Gurevich VV (2002) Arrestin2 and arrestin3 are differentially expressed in the rat brain during postnatal development. Neuroscience 109:421–436

Gurevich EV, Benovic JL, Gurevich VV (2004) Arrestin2 expression selectively increases during neural differentiation. J Neurochem 91:1404–1416

Gurevich VV, Hanson SM, Gurevich EV, Vishnivetskiy SA (2007) How rod arrestin achieved perfection: regulation of its availability and binding selectivity. pp 55–58. In: Fliesler SJ, Kisselev O (eds) Signal transduction in the retina. Methods in Signal Transduction Series, Boca Raton, FL, CRC Press

Han M, Gurevich VV, Vishnivetskiy SA, Sigler PB, Schubert C (2001) Crystal structure of beta-arrestin at 1.9 A: possible mechanism of receptor binding and membrane translocation. Structure 9:869–880

Hanson SM, Gurevich VV (2006) The differential engagement of arrestin surface charges by the various functional forms of the receptor. J Biol Chem 281:3458–3462

Hanson SM, Francis DJ, Vishnivetskiy SA, Klug CS, Gurevich VV (2006a) Visual arrestin binding to microtubules involves a distinct conformational change. J Biol Chem 281:9765–9772

Hanson SM, Francis DJ, Vishnivetskiy SA, Kolobova EA, Hubbell WL, Klug CS, Gurevich VV (2006b) Differential interaction of spin-labeled arrestin with inactive and active phosphorhodopsin. Proc Natl Acad Sci USA 103:4900–4905

Hanson SM, Gurevich EV, Vishnivetskiy SA, Ahmed MR, Song X, Gurevich VV (2007a) Each rhodopsin molecule binds its own arrestin. Proc Nat Acad Sci USA 104:3125–3128

Hanson SM, Cleghorn WM, Francis DJ, Vishnivetskiy SA, Raman D, Song S, Nair KS, Slepak VZ, Klug CS, Gurevich VV (2007b) Arrestin mobilizes signaling proteins to the cytoskeleton and redirects their activity. J Mol Biol 368:375–387

Hiller G, Weber K (1978) Radioimmunoassay for tubulin: a quantitative comparison of the tubulin content of different established tissue culture cells and tissues. Cell 14:795–804

Hirsch JA, Schubert C, Gurevich VV, Sigler PB (1999) The 2.8 A crystal structure of visual arrestin: a model for arrestin's regulation. Cell 97:257–269
Hunzicker-Dunn M, Gurevich VV, Casanova JE, Mukherjee S (2002) ARF6: a newly appreciated player in G protein-coupled receptor desensitization. FEBS Lett 521:3–8
Key TA, Foutz TD, Gurevich VV, Sklar LA, Prossnitz ER (2003) N-formyl peptide receptor phosphorylation domains differentially regulate arrestin and agonist affinity. J Biol Chem 278:4041–4047
Khoo DH, Parma J, Rajasoorya C, Ho SC, Vassart G (1999) A germline mutation of the thyrotropin receptor gene associated with thyrotoxicosis and mitral valve prolapse in a Chinese family. J Clin Endocrinol Metab 84:1459–1462
Kim YM, Benovic JL (2002) Differential roles of arrestin-2 interaction with clathrin and adaptor protein 2 in G protein-coupled receptor trafficking. J Biol Chem 277:30760–30768
Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H, Lefkowitz RJ (2005) Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. Proc Nat Acad Sci USA 102:1442–1447
Kovoor A, Celver J, Abdryashitov RI, Chavkin C, Gurevich VV (1999) Targeted construction of phosphorylation-independent b-arrestin mutants with constitutive activity in cells. J Biol Chem 274:6831–6834
Kuhn H (1978) Light-regulated binding of rhodopsin kinase and other proteins to cattle photoreceptor membranes. Biochemistry 17:4389–4395
Kuhn H, Hall SW, Wilden U (1984) Light-induced binding of 48-kDa protein to photoreceptor membranes is highly enhanced by phosphorylation of rhodopsin. FEBS Lett 176:473–478
Laporte SA, Oakley RH, Zhang J, Holt JA, Ferguson sSG, Caron MG, Barak LS (1999) The 2-adrenergic receptor/arrestin complex recruits the clathrin adaptor AP-2 during endocytosis. Proc Nat Acad Sci USA 96:3712–3717
Lefkowitz RJ, Shenoy SK (2005) Transduction of receptor signals by beta-arrestins. Science 308:512–517
Li J, Edwards PC, Burghammer M, Villa C, Schertler GF (2004) Structure of bovine rhodopsin in a trigonal crystal form. J Mol Biol 343:1409–1438
Liang Y, Fotiadis D, Filipek S, Saperstein DA, Palczewski K, Engel A (2003) Organization of the G protein-coupled receptors rhodopsin and opsin in native membranes. J Biol Chem 278:21655–21662
Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ (1990) Beta-arrestin: a protein that regulates beta-adrenergic receptor function. Science 248:1547–1550
Lohse MJ, Andexinger S, Pitcher J, Trukawinski S, Codina J, Faure JP, Caron MG, Lefkowitz RJ (1992) Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems. J Biol Chem 267:8558–8564
Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, Lefkowitz RJ (2001) Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. Proc Nat Acad Sci USA 98:2449–2454
McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ, Lefkowitz RJ (2000) Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. Science 290:1515–1518
Mukherjee S, Gurevich VV, Jones JC, Casanova JE, Frank SR, Maizels ET, Bader MF, Kahn RA, Palczewski K, Aktories K, Hunzicker-Dunn M (2000) The ADP ribosylation factor nucleotide exchange factor ARNO promotes beta-arrestin release necessary for luteinizing hormone/choriogonadotropin receptor desensitization. Proc Natl Acad Sci USA 97:5901–5906
Nair KS, Hanson SM, Kennedy MJ, Hurley JB, Gurevich VV, Slepak VZ (2004) Direct binding of visual arrestin to microtubules determines the differential subcellular localization of its splice variants in rod photoreceptors. J Biol Chem 279:41240–41248
Nair KS, Hanson SM, Mendez A, Gurevich EV, Kennedy MJ, Shestopalov VI, Vishnivetskiy SA, Chen J, Hurley JB, Gurevich VV, Slepak VZ (2005) Light-dependent redistribution of arrestin in vertebrate rods is an energy-independent process governed by protein–protein interactions. Neuron 46:555–567

Nelson CD, Perry SJ, Regier DS, Prescott SM, Topham MK, Lefkowitz RJ (2007) Targeting of diacylglycerol degradation to M1 muscarinic receptors by beta-arrestins. Science 315:663–666
Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS (2000) Differential affinities of visual arrestin, barrestin1, and barrestin2 for G protein-coupled receptors delineate two major classes of receptors. J Biol Chem 275:17201–17210
Ohguro H, Palczewski K, Walsh KA, Johnson RS (1994) Topographic study of arrestin using differential chemical modifications and hydrogen/deuterium exchange. Protein Sci:2428–2434
Orsini MJ, Benovic JL (1998) Characterization of dominant negative arrestins that inhibit beta2-adrenergic receptor internalization by distinct mechanisms. J Biol Chem 273:34616–34622
Palczewski K, Pulvermuller A, Buczylko J, Hofmann KP (1991) Phosphorylated rhodopsin and heparin induce similar conformational changes in arrestin. J Biol Chem 266:18649–18654
Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, LeTrong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. Science 289:739–745
Pals-Rylaarsdam R, Gurevich VV, Lee KB, Ptasienski J, Benovic JL, Hosey MM (1997) Internalization of the m2 muscarinic acetylcholine receptor: arrestin-independent and -dependent pathways. J Biol Chem 272:23682–23689
Pan L, Gurevich EV, Gurevich VV (2003) The nature of the arrestin × receptor complex determines the ultimate fate of the internalized receptor. J Biol Chem 278:11623–11632
Parnot C, Miserey-Lenkei S, Bardin S, Corvol P, Clauser E (2002) Lessons from constitutively active mutants of G protein-coupled receptors. Trends Endocrinol Metab 13:336–343
Paschke R (1996) Constitutively activating TSH receptor mutations as the cause of toxic thyroid adenoma, multinodular toxic goiter and autosomal dominant non autoimmune hyperthyroidism. Exp Clin Endocrinol Diabetes 104:129–132
Pulvermuller A, Schroder K, Fischer T, Hofmann KP (2000) Interactions of metarhodopsin II. Arrestin peptides compete with arrestin and transducin. J Biol Chem 275:37679–37685
Ren XR, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ (2005) Different G protein-coupled receptor kinases govern G protein and beta-arrestin mediated signaling of V2 vasopressin receptor. Proc Nat Acad Sci USA 102:1448–1453
Rockman HA, Koch WJ, Lefkowitz RJ (2002) Seven-transmembrane-spanning receptors and heart function. Nature 415:206–212
Schipani E, Kruse K, Juppner H (1995) A constitutively active mutant PTH-PTHrP receptor in Jansen-type metaphyseal chondrodysplasia. Science 268:98–100
Schleicher A, Kuhn H, Hofmann KP (1989) Kinetics, binding constant, and activation energy of the 48-kDa protein-rhodopsin complex by extra-metarhodopsin II. Biochemistry 28:1770–1775
Scott MG, Le Rouzic E, Perianin A, Pierotti V, Enslen H, Benichou S, Marullo S, Benmerah A (2002) Differential nucleocytoplasmic shuttling of beta-arrestins. Characterization of a leucine-rich nuclear export signal in beta-arrestin2. J Biol Chem 277:37693–37701
Shenoy SK, McDonald PH, Kohout TA, Lefkowitz RJ (2001) Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. Science 294:1307–1313
Shinohara T, Dietzschold B, Craft CM, Wistow G, Early JJ, Donoso LA, Horwitz J, Tao R (1987) Primary and secondary structure of bovine retinal S antigen (48-kDa protein). Proc Nat Acad Sci USA 84:6975–6979
Song X, Raman D, Gurevich EV, Vishnivetskiy SA, Gurevich VV (2006) Visual and both non-visual arrestins in their "inactive" conformation bind JNK3 and Mdm2 and relocalize them from the nucleus to the cytoplasm. J Biol Chem 281:21491–21499
Song X, Gurevich EV, Gurevich VV (2007) Cone arrestin binding to JNK3 and Mdm2: conformational preference and localization of binding sites. J Neurochem 103:1053–1062
Sterne-Marr R, Gurevich VV, Goldsmith P, Bodine RC, Sanders C, Donoso LA, Benovic JL (1993) Polypeptide variants of beta-arrestin and arrestin3. J Biol Chem 268:15640–15648
Sutton RB, Vishnivetskiy SA, Robert J, Hanson SM, Raman D, Knox BE, Kono M, Navarro J, Gurevich VV (2005) Crystal structure of cone arrestin at 2.3 Å: Evolution of receptor specificity. J Mol Biol 354:1069–1080

Vishnivetskiy SA, Paz CL, Schubert C, Hirsch JA, Sigler PB, Gurevich VV (1999) How does arrestin respond to the phosphorylated state of rhodopsin? J Biol Chem 274:11451–11454
Vishnivetskiy SA, Schubert C, Climaco GC, Gurevich YV, Velez M-G, Gurevich VV (2000) An additional phosphate-binding element in arrestin molecule: implications for the mechanism of arrestin activation. J Biol Chem 275:41049–41057
Vishnivetskiy SA, Hirsch JA, Velez M-G, Gurevich YV, Gurevich VV (2002) Transition of arrestin in the active receptor-binding state requires an extended interdomain hinge. J Biol Chem 277:43961–43968
Vishnivetskiy SA, Hosey MM, Benovic JL, Gurevich VV (2004) Mapping the arrestin–receptor interface: structural elements responsible for receptor specificity of arrestin proteins. J Biol Chem 279:1262–1268
Vishnivetskiy SA, Raman D, Wei J, Kennedy MJ, Hurley JB, Gurevich VV (2007) Rhodopsin phosphorylation level required for arrestin binding: a threshold mechanism. J Biol Chem 282: 32075–32083
Wang P, Wu Y, Ge X, Ma L, Pei G (2003) Subcellular localization of beta-arrestins is determined by their intact N domain and the nuclear export signal at the C terminus. J Biol Chem 278:11648–11653
Witherow DS, Garrison TR, Miller WE, Lefkowitz RJ (2004) beta-Arrestin inhibits NF-kappaB activity by means of its interaction with the NF-kappaB inhibitor IkappaBalpha. Proc Natl Acad Sci USA 101:8603–8607
Wu N, Hanson SM, Francis DJ, Vishnivetskiy SA, Thibonnier M, Klug CS, Shoham M, Gurevich VV (2006) Arrestin binding to calmodulin: a direct interaction between two ubiquitous signaling proteins. J Mol Biol 364:955–963
Xiao K, Shenoy SK, Nobles K, Lefkowitz RJ (2004) Activation-dependent conformational changes in {beta}-arrestin 2. J Biol Chem 279:55744–55753

A-Kinase Anchoring Proteins as the Basis for cAMP Signaling

K.L. Dodge-Kafka(✉), A. Bauman, and M.S. Kapiloff

Contents

1 Introduction . . . 4
1.1 Compartmentation of cAMP Signaling . . . 4
2 AKAPs . . . 5
3 Discovery of AKAP Function . . . 6
4 mAKAP as a Model for the Regulation of cAMP Signaling . . . 7
5 AKAPs as Drug Targets . . . 10
6 Future Directions . . . 12
References . . . 12

Abstract Common challenges to any cell are the processing of the extracellular stimuli it receives into intracellular signaling cascades that initiate a multitude of diverse biological functions. However, many of these stimuli act via a common signaling pathway, suggesting the cell must somehow discriminate between different stimuli and respond accordingly. Subcellular targeting through the association with adaptor and scaffolding proteins has emerged as a key mechanism by which cells maintain signaling specificity. Compartmentation of cAMP signaling is maintained by the clustering of cAMP signaling enzymes in discrete units by the scaffolding protein A-kinase anchoring proteins (AKAP). In doing so, AKAPs provide the molecular architecture for the cAMP micordomains that underlie the spacial-temporal control of cAMP signaling.

Abbreviations PKA: cAMP-dependent protein kinase; C: PKA catalytic subunit; RI: PKA regulatory subunit type I; RII: PKA regulatory subunit type II; AKAP: A-kinase anchoring protein; PDE: Phosphodiesterase; RyR: Ryanodine receptor; PP2A: Protein phosphatase 2A; Epac: Exchange protein activated by cAMP

K.L. Dodge-Kafka
Pat and Jim Calhoun Center for Cardiology, Department of Cell Biology, University of Connecticut Health Center, 263 Farmington Ave, Farmington, CT 06030, USA
dodge@uchc.edu

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

1 Introduction

The stimulation of cell membrane receptors provides specific information that is conveyed throughout the cell via a network of signal transduction pathways. One of the best-characterized pathways involves the diffusible second messenger cAMP. Since its discovery in 1958, many components of the cAMP pathway have been identified and characterized. Most often, cAMP signaling is initiated by hormone or neurotransmitter binding to G-protein-coupled receptors (GPCRs). Receptor stimulation causes the dissociation of G_{α} subunit from $G_{\beta\gamma}$ subunits. In turn, $G_{\alpha s}$ activates adenylyl cyclase, a family of integral membrane proteins that catalyzes the synthesis of cAMP from ATP. The most common target of cAMP is the cAMP-dependent protein kinase A (PKA), although other effectors such as cyclic nucleotide-gated channels and the Rap1-guanine nucleotide exchange factor protein directly activated by cAMP (Epac) should be noted. PKA catalyzes the phosphorylation of an array of proteins located throughout the cell. cAMP signaling is terminated by phosphodiesterases that hydrolyze cAMP to 5′-AMP and phosphatases that dephosphorylate target proteins. Additionally, cAMP generation is opposed by cell membrane receptor-mediated activation of $G_{\alpha i}$, which inhibits adenylyl cyclase. While this pathway clearly defines the basic mechanics of cAMP signaling, it also presents a picture of the PKA enzyme happily phosphorylating all targets under any conditions of elevated cAMP. Research over the last 30 years has decidedly demonstrated this is not the case, giving rise to the question of how specificity of phosphorylation is maintained. While the molecular mechanisms providing for specificity are not fully understood, new evidence suggests that individual signaling units or "signalosomes" consisting of adenylyl cyclases, cAMP effectors, and phosphodiesterases may account for the spacial-temporal activation of PKA. Importantly, the molecular glue that holds these signalsomes together are the scaffolding proteins A-kinase anchoring proteins (AKAPs). This chapter will highlight the role of AKAPs in the compartmentalization of cAMP and PKA signaling and will suggest how manipulation of AKAP complexes may be used to alter PKA signaling.

1.1 Compartmentation of cAMP Signaling

The idea of compartmentalized pools of PKA originated in the late 1970s when Corbin et al. (1977) showed the existence of both soluble and particulate fractions of PKA activity in the rat heart. Later experiments demonstrated that the two pools of PKA were differentially activated. While both particulate and soluble PKA were stimulated by β-adrenergic receptor agonists, ligand binding to prostaglandin receptors only activated soluble PKA (Hayes et al. 1979, 1980). Moreover, while both the β-AR agonist isoproterenol and prostaglandin E1 increased cAMP concentrations, only isoproterenol increased glycogen metabolism and phosphorylation of

troponin I (Brunton et al. 1979). These results illustrate that even though different hormones may act through the same second messenger, they can stimulate different pools of PKA and mediate distinct physiological responses.

More recent experiments have allowed the visualization of cAMP compartmentalization within a living cell. Elegant electrophysiological experiments by Jurevicius and Fischmeister (1996) revealed that local stimulation of β-adrenergic receptors on one side of a cardiac myocyte results in local cAMP production and the restricted stimulation of adjacent calcium channels. Zaccolo (2004) have extended these observations using a fluorescence resonance energy transfer (FRET) cAMP sensor based on the PKA holoenzyme. They found that β-adrenergic agonists preferentially generated cAMP at the myocyte transverse tubule and junctional sarcoplasmic reticulum membranes, while the adenylyl cyclase activator forskolin produced global increases in cAMP levels. Their results suggest that in the cardiac myocyte cAMP could be restricted to compartments as small as 1 μm across. Remarkably, activation of the cAMP sensor by β-adrenergic agonists was dependent on it being anchored by AKAPs and was enhanced by phosphodiesterase inhibition. Later, Mongillo et al. (2004) found that type-4 phosphodiesterases (PDE4) were specifically responsible for modulating the amplitude and duration of β-adrenergic-induced cAMP signaling, while PDE3 controlled other pools of cAMP. The results of these and an increasing number of other recent studies support the existence of distinct cAMP microdomains that control specialized PKA signaling. These signaling domains are, in part, due to AKAPs.

2 AKAPs

AKAPs are a family of over 50 functionally related proteins defined by their ability to bind PKA. The PKA holoenzyme is a tetramer consisting of two catalytic (C) subunits held in an inactive conformation by a regulatory (R) subunit dimer (Scott 1991). Binding of cAMP to the R subunits leads to the dissociation of the holoenzyme and release of active C subunits. The C subunits are multifunctional serine/threonine kinases that phosphorylate a number of downstream targets. To date, three C-subunit genes (Cα, Cβ, Cγ) and four R-subunit genes (RIα, RIβ RIIα, and RIIβ) have been identified (Taylor et al. 1990; Scott 1991). While the C-subunits display similar kinetic and physiological properties, the R-subunits exhibit distinct cAMP-binding affinities and sub-cellular localizations (Taylor et al. 1990).

AKAPs bind the R-subunits of PKA via an amphipathic helix motif within the AKAP (Carr et al. 1992; Newlon et al. 2001). The molecular details of this association are well characterized and will be discussed further in a subsequent chapter. Type-II PKA (containing RII subunits) binds to AKAPs with high affinity ($K_D = 10^{-9}$M) (Carr et al. 1992; Alto et al. 2003). While there are a few AKAPs that bind with high affinity to either type-I PKA alone or both type-I and type-II PKA, most AKAPs bind type-I PKA with a 1,000-fold lower affinity than type-II PKA (Stokka et al. 2006). As a result, type-I PKA is predominately cytosolic, whereas type-II

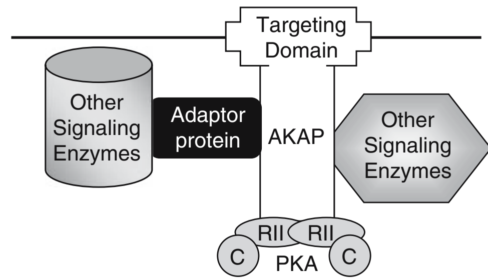

Fig. 1 Diagram depicting the four properties of AKAPs. AKAPs are defined based upon their ability to bind the R-subunit of PKA. Unique targeting domains contained within the individual AKAP determine their subcellular placement. One of the most intriguing properties of AKAPs is their ability to incorporate multiple signaling pathways, while the association with additional adaptor proteins allows AKAPs to integrate into multi-protein networks

PKA is typically associated with cellular structures and organelles. The importance of AKAPs is underscored by the fact that half of the kinase activity in the mammalian heart is associated with the particulate fraction (Corbin et al. 1977).

AKAPs exhibit several common properties (Fig. 1). By definition, all AKAPs bind PKA R-subunits and can mediate the immunoprecipitation of PKA catalytic activity from cells. The second property allows for the specific subcellular location of each AKAP. Distinct binding regions in each AKAP participate in protein/protein or lipid/protein interactions, allowing for the subcellular distribution of the AKAP (Fraser et al. 1998; Dodge and Scott 2000; Westphal et al. 2000). The third and most intriguing characteristic of AKAPs is that they coordinate the integration of enzymes from multiple signaling networks onto a specific substrate. These additional components may be almost any protein in the signal transduction toolbox, including protein kinases, phosphatases, phosphodiesterases, and adenlyl cyclases, as well as G-protein-coupled receptors and ion channels (Coghlan et al. 1995; Klauck et al. 1996; Fraser et al. 1998, 2000; Westphal et al. 1999; Dodge et al. 2001). Lastly, AKAPs are recruited into much larger multiprotein complexes through the interactions with other adapter molecules, such as PDZ and SH3 domain-containing proteins (Colledge et al. 2000; Westphal et al. 2000). These four properties of AKAPs allow the scaffolding proteins to integrate multiple signaling pathways, allowing for the convergence of signals onto a common target.

3 Discovery of AKAP Function

Historically, most AKAPs were initially identified in screens for PKA-binding proteins. Thus, these AKAPs tend to be better characterized. The function and/or identity of many other putative AKAPs, however, remains obscure. For cellular processes where it has been suspected that PKA anchoring may be involved, techniques to globally disrupt PKA anchoring in a cell have proven quite useful. A peptide that mimics the RII-binding domain of AKAP-Lbc (termed Ht31 peptide) competes with AKAP/PKA binding both in vivo and in vitro (Carr et al. 1992). Over-expression of

this peptide has been used to demonstrate the importance of PKA anchoring for several physiological functions (Rosenmund et al. 1994).

The use of the Ht31 peptide demonstrated the importance of AKAPs in non-insulin-dependent diabetes mellitus (NIDDM) (Lester et al. 1997). Over-expression of the anchoring inhibitor peptide in primary islets and cloned beta cell lines blocked the ability of the hormone glucagon-like peptide (GLP-1) to stimulate insulin secretion. Furthermore, expression of Ht31 in these cells disrupted cAMP-mediated increases in intracellular calcium. These data suggest that an anchored pool of PKA regulates both insulin secretion and calcium flux in these cells.

In another example of the importance of AKAPs, Fink et al. (2001) used adenoviral-mediated expression of the Ht31 peptide in cardiac myocytes. Global inhibition of PKA anchoring in myocytes resulted in an increased rate and amplitude of cell shortening and relaxation. Furthermore, Ht31 expression blocked PKA phosphorylation of troponin I and myosin-binding protein C, suggesting that AKAP-bound PKA regulates the phosphorylation of these targets. Both of these examples highlight the dependence of proper physiological function on anchored PKA signaling. Thus, screens to identify other unknown AKAPs and the physiological pathways they mediate are warranted.

4 mAKAP as a Model for the Regulation of cAMP Signaling

The scaffold protein mAKAP is an example of an AKAP that is well characterized. Moreover, mAKAP provides an example of how AKAPs function to link multiple signaling pathways and to control local cAMP levels. mAKAPβ is a 230-kDa protein present at the nuclear envelope in both cardiac and skeletal myocytes (Kapiloff et al. 1999; Michel et al. 2005). The alternatively spliced-form mAKAPα is present in neurons. mAKAP complexes can contain PKA, the phosphodiesterase PDE4D3, the calcium-activated calcium channel ryanodine receptor, the protein phosphatases PP2A, and calcineurin Aβ, nesprin-1α, Epac1, extracellular signal-regulated kinase 5 (ERK5), and its upstream activator MEK5, 3-phosphoinositide-dependent kinase-1 (PDK1), and p90RSK (Kapiloff et al. 1999; Marx et al. 2000; Dodge et al. 2001; Ruehr et al. 2003; Pare et al. 2004, 2005; Dodge-Kafka et al. 2005; Michel et al. 2005). Due to mAKAPβ's large size and its association with the scaffold protein nesprin-1α, additional mAKAPβ-binding partners likely will be identified.

The mAKAP signalosome is unique in that it regulates local cAMP levels through two conjoined negative feedback loops comprised of different signaling pathways (Fig. 2). These pathways converge on the associated phosphodiesterase, either stimulating or abating its activity, allowing for the precise tuning of the surrounding cAMP concentration. Phosphorylation of PDE4D3 at serine 13 enhances the binding affinity of the phosphodiesterase for mAKAP, while phosphorylation of serine 54 increases the catalytic activity of the phosphodiesterase (Sette and Conti 1996; Carlisle Michel et al. 2004). This complex interaction between PDE

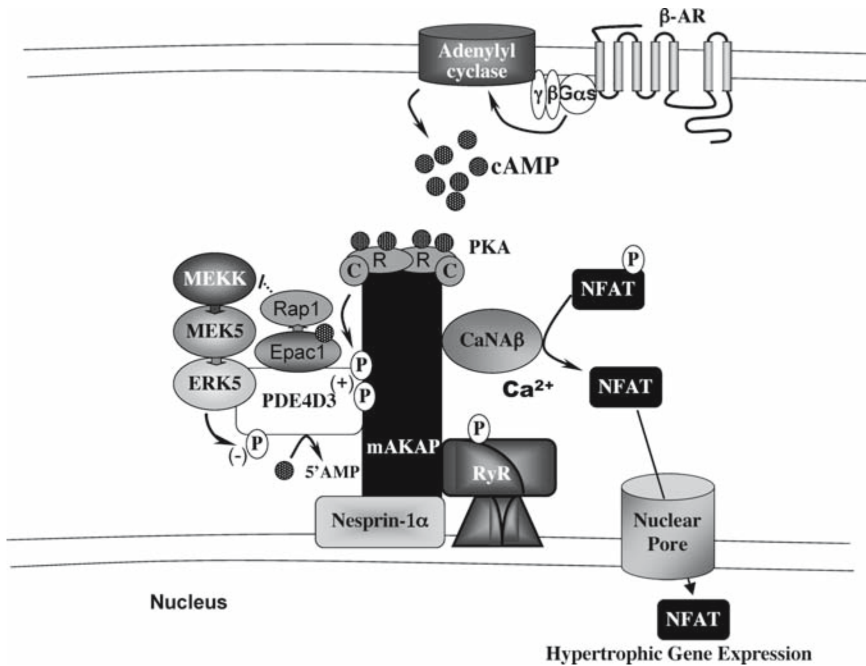

Fig. 2 Diagram depicting the mAKAPβ singalsome. The β-receptor stimulates the release of the Gαs subunit from the trimeric G-protein, initiating the activation of the adenylyl cyclase. The resulting increase in cAMP production stimulates the activation of PKA in the complex and phosphorylation of the two currently identified targets, PDE4D3 and RyR2. Phosphorylation of RyR2 increases the release of calcium, promoting the activation of calcineurin Aβ and dephosphorylation of the transcription factor NFAT. The now dephosphorylated NFAT translocates into the nucleus, where it promotes hypertrophic gene expression. The second target for PKA, PDE4D3, is responsible for turning off this pathway by decreasing the concentration of cAMP and promoting the formation of the PKA holoenzyme. However, stimulation of the map kinase ERK5 results in a decrease in cAMP hydrolysis by phosphorylating PDE4D3, lengthening the activation of PKA in the complex. Prolonged increases in cAMP concentration activate Epac within the complex, inhibiting ERK5

and kinase activity sets up a unique feedback mechanism allowing for the temporal control of kinase activity. In a myocyte, one expects that under basal conditions, mAKAPβ-bound PKA activity will be minimal due to the hydrolysis of ambient cAMP by bound PDE4D3. Upon β-adrenergic stimulation, cAMP concentrations increase, activating PKA. PDE4D3 is phosphorylated on both serine residues, increasing the binding affinity for mAKAPβ and PDE4D3 catalytic activity. These events form a negative feedback loop that inhibits the accumulation of local cAMP, resulting in the attenuation of PKA signaling.

Additional regulation of local cAMP levels is contributed by further phosphorylation of mAKAP-bound PDE4D3. Phosphorylation of serine 579 by the MAP-kinase ERK5 inhibits PDE4D3 catalytic activity (Hoffmann et al. 1999). In contrast to the

effects of PKA phosphorylation, ERK5 phosphorylation promotes the local accumulation of cAMP around the mAKAPβ signalosome, potentiating PKA activation (Dodge-Kafka et al. 2005). Furthermore, ERK5 is targeted to the mAKAP complex through direct interaction with PDE4D3, and mutation of the ERK-binding domain on PDE4D3 blocks ERK5 association with the complex (Dodge-Kafka et al. 2005). Due to its phosphorylation by both PKA and ERK5, PDE4D3 serves as the fulcrum for the two negative feedback loops. Therefore, by tethering these three enzymes into the same signaling complex, mAKAPβ permits the precise tuning of cAMP levels by both G_s-coupled and MAP-kinase signaling pathways.

mAKAP-associated PDE4D3 also directly binds to Epac1. This cAMP effector regulates the activity of the small G-protein Rap1, which in turn regulates ERK5 in the mAKAP complex. As cAMP concentrations climb due to ERK5 and adenylate cyclase activation, Epac1, which has a 100-fold lower affinity for cAMP than PKA, is activated. Epac1 activation results in Rap1 activation, which subsequently leads to MEK5 and ERK5 inhibition. As a result, in the presence of very high levels of cAMP, PDE4D3 is not inhibited by the ERK signaling pathway, and cAMP degradation is favored.

Another important target of mAKAP-associated PKA is the ryanodine receptor (RyR). The RyR is responsible for the bulk release of calcium ions from intracellular stores and plays an important role in excitation–contraction coupling. Importantly, the cardiac RyR2 isoform is a substrate for PKA phosphorylation. Phosphorylation of RyR2 serine 2808 increases the channel's sensitivity to calcium and the probability of channel opening (Marx 2003). A subset of nuclear RyR2 can be co-immunoprecipitated with mAKAPβ from cardiac myocytes, and β-AR stimulation of primary myocyte cultures increases the phosphorylation of mAKAPβ-associated RyR2 (Kapiloff et al. 2001). These findings suggest that within the local context of the mAKAPβ signalosome, PKA-mediated RyR2 phosphorylation could potentate the release of calcium. Importantly, PKA-phosphorylated RyR2 may be de-phosphorylated by the associated phosphatase PP2A (Marx et al. 2000; Kapiloff et al. 2001). This complex formation and regulation of the RyR2 by the mAKAPβ complex may have important clinical applications, as Marx and colleagues have shown that PKA phosphorylation of RyR2 is increased in heart failure due to the decreased expression of PDE4D3 and local phosphatases as well as sustained β-adrenergic signaling (Marx et al. 2000; Lehnart et al. 2005).

One function of the calcium ion released by mAKAPβ-associated RyR2 may be the activation of the associated calcium/calmodulin-dependent phosphatase calcineurin Aβ. In vivo, calcineurin Aβ is important for the activation of NFATc (nuclear factor of activated T-cells) transcription factors and is required for the induction of cardiac hypertrophy (Lehnart et al. 2005). The functional significance of mAKAPβ-orchestrated stimulation of calcineruin was demonstrated by Pare et al. (2005), who found activation of RyR2 in the complex led to stimulation of the associated phoshpatase and induction of cardiac hypertrophy. Furthermore, myocytes lacking mAKAPβ expression demonstrated a significant reduction of β-adrenergic-stimulation of cardiac hypertrophy. Importantly, regulation of the localized cAMP concentration

by the bound phosphodiesterase would affect the calcium release from the RyR2 and the activation of calcineurin Aβ and induction of cardiac disease.

5 AKAPs as Drug Targets

Due to the pleiotropic effects of cAMP signaling, it is no surprise that current therapies for some human diseases include the use of drugs that affect cAMP levels. For example, β-adrenergic agonists are a mainstay of asthma therapy due to cAMP-mediated relaxation of bronchial smooth muscle. In a complementary fashion, caffeine and theophylline have been used to inhibit phosphodiesterases. The problem with current cAMP-directed therapies is that many such drugs have unacceptable side effects. For example, β_2-specific agonist therapy results in an increased risk of heart attacks (Salpeter et al. 2004). The discovery of AKAPs has brought the promise of more selective therapies that may target specific cAMP pathways. Because cAMP signaling and AKAP function in the cardiac myocyte have been especially well studied, AKAP-targeted therapy may be first directed towards heart disease.

During the induction of cardiac disease, increased catecholaminergic stimulation will induce cAMP signaling and the phosphorylation of PKA targets in the heart. Several lines of research suggest that this increased PKA activity will ultimately exacerbate the underlying cardiac condition. For example, overexpression of the β1-adrenergic receptor, the $G_{\alpha s}$ subunit, and the PKA catalytic subunit in the hearts of transgenic mice resulted in dilated cardiomyopathy (Iwase et al. 1997; Engelhardt et al. 1999; Bisognano et al. 2000; Antos et al. 2001). Moreover, the use of β-blockers to inhibit β-adrenergic receptor signaling is standard therapy in the management of heart failure (Lehnart et al. 2005; Adamson and Gilbert 2006; Shin et al. 2007). However, other research supports the notion that an increase in cAMP signaling is beneficial in cardiac disease. For example, overexpression of the type-VI adenylate cyclase in the hearts of $G_{\alpha q}$ transgenic mice improved contractile function and increased survival of these mice (Roth et al. 1999, 2002). Further, the PDE3 inhibitor milrinone has been used for several years as a treatment for cardiac disease (Shin et al. 2007). This inhibitor increases cAMP concentration in the heart, resulting in an increase in contractility.

These seemingly contradictory results are hard to reconcile until one begins to look at the microdomains of cAMP signaling in the heart. When one compares the cAMP content in both the cytosolic and particulate fraction from the normal to the failing human myocardium, the reduction in cAMP content in the failing heart is much more pronounced in the particulate fraction than in the cyosolic (Movsesian 2004). Furthermore, phosphorylation of some PKA targets such as RyR2 is increased, while phosphorylation of other targets such as phospholamban is decreased in heart failure (Schwinger et al. 1999; Marx et al. 2000). Therefore, one goal for the treatment for heart disease might be to target individual PKA complexes, thereby affecting the phosphorylation of individual substrates. Given that AKAPs are distinguished by their binding partners and locations, it may be possible to invent therapeutic strategies that specifically manipulate AKAPs of interest.

To begin, perhaps the most elegant strategy for differential inhibition of PKA phosphorylation would be the expression of high-affinity Ht31-like molecules that would displace PKA from the AKAP complex. For example, Ht31 expression in myocytes was able to inhibit β-adrenergic-induced PKA phosphorylation of troponin I and myosin basic protein, but not β-adrenergic-induced phospholamban phosphorylation (Fink et al. 2001). One might imagine that Ht31 expression in the failing heart might reverse RyR2 hyper-phosphorylation, without further decrease of phospholamban phosphorylation. This concept was supported by the work of Pare et al. (2005). Disruption of PKA association with mAKAPβ in myocytes inhibited adrenergic-induced cellular hypertrophy. Other AKAPs may also be potential targets for this approach. For example, AKAP15/18-bound PKA is responsible for β-agonist-induced potentiation of the L-type calcium channel, such that direct PKA phosphorylation increases the open probability of the channel (Fraser et al. 1998; Gray et al. 1998). Calcium channel blockers are currently used for the treatment of left ventricular hypertrophy (Onose et al. 2001). Therefore, displacing PKA bound to AKAP15/18 would reduce β-AR stimulation of the L-type calcium channel, potentially having a similar effect in vivo as channel blockers.

AKAPs localize PKA to specific subcellular domains through their unique targeting domains, resulting in the efficient phosphorylation of local PKA substrates (see Fig. 1). Therefore, disruption of AKAP localization is a second potential therapeutic approach to limit the hyper-phosphorylation of PKA substrates in disease states. For example, mAKAPβ is directed to the nuclear envelope through binding to the integral membrane protein nesprin-1α (Kapiloff et al. 1999; Pare et al. 2004). Overexpression of the mAKAPβ spectrin-repeat targeting domain in cardiac myocytes displaces mAKAPβ from nesprin-1α and the nuclear envelope. Importantly, displacement of mAKAPβ from its normal cellular location is sufficient to block the induction of myocyte hypertrophy (Dodge-Kafka et al. 2005; Pare et al. 2005). Again, AKAP15/18 may be another useful example. AKAP15/18 associates with the L-type calcium channel through a leucine zipper motif (Hulme et al. 2003). A peptide mimicking this binding domain can compete with AKAP15/18 for channel binding, inhibiting β-adrenergic stimulation of the channel (Hulme et al. 2003).

Although AKAPs are defined by their ability to bind and localize PKA, most AKAPs are multivalent scaffolds that bind components of other signaling pathways (see Fig. 1). Just like Ht31 peptide will displace PKA from its anchoring proteins, other peptides may be derived that will displace other signaling enzymes from AKAP complexes. For example, the mAKAPβ signalosome includes ERK5, which is involved in the induction of cardiac disease (Takahashi et al. 2005). Displacement of ERK5 from the mAKAPβ signalosome by overexpression of a mAKAPβ-derived binding peptide may prove useful to inhibit the cardiac disease.

Finally, the advent of RNA interference foretells an era in which genes may be selectively inhibited in patients at will. For example, one might imagine that future treatment of heart failure will include the introduction of mAKAPβ-specific small interfering RNA that will attenuate the development of myocyte hypertrophy.

6 Future Directions

Although the second messenger cAMP and its major target PKA have been extensively studied, the idea that localized domains of cAMP result in the discrete activation of select pools of PKA is still relatively novel. Multiple lines of research have implicated the role of AKAPs in providing the molecular architecture of these microdomains. However, the analysis of the physiological significance of AKAPs is still incomplete. Undoubtedly, this field will benefit from genetic approaches utilizing knockout mice of the various AKAPs. These studies should not only provide insight into the molecular mechanisms of AKAP function, but also into the physiological roles of the different AKAP scaffolds. In particular, AKAP knock-out mice will be useful in determining whether individual AKAPs are potential targets for therapeutic intervention. These studies will complement existing information using the Ht31 peptide to globally disrupt PKA anchoring in a multitude of cells.

The paradox seen in the heart that cAMP can lead to both “good” and “bad” outcomes highlights the importance of cAMP micodomains and the ability of the cell to main these domains under stress conditions. Thus, one can imagine restoring the balance of cAMP signaling by modulating these localized domains. AKAPs would provide an ideal mechanism for the management of cAMP microdomains and may provide novel therapeutic strategies against heart failure. However, to utilize this approach would require an in-depth analysis of AKAP targets and their phoshorylation status in the heart disease.

The disadvantage of targeting PKA/AKAP interactions is that all known inhibitory reagents are not specific for a particular AKAP. Therefore, the development of anchoring inhibitory peptides that affect only one specific AKAP would be of great interest. For example, Alto et al. (2003) used both computer-based modeling and peptide-array analysis to design a peptide that is 10,000-fold more selective for disrupting AKAP/RII interactions over RI. Importantly, the complete understanding of the AKAP complex and its role in the regulation of cell biology will allow for the design of novel drugs, providing potential therapeutic strategies for the treatment and management of many diseases.

References

Adamson PB, Gilbert, EM (2006) Reducing the risk of sudden death in heart failure with beta-blockers. J Card Fail 12(9):734–746

Alto NM, Soderling SH et al (2003) Bioinformatic design of A-kinase anchoring protein-in silico: a potent and selective peptide antagonist of type II protein kinase A anchoring. Proc Natl Acad Sci USA 100(8):4445–4450

Antos CL, Frey N et al (2001) Dilated cardiomyopathy and sudden death resulting from constitutive activation of protein kinase a. Circ Res 89(11):997–1004

Bisognano JD, Weinberger HD et al (2000) Myocardial-directed overexpression of the human beta(1)-adrenergic receptor in transgenic mice. J Mol Cell Cardiol 32(5):817–830

Brunton LL, Hayes JS et al (1979) Hormonally specific phosphorylation of cardiac troponin I and activation of glycogen phosphorylase. Nature 280(5717):78–80

Carlisle Michel JJ, Dodge KL et al (2004) PKA-phosphorylation of PDE4D3 facilitates recruitment of the mAKAP signalling complex. Biochem J 381(Pt 3):587–592
Carr DW, Hausken ZE et al (1992) Association of the type II cAMP-dependent protein kinase with a human thyroid RII-anchoring protein. Cloning and characterization of the RII-binding domain. J Biol Chem 267(19):13376–13382
Coghlan VM, Perrino BA et al (1995) Association of protein kinase A and protein phosphatase 2B with a common anchoring protein. Science 267(5194):108–111
Colledge M, Dean RA et al (2000) Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. Neuron 27(1):107–119
Corbin JD, Sugden PH et al (1977) Compartmentalization of adenosine 3′:5′-monophosphate and adenosine 3′:5′-monophosphate-dependent protein kinase in heart tissue. J Biol Chem 252(11):3854–3861
Dodge K, Scott JD (2000) AKAP79 and the evolution of the AKAP model. FEBS Lett 476(1–2):58–61
Dodge KL, Khouangsathiene S et al (2001) mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module. EMBO J 20(8):1921–1930
Dodge-Kafka KL, Soughayer J et al (2005) The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. Nature 437(7058):574–578
Engelhardt S, Hein L et al (1999) Progressive hypertrophy and heart failure in beta1-adrenergic receptor transgenic mice. Proc Natl Acad Sci USA 96(12):7059–7064
Fink MA, Zakhary DR et al (2001) AKAP-mediated targeting of protein kinase a regulates contractility in cardiac myocytes. Circ Res 88(3):291–297
Fraser ID, Tavalin SJ et al (1998) A novel lipid-anchored A-kinase anchoring protein facilitates cAMP-responsive membrane events. EMBO J 17(8):2261–2272
Fraser ID, Cong M et al (2000) Assembly of an A kinase-anchoring protein-beta(2)-adrenergic receptor complex facilitates receptor phosphorylation and signaling. Curr Biol 10(7):409–412
Gray PC, Johnson BD et al (1998) Primary structure and function of an A kinase anchoring protein associated with calcium channels. Neuron 20(5):1017–1026
Hayes JS, Brunton LL et al (1979) Hormonally specific expression of cardiac protein kinase activity. Proc Natl Acad Sci USA 76(4):1570–1574
Hayes JS, Brunton LL et al (1980) Selective activation of particulate cAMP-dependent protein kinase by isoproterenol and prostaglandin E1. J Biol Chem 255(11):5113–5119
Hoffmann R, Baillie GS et al (1999) The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579. EMBO J 18(4):893–903
Hulme JT, Lin TW et al (2003) Beta-adrenergic regulation requires direct anchoring of PKA to cardiac CaV1.2 channels via a leucine zipper interaction with A kinase-anchoring protein 15. Proc Natl Acad Sci USA 100(22):13093–13098
Iwase M, Uechi M et al (1997) Cardiomyopathy induced by cardiac Gs alpha overexpression. Am J Physiol 272(1 Pt 2):H585–H589
Jurevicius J, Fischmeister R (1996) cAMP compartmentation is responsible for a local activation of cardiac Ca2+ channels by beta-adrenergic agonists. Proc Natl Acad Sci USA 93(1):295–299
Kapiloff MS, Schillace RV et al (1999) mAKAP: an A-kinase anchoring protein targeted to the nuclear membrane of differentiated myocytes. J Cell Sci 112(Pt 16):2725–2736
Kapiloff MS, Jackson N et al (2001) mAKAP and the ryanodine receptor are part of a multi-component signaling complex on the cardiomyocyte nuclear envelope. J Cell Sci 114(Pt 17):3167–3176
Klauck TM, Faux MC et al (1996) Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science 271(5255):1589–1592
Lehnart SE, Wehrens XH et al (2005) Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias. Cell 123(1):25–35
Lester LB, Langeberg LK et al (1997) Anchoring of protein kinase A facilitates hormone-mediated insulin secretion. Proc Natl Acad Sci USA 94(26):14942–14947

Marx SO (2003) Ion channel macromolecular compexes in the heart. J Mol Cell Cardiol 35:37–44
Marx SO, Reiken S et al (2000) PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. Cell 101(4):365–376
Michel JJ, Townley IK et al (2005) Spatial restriction of PDK1 activation cascades by anchoring to mAKAPalpha. Mol Cell 20(5):661–672
Mongillo M, McSorley T et al (2004) Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. Circ Res 95(1):67–75
Movsesian MA (2004) Altered cAMP-mediated signalling and it role in the pathogenesis of dilated cardiomyopathy. Cardiovasc Res 62:450–459
Newlon MG, Roy M et al (2001) A novel mechanism of PKA anchoring revealed by solution structures of anchoring complexes. EMBO J 20(7):1651–1662
Onose Y, Oki T et al (2001) Effect of cilnidipine on left ventricular diastolic function in hypertensive patients as assessed by pulsed Doppler echocardiography and pulsed tissue Doppler imaging. Jpn Circ J 65(4):305–309
Pare GC, Easlick JL et al (2004) Nesprin-1a contributes to the targeting of mAKAP to the cardiac myocyte nuclear envelope. Exp Cell Res 303:388–399
Pare GC, Bauman AL et al (2005) The mAKAP complex participates in the induction of cardiac myocyte hypertrophy by adrenergic receptor signaling. J Cell Sci 118(Pt 23):5637–5646
Rosenmund C, Carr DW et al (1994) Anchoring of protein kinase A is required for modulation of AMPA/kainate receptors on hippocampal neurons. Nature 368(6474):853–856
Roth DM, Gao MH et al (1999) Cardiac-directed adenylyl cyclase expression improves heart function in murine cardiomyopathy. Circulation 99(24):3099–3102
Roth DM, Bayat H et al (2002) Adenylyl cyclase increases survival in cardiomyopathy. Circulation 105(16):1989–1994
Ruehr ML, Russell MA et al (2003) Targeting of protein kinase A by muscle A kinase-anchoring protein (mAKAP) regulates phosphorylation and function of the skeletal muscle ryanodine receptor. J Biol Chem 278(27):24831–24836
Salpeter SR, Ormiston TM et al (2004) Cardiovascular effects of beta-agonists in patients with asthma and COPD: a meta-analysis. Chest 125(6):2309–2321
Schwinger RH, Munch G et al (1999) Reduced Ca(2+)-sensitivity of SERCA 2a in failing human myocardium due to reduced serin-16 phospholamban phosphorylation. J Mol Cell Cardiol 31(3):479–491
Scott JD (1991) Cyclic nucleotide-dependent protein kinases. Pharmacol Ther 50(1):123–145
Sette C, Conti M (1996) Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. Involvement of serine 54 in the enzyme activation. J Biol Chem 271(28):16526–16534
Shin DD, Brandimarte F et al (2007) Review of current and investigational pharmacologic agents for acute heart failure syndromes. Am J Cardiol 99(2A):S4–S23
Stokka AJ, Gesellchen F et al (2006) Characterization of A-kinase-anchoring disruptors using a solution-based assay. Biochem J 400(3):493–499
Takahashi N, Saito Y et al (2005) Hypertrophic responses to cardiotrophin-1 are not mediated by STAT3, but via a MEK5-ERK5 pathway in cultured cardiomyocytes. J Mol Cell Cardiol 38(1):185–192
Taylor SS, Buechler JA et al (1990) cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu Rev Biochem 59:971–1005
Westphal RS, Tavalin SJ et al (1999) Regulation of NMDA receptors by an associated phosphatase-kinase signaling complex. Science 285(5424):93–96
Westphal RS, Soderling SH et al (2000) Scar/WAVE-1, a Wiskott-Aldrich syndrome protein, assembles an actin-associated multi-kinase scaffold. EMBO J 19(17):4589–600
Zaccolo M (2004) Use of chimeric fluorescent proteins and fluorescence resonance energy transfer to monitor cellular responses. Circ Res 94(7):866–873

Role of Ena/VASP Proteins in Homeostasis and Disease

G. Pula and M. Krause(✉)

Contents

1 Domain Structure and Functions of the Ena/VASP Proteins 40
1.1 EVH1 Domain Structure and Function 40
1.2 Structure and Function of the Proline-Rich Domain 41
1.3 EVH2 Domain Structure and Function 41
2 Protein Complexes Containing Ena/VASP Proteins 43
2.1 Listeria 43
2.2 Focal Adhesions and Stress Fibres 43
2.3 Lamellipodia and Filopodia 44
2.4 Cell–Cell Junctions (Endothelia/Epithelia) 45
2.5 Immunological Synapse 45
2.6 Phagosome 46
3 Regulation of Ena/VASP Proteins by Phosphorylation 46
3.1 PKA- and PKG-Dependent Phosphorylation of Ena/VASP Proteins 46
3.2 PKA- and PKG-Independent Phosphorylation of Ena/VASP Proteins 47
3.3 Regulation of Ena/VASP Protein Functions by Phosphorylation 48
4 Pathophysiologic Relevance of Ena/VASP Proteins 49
4.1 Thrombosis 49
4.2 Cancer 52
4.3 Arteriosclerosis 54
4.4 Cardiomyopathy 54
4.5 Nephritis. 55
5 Ena/VASP Proteins and Drug Discovery 56
References. 57

Abstract The actin cytoskeleton is required for many important processes during embryonic development. In later stages of life, important homeostatic processes depend on the actin cytoskeleton, such as immune response, haemostasis and blood vessel preservation. Therefore, the function of the actin cytoskeleton must be tightly regulated, and aberrant regulation may cause disease. A growing number of proteins

M. Krause
Randall Division of Cell and Molecular Biophysics, King's College London, New Hunt's House, Guy's Campus, London SE1 1UL, UK
matthias.krause@kcl.ac.uk

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

have been described to bind and regulate the actin cytoskeleton. Amongst them, Ena/VASP proteins function as anti-capping proteins, thereby directly modulating the actin ultrastructure. Ena/VASP function is regulated by their recruitment into protein complexes downstream of plasma membrane receptors and by phosphorylation. As regulators of the actin ultrastructure, Ena/VASP proteins are involved in crucial cellular functions, such as shape change, adhesion, migration and cell–cell interaction and hence are important targets for therapeutic intervention. In this chapter, we will first describe the structure, function and regulation of Ena/VASP proteins. Then, we will review the involvement of Ena/VASP proteins in the development of human diseases. Growing evidence links Ena/VASP proteins to important human diseases, such as thrombosis, cancer, arteriosclerosis, cardiomyopathy and nephritis. Finally, present and future perspectives for the development of therapeutic molecules interfering with Ena/VASP-mediated protein–protein interactions are presented.

1 Domain Structure and Functions of the Ena/VASP Proteins

The Ena/VASP family in vertebrates consists of three family members: VASP (vasodilator stimulated phosphoprotein), Mena (mammalian enabled) and EVL (Ena VASP-like). Both *C. elegans* and *Drosophila* harbour only one ortholog, *unc-34* and *ena*, respectively. All Ena/VASP proteins have the same domain structure: an N-terminal Ena/VASP homology 1 (EVH1) domain, a central proline-rich domain and a C-terminal Ena/VASP homology 2 (EVH2) domain (Halbrugge and Walter 1989; Gertler et al. 1989, 1996).

1.1 EVH1 Domain Structure and Function

The EVH1 domain adopts a structure similar to pleckstrin homology (PH) domains. However, EVH1 domains do not bind to phospholipids. Instead, they mediate specific protein–protein interactions with proline-rich short peptide motifs. This interaction is important for the recruitment of EVH1 domain-harbouring proteins to specific subcellular locations and to membrane receptor complexes. According to their ligand specificity, they can be divided into four classes. Class-I EVH1 domains bind to a peptide with the consensus sequence (D/E)-(F/W/Y/L)-P-P-P-P-X-(D/E)-(D/E)-(D/E)-L. Class-II EVH1 domains found in Homer/Vesl proteins bind to the consensus sequence PPXX(F/W/Y)XD. Class-III EVH1 domains are found in N-WASP and WASP, and are also called WH1 domains. They mediate specific binding to a considerably longer EVH1-ligand found in WIP and WIP-related proteins (Volkman et al. 2002; Zettl and Way 2002; Peterson et al. 2007). The last class consists of the EVH1 domains found in Spred proteins, for which there are no known ligands. However, the crystal structure of the Spred EVH1 domain indicates that it represents a fourth distinct EVH1 class (Bundschu et al. 2006; Harmer et al. 2005; Wakioka et al. 2001). In this review, we will

concentrate on the class-I EVH1 domain that is found exclusively in Ena/VASP proteins.

The EVH1 domain is composed of two anti-parallel beta sheets followed by a C-terminal alpha helix. The proline-rich peptide adopts a left-handed poly-L-proline II helix that binds primarily to aromatic amino acids within a deep grove formed by beta-strands within the EVH1 domain (Fedorov et al. 1999; Prehoda et al. 1999). This binding modus is similar to the interaction of SH3 and WW domains and Profilin with their proline-rich ligands. However, the binding surfaces in these domains are flat compared to the deep grove found in the EVH1 domain. Furthermore, specificity in the binding of the class-I EVH1 domain to its ligands is ensured by the aromatic residue N-terminal of the poly-L-proline helix and the acidic residues flanking both sides of it (Niebuhr et al. 1997; Carl et al. 1999; Fedorov et al. 1999; Prehoda et al. 1999; Ball et al. 2000). EVH1 class-I domain binding sites are found in several proteins (see Table 1) and mediate recruitment of Ena/VASP proteins to specific subcellular sites and receptor complexes.

1.2 Structure and Function of the Proline-Rich Domain

The central proline-rich domain is the least conserved region within the Ena/VASP proteins. However, all Ena/VASP proteins harbour proline-rich binding sites that confer binding to profilin within the central region (Reinhard et al. 1995; Gertler et al. 1996; Lambrechts et al. 2000). Mena differs most from VASP and EVL within this domain because it contains a highly charged region of unknown function directly after the EVH1 domain (Gertler et al. 1996). Furthermore, different isoforms of Mena are expressed, and the additional exons are found within the central region. Mena has three additional exons, of which the longest is exclusively present in the 140-kDa neuronal Mena isoform (Gertler et al. 1996). Another Mena isoform, Mena(S), is unique among the Ena/VASP proteins by omitting most of the profilin-binding sites (Tani et al. 2003).

The central proline-rich domain of Ena/VASP proteins also harbours binding sites for SH3 and WW domain-containing proteins. It has been shown that EVL can interact with the SH3 domain of Lyn, nSrc, c-Abl, Spectrin, and Tuba (Lambrechts et al. 2000; Rotter et al. 2005; Salazar et al. 2003; Bournier et al. 2006). VASP interacts with the SH3 domain of LASP-1 and Tuba (Salazar et al. 2003; Keicher et al. 2004). An interaction of Mena with the SH3 domains of c-Abl, Tuba, and Src has been identified (Gertler et al. 1996; Salazar et al. 2003). Finally, the WW domain of FE65 can directly interact with proline-rich peptides in the central region of Mena and EVL (Ermekova et al. 1997; Lambrechts et al. 2000).

1.3 EVH2 Domain Structure and Function

The EVH2 domain contains binding sites for G-actin (Walders-Harbeck et al. 2002; Chereau and Dominguez 2006) and F-actin (Bachmann et al. 1999; Huttelmaier et al. 1999; Harbeck et al. 2000). The most C-terminal part of the EVH2 domain

Table 1 EVH1 class-I-domain-binding sites found in different ligand proteins

Protein (reference)	Binding sites
Listeria monocytogenes **ActA** (Niebuhr et al. 1997)	DFPPPPTDEEL EFPPPPTDEEL EFPPPPTEDEL DFPPIPTEEEL
Human Fat-1 (Moeller et al. 2004; Niebuhr et al. 1997; Tanoue and Takeichi 2004)	DFPPPPED
Human Fyb/SLAP (Krause et al. 2000)	IFPPPPDDDIYDG
Human Lamellipodin (Krause et al. 2004)	QFPPPPTPPAME DFPPPPPESSL VFPPPPPSPVP EFPSPPSDSDF DFPPPPPETDL DLPLPPIE MALPPPPPEL
Migfilin (Zhang et al. 2006)	DLLPPPPPPPPVLLPSEEE
Palladin (Boukhelifa et al. 2004)	PFPPPPPAFPEL DVFPLPPPPPPLP
Human RIAM (Lafuente et al. 2004)	DDLPPPPAD DLPLPPPPPEPL SLPPPPPVRR DDFLPPPPPPPPLDD ELPPPPPD DFVPPPPPSYA ELPPPPPPPAP
***Drosophila* Robo** (Bashaw et al. 2000)	EFLPPPPEHPPP
Human Vinculin (Brindle et al. 1996; Reinhard et al. 1996)	PDFPPPPPDLEQLR
Human Xin (van der Ven et al. 2006)	EDLPLPPPPALEDL
Human Zyxin (Drees et al. 2000)	EDFPLPPPPLAGDG GAFPPPPPPMIEEP ESFPPAPLEEEIFP EIFPSPPPPPEEEG

contains a coiled-coil motif that mediates tetramerisation of Ena/VASP proteins (Bachmann et al. 1999; Barzik et al. 2005; Kuhnel et al. 2004; Zimmermann et al. 2002). The main molecular function of Ena/VASP proteins is to allow elongation of actin filaments by protecting them from being capped by capping protein. The G- and F-actin-binding sites and the coiled-coil motif are required for this function

(Barzik et al. 2005; Bear et al. 2002). This anti-capping activity is potentiated by the recruitment of profilin to its binding site in the central proline-rich region (Barzik et al. 2005).

2 Protein Complexes Containing Ena/VASP Proteins

2.1 Listeria

The facultative intracellular pathogen *Listeria monocytogenes* can invade mammalian cells and once in the cytoplasm can use the host actin cytoskeleton to propel itself forward (Frischknecht and Way 2001; Gouin et al. 2005). When it reaches the plasma membrane, it can push into a neighbouring cell, thereby enabling cell-to-cell spread of this pathogen. The recruitment of host Ena/VASP proteins by FP4 motifs of the surface protein ActA is required for intracellular motility of *Listeria*. This uncovered for the first time the fact that Ena/VASP proteins are essential for the regulation of actin polymerisation (Chakraborty et al. 1995; Smith et al. 1996). Importantly, ActA can also bind to and activate the Arp2/3 complex. The activation of this seven-protein complex leads to the nucleation of new actin filaments from the sides of existing filaments, resulting in the formation of a dendritic array of actin filaments. The combination of the function of the Arp2/3 complex and Ena/VASP results in the formation of an F-actin tail that propels *Listeria* through the host cell cytoplasm (Frischknecht and Way 2001; Gouin et al. 2005).

2.2 Focal Adhesions and Stress Fibres

In fibroblastic cells and platelets, Ena/VASP proteins are recruited to large protein complexes that anchor long anti-parallel bundles of actin filaments (stress fibres) to cell-matrix adhesion sites called focal adhesions. Several FP4 motif-containing proteins such as zyxin, vinculin, palladin, migfillin and RIAM localise to focal adhesions and are required to recruit Ena/VASP proteins to these sites (Brindle et al. 1996; Reinhard et al. 1996; Drees et al. 2000; Boukhelifa et al. 2004; Han et al. 2006; Jenzora et al. 2005; Zhang et al. 2006). Interestingly, vinculin can recruit both Ena/VASP and Arp2/3 (see below) to focal adhesions where barbed end growth has been observed (DeMali et al. 2002; Machesky and Hall 1997). Zyxin and palladin are also able to recruit Ena/VASP proteins into a periodical pattern along stress fibres. Interestingly, VASP overexpression in endothelial cells led to an increased formation of stress fibres (Price and Brindle 2000). However, Ena/VASP proteins are not required for stress fibre formation, and the precise function of Ena/VASP in this process is unknown (Bear et al. 2000).

2.3 Lamellipodia and Filopodia

Binding of the EVH1 domain to FP4-containing proteins is sufficient for the recruitment of Ena/VASP proteins to the very edge of lamellipodia and filopodia (Bear et al. 2001). So far, only three proteins with FP4 motifs have been identified that co-localise with Ena/VASP at this location: lamellipodin (Lpd), RIAM and the cadherin Fat-1 (Jenzora et al. 2005; Krause et al. 2004; Lafuente et al. 2004; Moeller et al. 2004; Tanoue and Takeichi 2004). Lpd, RIAM and the sole ortholog in *C. elegans mig-10* belong to the MRL (MIG10, RIAM, Lpd) protein family. They all harbour a Ras-association and a PH domain. Both Lpd and RIAM contain several FP4 motifs through which they directly interact with Ena/VASP proteins (Krause et al. 2004; Lafuente et al. 2004). Lpd interacts with active Ras, whereas RIAM preferentially binds to active Rap1 (Krause et al. 2004; Lafuente et al. 2004; Rodriguez-Viciana et al. 2004). The PH domain of Lpd binds specifically to PI(3,4)P2, a phosphoinositol that is produced by dephosphorylation of the PI3 kinase product PI(3,4,5)P3 by Src homology 2 domain-containing inositol 5-phosphatase (SHIP), and it localises at PDGF-induced membrane ruffles. The leading edge localisation of Lpd is independent of Ena/VASP proteins, indicating that Lpd functions upstream of Ena/VASP. Importantly, knockdown of Lpd expression led to impairment of lamellipodia formation. Conversely, overexpression of Lpd led to faster lamellipodial protrusion and ruffle formation, a phenocopy of Ena/VASP overexpression. This Lpd overexpression phenotype was dependent on Ena/VASP, indicating that Lpd function is mediated by Ena/VASP (Krause et al. 2004).

Fat1 cadherin belongs to a distinct subfamily of transmembrane cadherin cell–cell adhesion proteins and is also found at the extremity of lamellipodia and filopodia. This localisation is independent of Ena/VASP proteins. Fat1 cadherins harbour FP4 motifs mediating direct interaction with Ena/VASP proteins. Knockdown of Fat1 expression resulted in decreased lamellipodial protrusion velocity, reduced speed of cell migration in a scratch (wound) healing assay and disturbed cell–cell contacts (see below). However, Ena/VASP recruitment to the leading edge was only reduced, but not abolished, indicating that Fat1 is not the only mechanism for recruitment of Ena/VASP (Moeller et al. 2004; Tanoue and Takeichi 2004).

Our current hypothesis for the molecular function of Ena/VASP proteins at the extremity of lamellipodia and filopodia is that Ena/VASP proteins can bind at or near the barbed end of actin filaments and protect them from being capped by capping proteins. These Ena/VASP-protected actin filaments can rapidly elongate. When Ena/VASP are overexpressed or artificially targeted to the leading edge, this leads to the creation of an array of long, less branched actin filaments. In fibroblasts, this leads to fast protruding, but unstable lamellipodia, whereas in the growth cones of primary neurons, this induces the formation of multiple filopodia. Conversely, when Ena/VASP localisation to the leading edge is prevented, this leads to an array of very short, highly branched actin filaments and slower, but productive (more adherent) lamellipodial protrusion in fibroblasts and highly reduced filopodia formation in primary neurons (Bear et al. 2002; Lebrand et al. 2004).

2.4 Cell–Cell Junctions (Endothelia/Epithelia)

The FP4 motif containing proteins zyxin, vinculin, migfilin and Fat1 colocalizes with Ena/VASP proteins at cell–cell junctions (Geiger 1979; Crawford and Beckerle 1991; Vasioukhin et al. 2000; Moeller et al. 2004; Tanoue and Takeichi 2004; Gkretsi et al. 2005). E-cadherin homophilic ligation is necessary to recruit Ena/VASP to cell–cell adhesions (Scott et al. 2006). Importantly, actin assembly at cell–cell contacts requires Ena/VASP activity (Baum and Perrimon 2001; Scott et al. 2006). Genetic evidence suggests that *Ena* and the tyrosine kinase *Abl* negatively regulate each other in the embryonic nervous system and during epithelial morphogenesis of *Drosophila* (Gertler et al. 1990, 1995; Baum and Perrimon 2001; Grevengoed et al. 2001). Furthermore, *Ena* genetically interacts with the adherens junction protein Armadillo (the *Drosophila* beta-catenin ortholog) during epithelial morphogenesis (Grevengoed et al. 2001). Taken together, this indicates that Ena/VASP proteins play an important role in epithelial cell–cell contact formation.

2.5 Immunological Synapse

The immunological synapse is a specialised cell–cell adhesion site between activated T-cells and antigen-presenting cells (Tseng and Dustin 2002). Activation of the T-cell receptor induces activation and recruitment of several tyrosine kinases including ZAP70, Lck and Fyn. The phosphorylation and subsequent recruitment of the Fyb/SLAP/ADAP, Slp-76, LAT and Nck adapter molecules results in the assembly of a protein complex that also includes WASP and Ena/VASP proteins at the immunological synapse (Jordan et al. 2003; Krause et al. 2000). Fyb/SLAP/ADAP is a haematopoietic-specific adapter molecule that harbours an FP4 motif that directly interacts with Ena/VASP proteins. Importantly, recruitment of both Arp2/3 complex, presumably through WASP, and Ena/VASP proteins was required for the induction of actin polymerization at the T-cell/anti-CD3-coated bead interface, mimicking antigen-presenting cells (Krause et al. 2000). Similarly, the bacterial pathogen *Listeria monocytogenes* requires the recruitment of both Ena/VASP and the Arp2/3 complex to its ActA surface protein for actin polymerization and efficient intracellular motility (Frischknecht and Way 2001). This showed for the first time that in mammalian cells both Ena/VASP and Arp2/3 are required for efficient actin polymerisation downstream of a cell surface receptor (Krause et al. 2000). In contrast to the bacterial protein ActA, the recruitment of Ena/VASP and Arp2/3 is mediated through the assembly of a protein complex, which allows spatial and temporal regulation. Obviously, this is not essential for *Listeria*, which requires the constitutive induction of actin polymerization for its own motility, cell-to-cell spread and pathogenicity.

Fyb/SLAP/ADAP knockout T-cells showed impaired proliferation and cytokine production in response to T-cell receptor stimulation. T-cell receptor stimulation is

coupled to an increase in integrin-dependent adhesion. Fyb/SLAP/ADAP knockout T-cells are defective in this response, indicating that this protein may couple TCR stimulation to integrin activation and increased adhesion. Surprisingly, actin polymerization at the T-cell antigen-presenting cell contact site was not defective (Griffiths et al. 2001; Peterson et al. 2001). However, Ena/VASP proteins may be recruited in Fyb/SLAP/ADAP KO T-cells to the immunological synapse through RIAM (Lafuente et al. 2004). Whether RIAM is part of the protein complex downstream of the T-cell receptor is currently not known. Interestingly, RIAM overexpression in Jurkat T-cells led to increased adhesion and integrin activation. This suggests that both RIAM and Fyb/SLAP/ADAP may be required for Ena/VASP recruitment to the immunological synapse and for the activation of integrins downstream of the T-cell receptor.

2.6 Phagosome

Phagocytosis is an essential component of the immune response in which phagocytes recognise and engulf foreign material. Opsonisation of foreign particles by immunoglobulins enables the recognition by Fc-receptors (Allen and Aderem 1996). This leads to an activation of Fc-receptors and the recruitment of a complex similar to the complex found downstream of the T-cell receptor: Slp-76, Fyb/SLAP/ADAP, Ena/VASP, Nck and WASP (Coppolino et al. 2001; Niedergang and Chavrier 2004). Subsequently, actin polymerisation and engulfment of the particle are induced. Importantly, inhibition of the recruitment of Ena/VASP proteins led to an impairment in actin polymerisation and phagocytosis of opsonised sheep red blood cells (Coppolino et al. 2001).

3 Regulation of Ena/VASP Proteins by Phosphorylation

3.1 PKA- and PKG-Dependent Phosphorylation of Ena/VASP Proteins

Ena/VASP proteins show tightly regulated and reversible phosphorylation. VASP was initially isolated from human platelets as a 46-kDa membrane-associated protein that is phosphorylated by PKA and PKG (Halbrugge and Walter 1989; Halbrugge et al. 1990). Three phosphorylation sites were identified on VASP: Ser157, Ser239 and Thr274, all of which can be phosphorylated by either PKA or PKG (Butt et al. 1994). The phosphorylation of VASP at Ser157 results in the apparent mobility shift from 46 to 50 kDa observed on SDS-PAGE. Ser157 is the preferred site of phosphorylation for PKA, which phosphorylates VASP at this site with faster kinetics than at Ser239 either in vitro or in vivo. Ser239 is the preferred phosphorylation site for PKG in vitro. Experiments in intact human platelets showed that PKG

phosphorylates either Ser157 or Ser239 with similar kinetics, but the level of Ser157 phosphorylation is only around 50% of that induced by PKA activation (Smolenski et al. 1998). This and further evidence suggested a predominant role of PKG in the Ser239 phosphorylation of VASP in platelets and other cells (Ibarra-Alvarado et al. 2002; Cook and Haynes 2007). On the other hand, opposing evidence has appeared in the literature (Li et al. 2003), which reports that in human platelets cGMP-dependent phosphorylation of VASP at Ser239 is predominantly PKA-dependent. Thr274 is phosphorylated by both PKA and PKG, but only following phosphorylation of VASP on Ser157 and Ser239.

PKA is responsible also for the phosphorylation of other members of the Ena/VASP family. EVL is phosphorylated on Ser156 (corresponding to VASP Ser157) in a PKA-dependent manner both in vitro and in vivo (Lambrechts et al. 2000). As for VASP Ser157, the phosphorylation of EVL on Ser156 induces a mobility shift in SDS-PAGE. The other two phosphorylation sites of VASP (Ser239 and Thr278) are not conserved in EVL, where the corresponding residues are instead mutated to glutamine and alanine, respectively. Mena, the other mammalian member of the Ena/VASP family, is also phosphorylated in a PKA-dependent manner (Gertler et al. 1996). Mena contains two serine-phosphorylation sites corresponding to VASP Ser157 and Ser239, but not the Thr-phosphorylation site, and their phosphorylation leads to a mobility shift similar to the one observed for VASP (Gertler et al. 1996; Lebrand et al. 2004).

3.2 PKA- and PKG-Independent Phosphorylation of Ena/VASP Proteins

In addition to PKA and PKG, PKC has also been reported to phosphorylate VASP at the Ser157 site. Chitaley et al. (2004) reported PKA- and PKG-independent phosphorylation of Ser157, but not Ser239, in cultured rat smooth muscle cells from the aorta. PKC appeared to be the kinase responsible for this post-translational modification, since phosphorylation was induced by phorbol 12-myristate 13-acetate (PMA) and inhibited by specific PKC inhibition. Moreover, Chitaley et al. (2004) showed that recombinant PKCα directly phosphorylated VASP at the residue Ser157. Wentworth et al. (2006) also showed PKC-dependent Ser157 phosphorylation of VASP. These authors reported PMA-dependent phosphorylation of VASP Ser157 in human platelets and inhibition of it by bisindolylmaleimide I (BIM I), a specific PKC inhibitor. Moreover, the activation of platelets by thrombin, a physiological platelet agonist, induced VASP Ser157 phosphorylation, which was partially PKC dependent. Finally, Pula et al. (2006) reported negative regulation of VASP Ser157 phosphorylation by PKCδ. The molecular mechanism of this regulation of VASP phosphorylation is not entirely clear, but it does not involve the control of PKA or PKG activity.

Finally, in contrast to the other members of the family, Mena is tyrosine-phosphorylated (Gertler et al. 1996). *Ena*, the *Drosophila* ortholog of Mena, is

tyrosine-phosphorylated by Abelson tyrosine kinase (Comer et al. 1998). Subsequently, Mena has also been reported to be phosphorylated by c-Abl kinase at the residue Tyr296, with Abi-1 (Abl interactor 1) binding to Mena and dramatically enhancing its tyrosine phosphorylation by c-Abl (Tani et al. 2003).

3.3 Regulation of Ena/VASP Protein Functions by Phosphorylation

The phosphorylation of Ena/VASP proteins has different functional consequences. First of all, the regulation of actin filament structure by Ena/VASP proteins is regulated by phosphorylation. In vitro phosphorylation of VASP resulted in the down-regulation of its filament bundling and anti-capping activities (Harbeck et al. 2000; Barzik et al. 2005). Profilin enhances VASP anti-capping activity in a dose-dependent manner. Phosphorylation also inhibits VASP anti-capping activity in the presence of profilin (Barzik et al. 2005). Similarly to VASP, PKA-dependent phosphorylation of EVL was shown to impair the ability of this protein to enhance actin polymerisation (Lambrechts et al. 2000). Finally, contrasting evidence has been reported regarding the regulation of VASP's ability to bind actin. PKA-dependent phosphorylation of VASP on Ser157 was reported to potentiate F-actin binding by Laurent et al. (1999), whilst Harbeck et al. (2000) described a reduction of F-actin binding as a consequence of the same post-translational modification. A possible explanation for this contradiction lies in the different salt concentrations used for the co-sedimentation assays in these two reports. Nonetheless, the acceptance of a negative regulatory role for the PKA-dependent phosphorylation of Ena/VASP proteins on their anti-capping activity suggests that this post-translational modification should also negatively regulate F-actin binding, therefore in agreement with Harbeck et al. (2000).

The PKA-dependent phosphorylation of Ena/VASP family members regulates their ability to interact with other proteins. Lambrechts et al. (2000) showed that the interaction of EVL with Abl and n-Src SH3 domains is selectively abolished by PKA-dependent phosphorylation of this Ena/VASP protein. Other protein–protein interactions (e.g. with Lyn SH3 domain, Fe65 WW domain or profilin) were not affected by the PKA-dependent phosphorylation of EVL. Moreover, the PKA-dependent phosphorylation of VASP was shown to inhibit its interaction with Abl tyrosine kinase (Howe et al. 2002). On the other hand, the interaction of VASP with profilin, vinculin and zyxin is not affected by PKA-dependent phosphorylation (Harbeck et al. 2000). Finally, the Tyr-phosphorylation of Drosophila Ena was shown to inhibit its interaction with Abelson kinase (Comer et al. 1998).

Importantly, the phosphorylation state of Ena/VASP appears to play an important role in the regulation of cell morphology and motility. In neurons, PKA-dependent phosphorylation of Ena/VASP proteins, and in particular the phosphorylation of Mena Ser236, leads to filopodia production and neurite growth (Lebrand et al. 2004). Hyperphosphorylation of VASP Ser157 in human platelets is associated with hyperproduction of filopodia and increased platelet aggregation in response to collagen

(Pula et al. 2006). In neutrophils, activation of PKG induced both VASP phosphorylation and cell spreading (Lawrence and Pryzwansky 2001). On the contrary, in cultured fibroblastic cells, there is evidence that the PKA/PKG-dependent phosphorylation of Mena negatively regulates fibroblast migration (Loureiro et al. 2002). Finally, in human umbilical vein endothelial cells (HUVECs), there is evidence that PKG activation induces VASP phosphorylation and reduces cell migration (Smolenski et al. 2000).

4 Pathophysiologic Relevance of Ena/VASP Proteins

In this section, the involvement of Ena/VASP proteins in the aetiology of human diseases and the resulting potential of these proteins as therapeutic targets will be discussed. Besides their crucial role in the development of the vertebrate nervous system (Lanier et al. 1999; Menzies et al. 2004), Ena/VASP proteins are involved in the healthy maintenance of the human body throughout life, and the alteration of their cellular functions is associated with serious human diseases. The involvement of Ena/VASP proteins in platelet-dependent haemostasis is the best-characterised physiological role for these proteins. The link between thrombotic diseases and Ena/VASP proteins has been described, making the proteins of these families likely targets for antithrombotic drugs. This is described in Sect. 4.1. Ena/VASP proteins also appear to play a role in the generation and development of cancer, and this is the subject of Sect. 4.2. Finally, a more modest number of publications suggest a novel link between the alteration of Ena/VASP protein activity and different human diseases, such as arteriosclerosis, cardiomyopathy and nephritis (Sects. 4.3, 4.4 and 4.5, respectively).

4.1 Thrombosis

Thrombotic diseases are caused by abnormal development of thrombi (blood clots) in the circulatory system, leading to blood vessel occlusion. Whole blood clots, or blood clot fragments, can also become detached from the site of formation and transported by the bloodstream to other sites (thromboembolism). Depending on the site of occlusion, abnormal thrombus formation can lead to life-threatening conditions such as pulmonary embolism, heart attack and stroke.

VASP is a key component of the haemostatic apparatus. In contrast, Mena is not significantly expressed in platelets, at least in mice (Aszodi et al. 1999; Hauser et al. 1999). Evl, despite its expression in murine platelets (Aszodi et al. 1999; Hauser et al. 1999), has not been investigated for its direct involvement in haemostasis. As a result, this section focuses exclusively on VASP.

The first significant observation of a possible role for VASP in haemostasis was the correlation between the PKA- and PKG-dependent phosphorylation of this protein and the responsivity to thrombin of human platelets. Horstrup et al. (1994) observed

that cAMP- and cGMP-elevating agents [prostaglandin E1 and forskolin, and 3-morpholinosydnonimine hydrochloride (SIN1) and sodium nitroprusside, respectively] induce both phosphorylation of VASP Ser157 and inhibition of the major platelet fibrinogen receptor $\alpha_{IIb}\beta_3$. Membrane-permeant selective activators of PKA or PKG inhibited $\alpha_{IIb}\beta_3$ and platelet activity, suggesting that these kinases were the effectors of the cyclic nucleotide-dependent regulation. Nonetheless, these authors could not prove that the PKA- or PKG-dependent phosphorylation of VASP was responsible for this regulatory event. The first evidence of a direct role of VASP in platelet regulation was obtained in VASP$^{-/-}$ knockout mice, where both thrombin-induced fibrinogen binding and P-selectin surface expression (a marker of platelet α-granule secretion) were enhanced, compared to wild-type mice (Hauser et al 1999). The cAMP- and cGMP-dependent phosphorylation of VASP is also responsible for the negative regulation of collagen-induced fibrinogen binding and platelet aggregation (Aszodi et al. 1999). Interestingly, other platelet responses to collagen, such as intracellular calcium mobilisation and dense granule secretion, were not affected by genetic ablation of VASP.

The phosphorylation of VASP could therefore represent a general negative feedback regulatory mechanism for the control of platelet aggregation. In fact, different platelet agonists, such as thrombin, collagen-related peptide (CRP) and collagen, have been reported to induce VASP phosphorylation at Ser157 (Wentworth et al. 2006; Pula et al. 2006), whilst others, such as von Willebrand factor (VWF), induce the phosphorylation of Ser239 (Riba et al. 2006). On the other hand, some platelet agonists, such as ADP, epinephrine and thromboxane A_2, reduce PKG-dependent phosphorylation of VASP Ser239 (Schwarz et al. 1999), suggesting that VASP-mediated regulation of platelet aggregation is complex and largely agonist-specific.

Regarding the molecular mechanism of platelet regulation, it has been suggested that VASP regulates fibrinogen binding by modulating the inside-out signalling of integrin $\alpha_{IIb}\beta_3$, the major fibrinogen receptor in platelets and the driving force of aggregation (Shattil et al. 1998; Aszodi et al. 1999). The PKA- and PKG-dependent phosphorylation could impair the linkage of $\alpha_{IIb}\beta_3$ with the actin cytoskeleton and, ultimately, reduce the activation of this fibrinogen receptor. Alternatively, VASP could play a role in the PKG-dependent inhibition of Rap 1, an abundant GTP-binding protein in platelets involved in the regulation of $\alpha_{IIb}\beta_3$ (Crittenden et al. 2004). The activation of Rap 1 in platelets is negatively regulated by the NO/cGMP/PKG pathway, which has VASP as an established substrate (Danielewski et al. 2005). A potential link between Rap1 and Ena/VASP is the Rap1 effector and Ena/VASP-binding protein RIAM. RIAM is expressed in platelets and is required for the Rap1-dependent activation of integrins (Lafuente et al. 2004; Han et al. 2006).

Besides regulating fibrinogen binding and α-degranulation, VASP also plays a pivotal role in the control of actin dynamics and cell shape in human platelets. Similarly to what happens in other cell types, during the reorganisation of actin cytoskeleton, VASP plays a major role in the anti-capping and bundling of growing actin filaments, leading to the formation of filopodia (Mejillano et al. 2004). Recently, the hyperphosphorylation of VASP Ser157 in the absence of PKCδ activity was shown to enhance actin polymerization, filopodia formation and platelet

aggregation (Pula et al. 2006). The critical importance of filopodia formation for platelet aggregation has previously been reported (Isenberg et al. 1990; Frojmovic et al. 1990). Therefore, the investigation of the role of VASP and other scaffolding proteins in the regulation of cytoskeletal rearrangements during platelet activation is a promising field for the discovery of drugs able to modulate haemostasis and treat thrombotic diseases.

Platelet hyperactivation is a feature of cardiovascular diseases, such as heart failure (Gibbs et al. 2001; Schafer et al. 2005), acute coronary syndrome (Gurbel et al. 2000; Heeschen et al. 2003) and cardiovascular complications of diabetes (Winocour 1992; Tschoepe et al. 1993), hypercholesterolemia (Nimpf et al. 1986) and hypertension (Schafer et al. 2004). Platelet activation is the essential step in promoting leukocyte adhesion, inflammation and the progression of atherosclerotic lesions (Huo et al. 2003; Massberg et al. 2002). Tonic production of NO and prostacyclin (PGI_2) in endothelial cells and their release in the bloodstream negatively regulate the reactivity of platelets and prevent thrombosis (Jin et al. 2005; Egbrink et al. 2005). The hypothesis that NO and prostacyclin locally released by endothelial cells are involved in the control of vascular thromboembolism in vivo has been suggested by studies in different models (FitzGerald et al. 1984; Lindberg et al. 1994; Broeders et al. 1998; Massberg et al. 1999; Cheng et al. 2002; Azizzadeh et al. 2003). It has been shown that increased bioavailability of endothelium-derived NO in the blood stream induced PKG-dependent phosphorylation of VASP on Ser157 and Ser239 in endothelial cells (Sporbert et al. 1999; Schafer et al. 2003) and in platelets (Schafer et al. 2006). On the other hand, co-incubation of endothelial cells and platelets has been shown to induce the PKA-dependent phosphorylation of platelet VASP Ser157 as a consequence of release from endothelial cells of different factors, including prostacyclin, which activates the cAMP/PKA pathway in platelets (Nolte et al. 1991b). Direct treatment of platelets with prostacyclin induced VASP phosphorylation at Ser157 (Nolte et al. 1991a). Taken together, these observations suggested that VASP could be the effector of the regulation of platelet activity by endothelium-derived NO and prostacyclin. This hypothesis was confirmed in an elegant study from Massberg et al. (2004), where the genetic ablation of VASP resulted in the enhancement of platelet adhesion to the endothelium of blood vessels under both physiological and pathophysiological conditions. P-selectin and integrin $\alpha_{IIb}\beta_3$ are critical mediators of the platelet/endothelium adhesion, and their modulation by VASP is the key event that regulates this cellular interaction. Since the interaction of platelets and endothelial cells is the first step towards thrombus formation in both healthy and thrombotic conditions, VASP is a promising target for the development of both haemostatic and antithrombotic drugs.

In this respect, it is noteworthy that Clopidogrel, an antiplatelet therapy for the prevention of adverse consequences during coronary intervention, is a $P2Y_{12}$ antagonist that inhibits the Gi protein-dependent signalling, potentiates the cAMP/PKA pathway and ultimately enhances VASP Ser239 phosphorylation in platelets (Schwarz et al. 1999). Cilostazol, a cyclic nucleotide phosphodiesterase (PDE) inhibitor used for the treatment of chronic peripheral arterial occlusion and stroke, also enhances the phosphorylation of VASP at both Ser157 and Ser239 (Sudo et al. 2003). Finally, dipyridamole, another PDE inhibitor that in combination with aspirin is very effective in preventing recurrent stroke, amplifies the NO/cGMP-dependent phosphorylation

of VASP (Aktas et al. 2003). In summary, significant evidence has accumulated in the literature about the importance of VASP and its PKA- and PKG-dependent regulation in the pathophysiology of thrombotic disorders. Pharmacological agents able to modulate VASP expression, localisation or post-translational modification can be expected to affect the responsivity of platelets and to alter the haemostasis/thrombosis equilibrium in vivo. In particular, pharmacological agents able to enhance the activity of VASP in platelets are potentially very interesting as anti-thrombotic tools.

4.2 Cancer

Cancer is a group of diseases characterised by abnormal and uncontrolled cell proliferation and, in some cases, metastasis. Metastasis is the spreading of cancer to different organs, and it is the consequence of detachment of cancerous cells from the primary tumour, intravasation, dissemination via blood and/or lymphatic vessels and invasion of new organs. Alteration of cell adhesion, loss of contact inhibition and consequent proliferation, and aberrant cell migration characterises the aggressive phenotype of cancer malignancies.

The actin cytoskeleton plays a fundamental role in the cellular properties of oncogenic cells (Pawlak and Helfman 2001; Rao and Li 2004; Hayot et al. 2006). Consequently, proteins functionally linked to the actin cytoskeleton are likely to play a role in the increased invasiveness of cancer cells. The expression levels of different actin-binding proteins, such as Arp2/3 complex subunits, cofilin, gelsolin, profilin, thymosin, WASP and WAVE, are altered in cancer cells (for a review, see Lambrechts et al. 2004). In a recent comprehensive study, the analysis of protein expression in highly invasive carcinoma cells showed overexpression of motility-related genes, and in particular of proteins linked to the actin cytoskeleton, such as capping proteins α1 and α2, Arp2/3 complex subunits p16 and p21, zyxin, vinculin, cofilin and, most importantly, Mena (Wang et al. 2004).

On the other hand, the down-regulation of VASP by a random homozygous knock-out strategy has been shown to result in loss of contact inhibition in NIH 3T3 fibroblasts, resulting in continued cell division and potential tumorigenesis (Liu et al. 1999). Interestingly, also the overexpression of VASP-induced neoplastic transformation of NIH 3T3 cells, suggesting that normal cell growth may require VASP expression within a narrow range (Liu et al. 1999). The tumorigenic transformation of epithelial cells by constitutively active rac1 is accompanied by up-regulation of VASP, whilst the reversion of the tumorigenic phenotype by dominant negative rac1 resulted in the downregulation of VASP (Quinlan 2004). In agreement with this observation, VASP has been reported significantly overexpressed in cancerous lung adrenocarcinoma cells, compared to normal lung cells (Dertsiz et al. 2005). Besides the level of expression, the phosphorylation of VASP appears to be also impaired in some malignancies. For example, Eigenthaler et al. (1993) reported decreased expression of PKG in platelets from patients with chronic myelocytic leukaemia (CML). One of the consequences of this was the severe impairment of VASP phosphorylation.

Other members of the Ena/VASP family have been suggested to play a role in the establishment of the cancerous cell phenotype. Mena is overexpressed in the majority of breast cancer cell lines and tissue samples (Di Modugno et al. 2004). Furthermore, anti-Mena antibodies have been observed in the serum of some breast cancer patients, but not in healthy controls, suggesting that a specific antitumour immune response was directed against Mena-expressing cancerous cells (Di Modugno et al. 2004). In contrast, a gene sharing 80% homology with Mena has been shown to be down-regulated in nickel-transformed embryonic lung cells and small-cell lung cancer SBC-2 cells (Mao et al. 2000). Finally, as a confirmation of the importance of Ena/VASP-mediated actin regulation in tumorigenic cell transformation, the expression of lamellipodin, a molecular interactor of Ena/VASP, is significantly down-regulated in breast and ovarian cancer samples compared to normal tissues (Dahl et al. 2005) and in osteosarcoma metastatic cells compared to primary osteosarcoma tumour cells (Eppert et al. 2005). Taken together, different lines of evidence link Ena/VASP proteins to cancerous transformation of cells and metastasis. At the moment, the exact relevance of these observations and the molecular mechanism underlying the potential role of Ena/VASP in cell transformation and tumour formation is not clear. Nonetheless, considering the potential of actin cytoskeleton regulation in the establishment of altered cell motility and cell division behaviours, the involvement of Ena/VASP protein in tumour formation seems a promising subject towards both understanding of cancer biology and drug discovery.

Another important aspect of cancer biology is the formation of new blood vessels (angiogenesis) during tumour progression. Angiogenesis is essential for the progressive growth and expansion of solid tumours. Without sufficient vascularisation, solid tumours beyond the size of 1–2 mm face necrosis and/or apoptosis. The inhibition of angiogenesis has been shown to significantly reduce tumour progression, and antiangiogenic therapy is an effective anticancer strategy (Parangi et al. 1996). In this light, the role that VASP plays in the regulation of endothelial cell proliferation and vasculogenesis is very interesting. In vitro experiments with HUVECs suspended in three-dimensional collagen matrices revealed that VASP, together with gelsolin and profilin, two actin regulatory proteins, is significantly up-regulated during capillary morphogenesis (Salazar et al. 1999). The expression of VASP in vasculogenic sites of human placenta is spatially and temporally regulated during pregnancy, suggesting a role for this protein in placental vasculogenesis. Vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) seem responsible for the up-regulation of VASP, since areas of strong VEGF and IL-8 expression correspond to areas of high VASP expression in placental samples, and, in vitro, these two growth factors show a stimulatory effect on VASP expression in placental explants (Kayisli et al. 2002). At the cellular level, VASP was shown to induce stress-fibre and membrane ruffle formation in aortic endothelium (Price and Brindle 2000). This suggests that VASP might positively regulate capillary formation via effects on endothelial motility and contractility. Finally, besides its regulatory effect on endothelial cells, VASP also regulates vascular smooth muscle cell proliferation (Chen et al. 2004). These authors showed that VASP overexpression in aortic smooth muscle cells deriving from VASP-deficient mice significantly increased proliferation (Chen et al. 2004). By the use of phospho-null

mutants, the same authors also suggest that phosphorylation of Ser157 has growth-stimulatory effects, whilst phosphorylation of Ser239 is critical for the NO-dependent inhibition of vascular smooth muscle cell proliferation. In summary, it seems plausible to hypothesise that VASP plays a significant role in vasculogenesis and, in this context, in the regulation of solid tumour vascularisation. Therefore, in addition to their potential as cell transformation determinants, Ena/VASP proteins could become a therapeutic target for the anti-angiogenic treatment of solid malignancies.

4.3 Arteriosclerosis

The expression of VASP in endothelial cells and its role in endothelial proliferation have already been discussed in this chapter. Nevertheless, it seems important to underscore the possible role of VASP in the regulation of neointimal cell proliferation, which is regarded as a major factor in the development of arteriosclerotic diseases, such as spontaneous atherosclerosis, post-angioplasty restenosis and vein graft atherosclerosis. The *tunica intima* is the innermost endothelial layer of blood vessels, and neointimal cells are those endothelial cells that migrate and proliferate at injury sites in order to replenish the intimal layer. Abnormal neointimal proliferation is a key step towards the formation of the neointimal plaque: a pathological mass of endothelial and smooth muscle cells, macrophages, lymphocytes and lipid that causes the blood vessel restriction at the basis of arteriosclerosis. VASP has been shown to be overexpressed in the neointimal cell layer, resulting from the surgical endothelial denudation of rat carotid arteries (Monks et al. 1998). Moreover, a reduction of PKG expression and PKG-dependent phosphorylation of VASP has been reported in neointimal cells of atherosclerotic rabbit aortas (Melichar et al. 2004). Interestingly, overexpression of constitutively active PKG and the consequent hyperphosphorylation of VASP in rat aorta resulted in the reduction of neointimal proliferation following artery denudation (Sinnaeve et al. 2002). Taken together, the above studies suggest a rationale for the involvement of VASP in the endothelium-driven development of arteriosclerotic diseases. The modulation of VASP expression or phosphorylation in proliferating endothelial cells could therefore be regarded as an opportunity for therapeutic intervention, not only against neoplastic angiogenesis, but also against arteriosclerosis. Notably, the anti-platelet drug clopidogrel, already described in this report as an enhancer of VASP phosphorylation, has also been shown to improve endothelial function and flow-mediated artery dilation in coronary disease patients (Warnholtz et al. 2007). This effect of clopidogrel on artery function could be mediated by regulation of VASP and its function as modulator of neointimal proliferation.

4.4 Cardiomyopathy

Besides platelets, vascular smooth muscle and endothelial cells, VASP is expressed in the human heart (Markert et al. 1996). Both VASP and Mena are expressed in

mouse heart, where they are associated with intercalated discs of cardiomyocytes (Gambaryan et al. 2001). Intercalated discs are complex membrane structures separating adjacent cardiomyocytes. Their intracellular side is characterised by a highly organised cytoskeleton rich in N-cadherin, catenins, vinculin and connexins. The displacement of VASP and Mena from cardiac intercalated discs via heart-targeted expression of EVH1 domain in transgenic mice resulted in the disorganisation of intercalated discs (Eigenthaler et al. 2003). The severe consequences of this histological abnormality were myocyte hypertrophy, cardiac dilatation and bradycardia, which led to early postnatal mortality. Mena and VASP were also shown to co-localise in vivo and interact in vitro with Xin, the protein encoded by the human gene ‘cardiomyopathy-associated 1’ or CMYA1 (van der Ven et al. 2006), and migfilin, a protein that serves the dual function of structural cytoskeletal organiser and transcriptional regulator in cardiomyocytes (Wu 2005). In summary, the scaffolding activity of Ena/VASP proteins seems to be crucial for the correct organisation of the cytoskeleton underlying regions of physical and functional contact between cardiomyocytes. Their important role in the establishment and maintenance of the structural integrity of cardiomyocytes and myocardium makes Ena/VASP proteins interesting candidates for cardiac diseases, in particular congenital cardiomyopathies associated with developmental defects and hypertrophy.

4.5 Nephritis

Another organ displaying abundant VASP and Mena expression is the kidney. The expression of both proteins in glomeruli and tubulointerstitium of healthy mice has been described (Gambaryan et al. 2001). Recently, the response of $VASP^{-/-}$ knockout mice to the experimental induction of passive nephrotoxic nephritis was studied, and the ablation of VASP was associated with increased resistance to the disease (Hohenstein et al. 2005). Nephritis is an inflammatory disease that leads to acute damage of glomerular cells and, at later stages, to glomerulosclerosis, tubulointerstitial fibrosis, progressive loss of glomerular and tubulointerstitial capillaries, and loss of renal function. Interestingly, the expression of VASP is increased in glomeruli and tubulointerstitium as a consequence of nephrotoxic nephritis. The histological analysis of $VASP^{-/-}$ mice revealed that in these animals, the inflammatory tissue injury was enhanced at early disease stages, but significantly reduced at later disease stages, compared to control wild type animals. In view of the role of platelets in kidney inflammatory diseases (Johnson 1991) and the potentiation of platelet activity in VASP knockouts (see Sect. 4.1), enhanced platelet binding and activation at the sites of injury are likely to explain the adverse effect of VASP ablation at early disease stages. Improved endothelial preservation and proliferation, and the resulting improved renal vascularisation, are proposed to be the reason for the protection against the progression of the disease in $VASP^{-/-}$ animals. However, two aspects of this work remain unexplained. First, it is difficult to understand how protection against tissue injury does not correspond to protection against the deterioration of renal function. Second, the authors propose a negative

regulatory role of vasculogenesis and capillary preservation, whilst amounting evidence suggests that VASP should play a positive regulatory role in these processes.

5 Ena/VASP Proteins and Drug Discovery

Because of their role in the aetiology of different human diseases, Ena/VASP proteins represent interesting drug targets, whose potential has not been fully explored. Only relatively recently have there been efforts towards the development of inhibitors of Ena/VASP activity. In particular, the Ena/VASP EVH1 domains and their ability to establish interactions with proteins containing the FP4 consensus motif have been targeted. The natural protein with the highest affinity for EVH1 domains is the ActA protein of *Listeria monocytogenes*. Peptides bearing the FP4 motif of ActA have long been shown to bind to the EVH1 domain and compete with its ligands (Niebuhr et al. 1997; Prehoda et al. 1999). The modification of the ActA domain led to the synthesis of a miniature protein (pGolemi) able to bind Mena EVH1 domain with an affinity ten-fold higher than ActA (Golemi-Kotra et al. 2004). The opposite strategy seems also possible, which is displacing the interaction of Ena/VASP proteins and their ligands with peptides mimicking small regions of the EVH1 domain. Using this approach, a series of peptides inhibiting the protein–protein interaction of Mena EVH1 and its ligands were identified (Hunke et al. 2006). Unfortunately, peptide drugs have been proven to have limited potential as therapeutic tools because of their poor bioavailability, short half-life (due to susceptibility to proteolysis and potential immunogenicity) and poor pharmacokinetics. In the case of Ena/VASP proteins, a promising way to get around the limitations of peptide drugs may be through the use of peptoids and peptomers. Peptoids are non-peptide agents designed to simulate a peptide structure, and the typical structure of peptoids is that of poly-N-substituted glycines. Peptomers are peptide–peptoid hybrid polymers. Zimmermann and colleagues (2003) described the synthesis of a peptomer by incorporation of peptoid building blocks into the peptide SFEFPPPPTEDEL from Listerial ActA. This peptomer maintained the ability to bind to VASP EVH1 in the micromolar range, and it represents a promising drug candidate in the generation of novel non-peptide therapeutics able to impair EVH1-mediated protein–protein interactions. The advantages of peptoids over peptides include improved chemical versatility and variability in the drug-design phase (Kirshenbaum et al. 1998), besides increased resistance to proteolysis and better metabolic stability, absorption and disposition (Wang et al. 1999). Another interesting feature of peptoid drugs is their potential specificity. In fact, the design of peptoids closely mimicking the binding domain of a particular interacting protein could enable the modulation of Ena/VASP activity in a tissue-, cell type- and subcellular structure-specific manner. Therefore, via attenuation of specific protein–protein interactions, and regulation of subcellular targeting of Ena/VASP proteins, peptoid drugs could achieve fine tuning of actin cytoskeleton-dependent processes.

In summary, Ena/VASP proteins, because of their role as actin cytoskeleton regulators and their growing relevance in important human diseases, are promising targets for biomedical research. Despite initial efforts, the absence of therapeutic tools able to target Ena/VASP proteins makes these proteins a fully exploitable field for drug discovery.

Acknowledgements We would like to thank Mark J. Belsey (Datamonitor Healthcare, London, UK) and Sue Perera (Randall Division of Cell and Molecular Biophysics, King's College London, UK) for critical reading of the manuscript. Our work is supported by CRUK grant C22104/A7155 and Wellcome Trust grant 077429.

References

Aktas B, Utz A, Hoenig-Liedl P, Walter U, Geiger J (2003) Dipyridamole enhances NO/cGMP-mediated vasodilator-stimulated phosphoprotein phosphorylation and signaling in human platelets: in vitro and in vivo/ex vivo studies. Stroke 34:764–769

Allen LA, Aderem A (1996) Mechanisms of phagocytosis. Curr Opin Immunol 8:36–40

Aszodi A, Pfeifer A, Ahmad M, Glauner M, Zhou XH, Ny L, Andersson KE, Kehrel B, Offermanns S, Fassler R (1999) The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. Embo J 18:37–48

Azizzadeh B, Buga GM, Berke GS, Larian B, Ignarro LJ, Blackwell KE (2003) Inhibitors of nitric oxide promote microvascular thrombosis. Arch Facial Plast Surg 5:31–35

Bachmann C, Fischer L, Walter U, Reinhard M (1999) The EVH2 domain of the vasodilator-stimulated phosphoprotein mediates tetramerization, F-actin binding, and actin bundle formation. J Biol Chem 274:23549–23557

Ball LJ, Kuhne R, Hoffmann B, Hafner A, Schmieder P, Volkmer-Engert R, Hof M, Wahl M, Schneider-Mergener J, Walter U, Oschkinat H, Jarchau T (2000) Dual epitope recognition by the VASP EVH1 domain modulates polyproline ligand specificity and binding affinity. EMBO J 19:4903–4914

Barzik M, Kotova TI, Higgs HN, Hazelwood L, Hanein D, Gertler FB, Schafer DA (2005) Ena/VASP proteins enhance actin polymerization in the presence of barbed end capping proteins. J Biol Chem 280:28653–28662

Bashaw GJ, Kidd T, Murray D, Pawson T, Goodman CS (2000) Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. Cell 101:703–715

Baum B, Perrimon N (2001) Spatial control of the actin cytoskeleton in Drosophila epithelial cells. Nat Cell Biol 3:883–890

Bear JE, Loureiro JJ, Libova I, Fassler R, Wehland J, Gertler FB (2000) Negative regulation of fibroblast motility by Ena/VASP proteins. Cell 101:717–728

Bear JE, Krause M, Gertler FB (2001) Regulating cellular actin assembly. Curr Opin Cell Biol 13:158–166

Bear JE, Svitkina TM, Krause M, Schafer DA, Loureiro JJ, Strasser GA, Maly IV, Chaga OY, Cooper JA, Borisy GG, Gertler FB (2002) Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. Cell 109:509–521

Boukhelifa M, Parast MM, Bear JE, Gertler FB, Otey CA (2004) Palladin is a novel binding partner for Ena/VASP family members. Cell Motil Cytoskeleton 58:17–29

Bournier O, Kroviarski Y, Rotter B, Nicolas G, Lecomte MC, Dhermy D (2006) Spectrin interacts with EVL (Enabled/vasodilator-stimulated phosphoprotein-like protein), a protein involved in actin polymerization. Biol Cell 98:279–293

Brindle NP, Holt MR, Davies JE, Price CJ, Critchley DR (1996) The focal-adhesion vasodilator-stimulated phosphoprotein (VASP) binds to the proline-rich domain in vinculin. Biochem J 318:753–757

Broeders MA, Tangelder GJ, Slaaf DW, Reneman RS, Egbrink MG (1998) Endogenous nitric oxide protects against thromboembolism in venules but not in arterioles. Arterioscler Thromb Vasc Biol 18:139–145

Bundschu K, Walter U, Schuh K (2006) The VASP-Spred-Sprouty domain puzzle. J Biol Chem 281:36477–36481

Butt E, Abel K, Krieger M, Palm D, Hoppe V, Hoppe J, Walter U (1994) cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. J Biol Chem 269:14509–14517

Carl UD, Pollmann M, Orr E, Gertlere FB, Chakraborty T, Wehland J (1999) Aromatic and basic residues within the EVH1 domain of VASP specify its interaction with proline-rich ligands. Curr Biol 9:715–718

Chakraborty T, Ebel F, Domann E, Niebuhr K, Gerstel B, Pistor S, Temm-Grove CJ, Jockusch BM, Reinhard M, Walter U et al (1995) A focal adhesion factor directly linking intracellularly motile Listeria monocytogenes and Listeria ivanovii to the actin-based cytoskeleton of mammalian cells. EMBO J 14:1314–1321

Chen L, Daum G, Chitaley K, Coats SA, Bowen-Pope DF, Eigenthaler M, Thumati NR, Walter U, Clowes AW (2004) Vasodilator-stimulated phosphoprotein regulates proliferation and growth inhibition by nitric oxide in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 24:1403–1408

Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T, Lawson JA, FitzGerald GA (2002) Role of prostacyclin in the cardiovascular response to thromboxane A2. Science 296:539–541

Chereau D, Dominguez R (2006) Understanding the role of the G-actin-binding domain of Ena/VASP in actin assembly. J Struct Biol 155:195–201

Chitaley K, Chen L, Galler A, Walter U, Daum G, Clowes AW (2004) Vasodilator-stimulated phosphoprotein is a substrate for protein kinase C. FEBS Lett 556:211–215

Comer AR, Ahern-Djamali SM, Juang JL, Jackson PD, Hoffmann FM (1998) Phosphorylation of Enabled by the Drosophila Abelson tyrosine kinase regulates the in vivo function and protein–protein interactions of Enabled. Mol Cell Biol 18:152–160

Cook AL, Haynes JM (2007) Phosphorylation of the PKG substrate, vasodilator-stimulated phosphoprotein (VASP), in human cultured prostatic stromal cells. Nitric Oxide 16:10–17

Coppolino MG, Krause M, Hagendorff P, Monner DA, Trimble W, Grinstein S, Wehland J, Sechi AS (2001) Evidence for a molecular complex consisting of Fyb/SLAP, SLP-76, Nck, VASP and WASP that links the actin cytoskeleton to Fcgamma receptor signalling during phagocytosis. J Cell Sci 114:4307–4318

Crawford AW, Beckerle MC (1991) Purification and characterization of zyxin, an 82,000-dalton component of adherens junctions. J Biol Chem 266:5847–5853

Crittenden JR, Bergmeier W, Zhang Y, Piffath CL, Liang Y, Wagner DD, Housman DE, Graybiel AM (2004) CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. Nat Med 10:982–986

Dahl E, Sadr-Nabavi A, Klopocki E, Betz B, Grube S, Kreutzfeld R, Himmelfarb M, An HX, Gelling S, Klaman I, Hinzmann B, Kristiansen G, Grutzmann R, Kuner R, Petschke B, Rhiem K, Wiechen K, Sers C, Wiestler O, Schneider A, Hofler H, Nahrig J, Dietel M, Schafer R, Rosenthal A, Schmutzler R, Durst M, Meindl A, Niederacher D (2005) Systematic identification and molecular characterization of genes differentially expressed in breast and ovarian cancer. J Pathol 205:21–28

Danielewski O, Schultess J, Smolenski A (2005) The NO/cGMP pathway inhibits Rap 1 activation in human platelets via cGMP-dependent protein kinase I. Thromb Haemost 93:319–325

DeMali KA, Barlow CA, Burridge K (2002) Recruitment of the Arp2/3 complex to vinculin: coupling membrane protrusion to matrix adhesion. J Cell Biol 159:881–891

Dertsiz L, Ozbilim G, Kayisli Y, Gokhan GA, Demircan A, Kayisli UA (2005) Differential expression of VASP in normal lung tissue and lung adenocarcinomas. Thorax 60:576–581
Di Modugno F, Bronzi G, Scanlan MJ, Del Bello D, Cascioli S, Venturo I, Botti C, Nicotra MR, Mottolese M, Natali PG, Santoni A, Jager E, Nistico P (2004) Human Mena protein, a serex-defined antigen overexpressed in breast cancer eliciting both humoral and CD8+ T-cell immune response. Int J Cancer 109:909–918
Drees B, Friederich E, Fradelizi J, Louvard D, Beckerle MC, Golsteyn RM (2000) Characterization of the interaction between zyxin and members of the Ena/vasodilator-stimulated phosphoprotein family of proteins. J Biol Chem 275:22503–22511
Egbrink MG, Van Gestel MA, Broeders MA, Tangelder GJ, Heemskerk JM, Reneman RS, Slaaf DW (2005) Regulation of microvascular thromboembolism in vivo. Microcirculation 12:287–300
Eigenthaler M, Ullrich H, Geiger J, Horstrup K, Honig-Liedl P, Wiebecke D, Walter U (1993) Defective nitrovasodilator-stimulated protein phosphorylation and calcium regulation in cGMP-dependent protein kinase-deficient human platelets of chronic myelocytic leukemia. J Biol Chem 268:13526–13531
Eigenthaler M, Engelhardt S, Schinke B, Kobsar A, Schmitteckert E, Gambaryan S, Engelhardt CM, Krenn V, Eliava M, Jarchau T, Lohse MJ, Walter U, Hein L (2003) Disruption of cardiac Ena-VASP protein localization in intercalated disks causes dilated cardiomyopathy. Am J Physiol Heart Circ Physiol 285:H2471–H2481
Eppert K, Wunder JS, Aneliunas V, Tsui LC, Scherer SW, Andrulis IL (2005) Altered expression and deletion of RMO1 in osteosarcoma. Int J Cancer 114:738–746
Ermekova KS, Zambrano N, Linn H, Minopoli G, Gertler F, Russo T, Sudol M (1997) The WW domain of neural protein FE65 interacts with proline-rich motifs in Mena, the mammalian homolog of Drosophila enabled. J Biol Chem 272:32869–32877
Fedorov AA, Fedorov E, Gertler F, Almo SC (1999) Structure of EVH1, a novel proline-rich ligand-binding module involved in cytoskeletal dynamics and neural function. Nat Struct Biol 6:661–665
FitzGerald GA, Smith B, Pedersen AK, Brash AR (1984) Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation. N Engl J Med 310:1065–1068
Frischknecht F, Way M (2001) Surfing pathogens and the lessons learned for actin polymerization. Trends Cell Biol 11:30–38
Frojmovic M, Longmire K, van de Ven TG (1990) Long-range interactions in mammalian platelet aggregation. II. The role of platelet pseudopod number and length. Biophys J 58:309–318
Gambaryan S, Hauser W, Kobsar A, Glazova M, Walter U (2001) Distribution, cellular localization, and postnatal development of VASP and Mena expression in mouse tissues. Histochem Cell Biol 116:535–543
Geiger B (1979) A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. Cell 18:193–205
Gertler FB, Bennett RL, Clark MJ, Hoffmann FM (1989) Drosophila abl tyrosine kinase in embryonic CNS axons: a role in axonogenesis is revealed through dosage-sensitive interactions with disabled. Cell 58:103–113
Gertler FB, Doctor JS, Hoffmann FM (1990) Genetic suppression of mutations in the Drosophila abl proto-oncogene homolog. Science 248:857–860
Gertler FB, Comer AR, Juang JL, Ahern SM, Clark MJ, Liebl EC, Hoffmann FM (1995) Enabled, a dosage-sensitive suppressor of mutations in the Drosophila Abl tyrosine kinase, encodes an Abl substrate with SH3 domain-binding properties. Genes Dev 9:521–533
Gertler FB, Niebuhr K, Reinhard M, Wehland J, Soriano P (1996) Mena, a relative of VASP and Drosophila Enabled, is implicated in the control of microfilament dynamics. Cell 87:227–239
Gibbs CR, Blann AD, Watson RD, Lip GY (2001) Abnormalities of hemorheological, endothelial, and platelet function in patients with chronic heart failure in sinus rhythm: effects of angiotensin-converting enzyme inhibitor and beta-blocker therapy. Circulation 103:1746–1751
Gkretsi V, Zhang Y, Tu Y, Chen K, Stolz DB, Yang Y, Watkins SC, Wu C (2005) Physical and functional association of migfilin with cell–cell adhesions. J Cell Sci 118:697–710

Golemi-Kotra D, Mahaffy R, Footer MJ, Holtzman JH, Pollard TD, Theriot JA, Schepartz A (2004) High affinity, paralog-specific recognition of the Mena EVH1 domain by a miniature protein. J Am Chem Soc 126:4–5
Gouin E, Welch MD, Cossart P (2005) Actin-based motility of intracellular pathogens. Curr Opin Microbiol 8:35–45
Grevengoed EE, Loureiro JJ, Jesse TL, Peifer M (2001) Abelson kinase regulates epithelial morphogenesis in Drosophila. J Cell Biol 155:1185–1198.
Griffiths EK, Krawczyk C, Kong YY, Raab M, Hyduk SJ, Bouchard D, Chan VS, Kozieradzki I, Oliveira-Dos-Santos AJ, Wakeham A, Ohashi PS, Cybulsky MI, Rudd CE, Penninger JM (2001) Positive regulation of T cell activation and integrin adhesion by the adapter Fyb/Slap. Science 293:2260–2263
Gurbel PA, Kereiakes DJ, Dalesandro MR, Bahr RD, O'Connor CM, Serebruany VL (2000) Role of soluble and platelet-bound P-selectin in discriminating cardiac from noncardiac chest pain at presentation in the emergency department. Am Heart J 139:320–328
Halbrugge M, Walter U (1989) Purification of a vasodilator-regulated phosphoprotein from human platelets. Eur J Biochem 185:41–50
Halbrugge M, Friedrich C, Eigenthaler M, Schanzenbacher P, Walter U (1990) Stoichiometric and reversible phosphorylation of a 46-kDa protein in human platelets in response to cGMP- and cAMP-elevating vasodilators. J Biol Chem 265:3088–3093
Han J, Lim CJ, Watanabe N, Soriani A, Ratnikov B, Calderwood DA, Puzon-McLaughlin W, Lafuente EM, Boussiotis VA, Shattil SJ, Ginsberg MH (2006) Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. Curr Biol 16:1796–1806
Harbeck B, Huttelmaier S, Schluter K, Jockusch BM, Illenberger S (2000) Phosphorylation of the vasodilator-stimulated phosphoprotein regulates its interaction with actin. J Biol Chem 275:30817–30825
Harmer NJ, Sivak JM, Amaya E, Blundell TL (2005) 1.15 A crystal structure of the X. tropicalis Spred1 EVH1 domain suggests a fourth distinct peptide-binding mechanism within the EVH1 family. FEBS Lett 579:1161–1166
Hauser W, Knobeloch KP, Eigenthaler M, Gambaryan S, Krenn V, Geiger J, Glazova M, Rohde E, Horak I, Walter U, Zimmer M (1999) Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. Proc Natl Acad Sci USA 96:8120–8125
Hayot C, Debeir O, Van Ham P, Van Damme M, Kiss R, Decaestecker C (2006) Characterization of the activities of actin-affecting drugs on tumor cell migration. Toxicol Appl Pharmacol 211:30–40
Heeschen C, Dimmeler S, Hamm CW, van den Brand MJ, Boersma E, Zeiher AM, Simoons ML (2003) Soluble CD40 ligand in acute coronary syndromes. N Engl J Med 348:1104–1111
Hohenstein B, Kasperek L, Kobelt DJ, Daniel C, Gambaryan S, Renne T, Walter U, Amann KU, Hugo CP (2005) Vasodilator-stimulated phosphoprotein-deficient mice demonstrate increased platelet activation but improved renal endothelial preservation and regeneration in passive nephrotoxic nephritis. J Am Soc Nephrol 16:986–996
Horstrup K, Jablonka B, Honig-Liedl P, Just M, Kochsiek K, Walter U (1994) Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition. Eur J Biochem 225:21–27
Howe AK, Hogan BP, Juliano RL (2002) Regulation of vasodilator-stimulated phosphoprotein phosphorylation and interaction with Abl by protein kinase A and cell adhesion. J Biol Chem 277:38121–38126
Hunke C, Hirsch T, Eichler J (2006) Structure-based synthetic mimicry of discontinuous protein binding sites: inhibitors of the interaction of Mena EVH1 domain with proline-rich ligands. Chembiochem 7:1258–1264
Huo Y, Schober A, Forlow SB, Smith DF, Hyman MC, Jung S, Littman DR, Weber C, Ley K (2003) Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. Nat Med 9:61–67

Huttelmaier S, Harbeck B, Steffens O, Messerschmidt T, Illenberger S, Jockusch BM (1999) Characterization of the actin binding properties of the vasodilator-stimulated phosphoprotein VASP. FEBS Lett 451:68–74
Ibarra-Alvarado C, Galle J, Melichar VO, Mameghani A, Schmidt HH (2002) Phosphorylation of blood vessel vasodilator-stimulated phosphoprotein at serine 239 as a functional biochemical marker of endothelial nitric oxide/cyclic GMP signaling. Mol Pharmacol 61:312–319
Isenberg WM, Bainton DF, Newman PJ (1990) Monoclonal antibodies bound to subunits of the integrin GPIIb-IIIa are internalized and interfere with filopodia formation and platelet aggregation. Blood 76:1564–1571
Jenzora A, Behrendt B, Small JV, Wehland J, Stradal TE (2005) PREL1 provides a link from Ras signalling to the actin cytoskeleton via Ena/VASP proteins. FEBS Lett 579:455–463
Jin RC, Voetsch B, Loscalzo J (2005) Endogenous mechanisms of inhibition of platelet function. Microcirculation 12:247–258
Johnson RJ (1991) Platelets in inflammatory glomerular injury. Semin Nephrol 11:276–284
Jordan MS, Singer AL, Koretzky GA (2003) Adaptors as central mediators of signal transduction in immune cells. Nat Immunol 4:110–116
Kayisli UA, Demir R, Erguler G, Arici A (2002) Vasodilator-stimulated phosphoprotein expression and its cytokine-mediated regulation in vasculogenesis during human placental development. Mol Hum Reprod 8:1023–1030
Keicher C, Gambaryan S, Schulze E, Marcus K, Meyer HE, Butt E (2004) Phosphorylation of mouse LASP-1 on threonine 156 by cAMP- and cGMP-dependent protein kinase. Biochem Biophys Res Commun 324:308–316
Kirshenbaum K, Barron AE, Goldsmith RA, Armand P, Bradley EK, Truong KT, Dill KA, Cohen FE, Zuckermann RN (1998) Sequence-specific polypeptoids: a diverse family of heteropolymers with stable secondary structure. Proc Natl Acad Sci USA 95:4303–4308
Krause M, Sechi AS, Konradt M, Monner D, Gertler FB, Wehland J (2000) Fyn-binding protein (Fyb)/SLP-76-associated protein (SLAP), Ena/vasodilator-stimulated phosphoprotein (VASP) proteins and the Arp2/3 complex link T cell receptor (TCR) signaling to the actin cytoskeleton. J Cell Biol 149:181–194
Krause M, Leslie JD, Stewart M, Lafuente EM, Valderrama F, Jagannathan R, Strasser GA, Rubinson DA, Liu H, Way M, Yaffe MB, Boussiotis VA, Gertler FB (2004) Lamellipodin, an Ena/VASP ligand, is implicated in the regulation of lamellipodial dynamics. Dev Cell 7:571–583
Kuhnel K, Jarchau T, Wolf E, Schlichting I, Walter U, Wittinghofer A, Strelkov SV (2004) The VASP tetramerization domain is a right-handed coiled coil based on a 15-residue repeat. Proc Natl Acad Sci USA 101:17027–17032
Lafuente EM, van Puijenbroek AA, Krause M, Carman CV, Freeman GJ, Berezovskaya A, Constantine E, Springer TA, Gertler FB, Boussiotis VA (2004) RIAM, an Ena/VASP and Profilin ligand, interacts with Rap1-GTP and mediates Rap1-induced adhesion. Dev Cell 7:585–595
Lambrechts A, Verschelde JL, Jonckheere V, Goethals M, Vandekerckhove J, Ampe C (1997) The mammalian profilin isoforms display complementary affinities for PIP2 and proline-rich sequences. Embo J 16:484–494
Lambrechts A, Kwiatkowski AV, Lanier LM, Bear JE, Vandekerckhove J, Ampe C, Gertler FB (2000) cAMP-dependent protein kinase phosphorylation of EVL, a Mena/VASP relative, regulates its interaction with actin and SH3 domains. J Biol Chem 275:36143–36151
Lambrechts A, Van Troys M, Ampe C (2004) The actin cytoskeleton in normal and pathological cell motility. Int J Biochem Cell Biol 36:1890–1909
Lanier LM, Gates MA, Witke W, Menzies AS, Wehman AM, Macklis JD, Kwiatkowski D, Soriano P, Gertler FB (1999) Mena is required for neurulation and commissure formation. Neuron 22:313–325
Laurent V, Loisel TP, Harbeck B, Wehman A, Grobe L, Jockusch BM, Wehland J, Gertler FB, Carlier MF (1999) Role of proteins of the Ena/VASP family in actin-based motility of Listeria monocytogenes. J Cell Biol 144:1245–1258

Lawrence DW, Pryzwansky KB (2001) The vasodilator-stimulated phosphoprotein is regulated by cyclic GMP-dependent protein kinase during neutrophil spreading. J Immunol 166:5550–5556
Lawrence DW, Comerford KM, Colgan SP (2002) Role of VASP in reestablishment of epithelial tight junction assembly after Ca2+ switch. Am J Physiol Cell Physiol 282:C1235–C1245
Lebrand C, Dent EW, Strasser GA, Lanier LM, Krause M, Svitkina TM, Borisy GG, Gertler FB (2004) Critical role of Ena/VASP proteins for filopodia formation in neurons and in function downstream of netrin-1. Neuron 42:37–49
Li Z, Ajdic J, Eigenthaler M, Du X (2003) A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. Blood 101:4423–4429
Lindberg RA, Slaaf DW, Lentsch AB, Miller FN (1994) Involvement of nitric oxide and cyclooxygenase products in photoactivation-induced microvascular occlusion. Microvasc Res 47:203–221
Liu K, Li L, Nisson PE, Gruber C, Jessee J, Cohen SN (1999) Reversible tumorigenesis induced by deficiency of vasodilator-stimulated phosphoprotein. Mol Cell Biol 19:3696–3703
Loureiro JJ, Rubinson DA, Bear JE, Baltus GA, Kwiatkowski AV, Gertler FB (2002) Critical roles of phosphorylation and actin binding motifs, but not the central proline-rich region, for Ena/vasodilator-stimulated phosphoprotein (VASP) function during cell migration. Mol Biol Cell 13:2533–2546
Machesky LM, Hall A (1997) Role of actin polymerization and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization. J Cell Biol 138:913–926
Mao X, Kashii T, Hayashi R, Sassa K, Fujishita T, Maruyama M, Kobayashi M, Liu S (2000) Cloning of differentially expressed sequence tags from nickel-transformed human embryonic lung cells. Cancer Lett 161:57–62
Markert T, Krenn V, Leebmann J, Walter U (1996) High expression of the focal adhesion- and microfilament-associated protein VASP in vascular smooth muscle and endothelial cells of the intact human vessel wall. Basic Res Cardiol 91:337–343
Massberg S, Sausbier M, Klatt P, Bauer M, Pfeifer A, Siess W, Fassler R, Ruth P, Krombach F, Hofmann F (1999) Increased adhesion and aggregation of platelets lacking cyclic guanosine 3',5'-monophosphate kinase I. J Exp Med 189:1255–1264
Massberg S, Brand K, Gruner S, Page S, Muller E, Muller I, Bergmeier W, Richter T, Lorenz M, Konrad I, Nieswandt B, Gawaz M (2002) A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. J Exp Med 196:887–896
Massberg S, Gruner S, Konrad I, Garcia Arguinzonis MI, Eigenthaler M, Hemler K, Kersting J, Schulz C, Muller I, Besta F, Nieswandt B, Heinzmann U, Walter U, Gawaz M (2004) Enhanced in vivo platelet adhesion in vasodilator-stimulated phosphoprotein (VASP)-deficient mice. Blood 103:136–142
Mejillano MR, Kojima S, Applewhite DA, Gertler FB, Svitkina TM, Borisy GG (2004) Lamellipodial versus filopodial mode of the actin nanomachinery: pivotal role of the filament barbed end. Cell 118:363–373
Melichar VO, Behr-Roussel D, Zabel U, Uttenthal LO, Rodrigo J, Rupin A, Verbeuren TJ, Kumar HSA, Schmidt HH (2004) Reduced cGMP signaling associated with neointimal proliferation and vascular dysfunction in late-stage atherosclerosis. Proc Natl Acad Sci USA 101:16671–16676
Menzies AS, Aszodi A, Williams SE, Pfeifer A, Wehman AM, Goh KL, Mason CA, Fassler R, Gertler FB (2004) Mena and vasodilator-stimulated phosphoprotein are required for multiple actin-dependent processes that shape the vertebrate nervous system. J Neurosci 24:8029–8038
Moeller MJ, Soofi A, Braun GS, Li X, Watzl C, Kriz W, Holzman LB (2004) Protocadherin FAT1 binds Ena/VASP proteins and is necessary for actin dynamics and cell polarization. EMBO J 23:3769–3779
Monks D, Lange V, Silber RE, Markert T, Walter U, Nehls V (1998) Expression of cGMP-dependent protein kinase I and its substrate VASP in neointimal cells of the injured rat carotid artery. Eur J Clin Invest 28:416–423

Niebuhr K, Ebel F, Frank R, Reinhard M, Domann E, Carl UD, Walter U, Gertler FB, Wehland J, Chakraborty T (1997) A novel proline-rich motif present in ActA of Listeria monocytogenes and cytoskeletal proteins is the ligand for the EVH1 domain, a protein module present in the Ena/VASP family. EMBO J 16:5433–5444

Niedergang F, Chavrier P (2004) Signaling and membrane dynamics during phagocytosis: many roads lead to the phagos(R)ome. Curr Opin Cell Biol 16:422–428

Nimpf J, Wurm H, Kostner GM, Kenner T (1986) Platelet activation in normo- and hyperlipoproteinemias. Basic Res Cardiol 81:437–453

Nolte C, Eigenthaler M, Schanzenbacher P, Walter U (1991a) Comparison of vasodilatory prostaglandins with respect to cAMP-mediated phosphorylation of a target substrate in intact human platelets. Biochem Pharmacol 42:253–262

Nolte C, Eigenthaler M, Schanzenbacher P, Walter U (1991b) Endothelial cell-dependent phosphorylation of a platelet protein mediated by cAMP- and cGMP-elevating factors. J Biol Chem 266:14808–14812

Parangi S, O'Reilly M, Christofori G, Holmgren L, Grosfeld J, Folkman J, Hanahan D (1996) Antiangiogenic therapy of transgenic mice impairs de novo tumor growth. Proc Natl Acad Sci USA 93:2002–2007

Pawlak G, Helfman DM (2001) Cytoskeletal changes in cell transformation and tumorigenesis. Curr Opin Genet Dev 11:41–47

Peterson EJ, Woods ML, Dmowski SA, Derimanov G, Jordan MS, Wu JN, Myung PS, Liu QH, Pribila JT, Freedman BD, Shimizu Y, Koretzky GA (2001) Coupling of the TCR to integrin activation by Slap-130/Fyb. Science 293:2263–2265

Peterson FC, Deng Q, Zettl M, Prehoda KE, Lim WA, Way M, Volkman BF (2007) Multiple WIP recognition motifs are required for a functional interaction with N-WASP. J Biol Chem 282(11):8446–8453.

Prehoda KE, Lee DJ, Lim WA (1999) Structure of the enabled/VASP homology 1 domain-peptide complex: a key component in the spatial control of actin assembly. Cell 97:471–480

Price CJ, Brindle NP (2000) Vasodilator-stimulated phosphoprotein is involved in stress-fiber and membrane ruffle formation in endothelial cells. Arterioscler Thromb Vasc Biol 20:2051–2056

Pula G, Schuh K, Nakayama K, Nakayama KI, Walter U, Poole AW (2006) PKCδ regulates collagen-induced platelet aggregation through inhibition of VASP-mediated filopodia formation. Blood 108:4035–4044

Quinlan MP (2004) Suppression of epithelial cell transformation and induction of actin dependent differentiation by dominant negative Rac1, but not Ras, Rho or Cdc42. Cancer Biol Ther 3:65–70

Rao J, Li N (2004) Microfilament actin remodeling as a potential target for cancer drug development. Curr Cancer Drug Targets 4:345–354

Reinhard M, Giehl K, Abel K, Haffner C, Jarchau T, Hoppe V, Jockusch BM, Walter U (1995) The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. Embo J 14:1583–1589

Reinhard M, Rudiger M, Jockusch BM, Walter U (1996) VASP interaction with vinculin: a recurring theme of interactions with proline-rich motifs. FEBS Lett 399:103–107

Riba R, Oberprieler NG, Roberts W, Naseem KM (2006) Von Willebrand factor activates endothelial nitric oxide synthase in blood platelets by a glycoprotein Ib-dependent mechanism. J Thromb Haemost 4:2636–2644

Rodriguez-Viciana P, Sabatier C, McCormick F (2004) Signaling specificity by Ras family GTPases is determined by the full spectrum of effectors they regulate. Mol Cell Biol 24:4943–4954

Rotter B, Bournier O, Nicolas G, Dhermy D, Lecomte MC (2005) AlphaII-spectrin interacts with Tes and EVL, two actin-binding proteins located at cell contacts. Biochem J 388:631–638

Salazar R, Bell SE, Davis GE (1999) Coordinate induction of the actin cytoskeletal regulatory proteins gelsolin, vasodilator-stimulated phosphoprotein, and profilin during capillary morphogenesis in vitro. Exp Cell Res 249:22–32

Salazar MA, Kwiatkowski AV, Pellegrini L, Cestra G, Butler MH, Rossman KL, Serna DM, Sondek J, Gertler FB, De Camilli P (2003) Tuba: A novel protein containing Bin/Amphiphysin/Rvs (BAR) and Dbl homology domains links dynamin to regulation of the actin cytoskeleton. J Biol Chem 278:49031–49043

Schafer A, Burkhardt M, Vollkommer T, Bauersachs J, Munzel T, Walter U, Smolenski A (2003) Endothelium-dependent and -independent relaxation and VASP serines 157/239 phosphorylation by cyclic nucleotide-elevating vasodilators in rat aorta. Biochem Pharmacol 65:397–405

Schafer A, Widder J, Eigenthaler M, Ertl G, Bauersachs J (2004) Reduced basal nitric oxide bioavailability and platelet activation in young spontaneously hypertensive rats. Biochem Pharmacol 67:2273–2279

Schafer A, Fraccarollo D, Eigenthaler M, Tas P, Firnschild A, Frantz S, Ertl G, Bauersachs J (2005) Rosuvastatin reduces platelet activation in heart failure: role of NO bioavailability. Arterioscler Thromb Vasc Biol 25:1071–1077

Schafer A, Flierl U, Kobsar A, Eigenthaler M, Ertl G, Bauersachs J (2006) Soluble guanylyl cyclase activation with HMR1766 attenuates platelet activation in diabetic rats. Arterioscler Thromb Vasc Biol 26:2813–2818

Schwarz UR, Geiger J, Walter U, Eigenthaler M (1999) Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets – definition and detection of ticlopidine/clopidogrel effects. Thromb Haemost 82:1145–1152

Scott JA, Shewan AM, den Elzen NR, Loureiro JJ, Gertler FB, Yap AS (2006) Ena/VASP proteins can regulate distinct modes of actin organization at cadherin-adhesive contacts. Mol Biol Cell 17:1085–1095

Shattil SJ, Kashiwagi H, Pampori N (1998) Integrin signaling: the platelet paradigm. Blood 91:2645–2657

Sinnaeve P, Chiche JD, Gillijns H, Van Pelt N, Wirthlin D, Van De Werf F, Collen D, Bloch KD, Janssens S (2002) Overexpression of a constitutively active protein kinase G mutant reduces neointima formation and in-stent restenosis. Circulation 105:2911–2916

Smith GA, Theriot JA, Portnoy DA (1996) The tandem repeat domain in the Listeria monocytogenes ActA protein controls the rate of actin-based motility, the percentage of moving bacteria, and the localization of vasodilator-stimulated phosphoprotein and profilin. J Cell Biol 135:647–660

Smolenski A, Bachmann C, Reinhard K, Honig-Liedl P, Jarchau T, Hoschuetzky H, Walter U (1998) Analysis and regulation of vasodilator-stimulated phosphoprotein serine 239 phosphorylation in vitro and in intact cells using a phosphospecific monoclonal antibody. J Biol Chem 273:20029–20035

Smolenski A, Poller W, Walter U, Lohmann SM (2000) Regulation of human endothelial cell focal adhesion sites and migration by cGMP-dependent protein kinase I. J Biol Chem 275:25723–25732

Sporbert A, Mertsch K, Smolenski A, Haseloff RF, Schonfelder G, Paul M, Ruth P, Walter U, Blasig IE (1999) Phosphorylation of vasodilator-stimulated phosphoprotein: a consequence of nitric oxide- and cGMP-mediated signal transduction in brain capillary endothelial cells and astrocytes. Brain Res Mol Brain Res 67:258–266

Sudo T, Ito H, Kimura Y (2003) Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) by the anti-platelet drug, cilostazol, in platelets. Platelets 14:381–390

Tani K, Sato S, Sukezane T, Kojima H, Hirose H, Hanafusa H, Shishido T (2003) Abl interactor 1 promotes tyrosine 296 phosphorylation of mammalian enabled (Mena) by c-Abl kinase. J Biol Chem 278:21685–21692

Tanoue T, Takeichi M (2004) Mammalian Fat1 cadherin regulates actin dynamics and cell–cell contact. J Cell Biol 165:517–528

Tschoepe D, Roesen P, Schwippert B, Gries FA (1993) Platelets in diabetes: the role in the hemostatic regulation in atherosclerosis. Semin Thromb Hemost 19:122–128

Tseng SY, Dustin ML (2002) T-cell activation: a multidimensional signaling network. Curr Opin Cell Biol 14:575–580

van der Ven PF, Ehler E, Vakeel P, Eulitz S, Schenk JA, Milting H, Micheel B, Furst DO (2006) Unusual splicing events result in distinct Xin isoforms that associate differentially with filamin c and Mena/VASP. Exp Cell Res 312:2154–2167
Vasioukhin V, Bauer C, Yin M, Fuchs E (2000) Directed actin polymerization is the driving force for epithelial cell–cell adhesion. Cell 100:209–219
Volkman BF, Prehoda KE, Scott JA, Peterson FC, Lim WA (2002) Structure of the N-WASP EVH1 domain-WIP complex: insight into the molecular basis of Wiskott-Aldrich syndrome. Cell 111:565–576
Wakioka T, Sasaki A, Kato R, Shouda T, Matsumoto A, Miyoshi K, Tsuneoka M, Komiya S, Baron R, Yoshimura A (2001) Spred is a Sprouty-related suppressor of Ras signalling. Nature 412:647–651
Walders-Harbeck B, Khaitlina SY, Hinssen H, Jockusch BM, Illenberger S (2002) The vasodilator-stimulated phosphoprotein promotes actin polymerisation through direct binding to monomeric actin. FEBS Lett 529:275–280.
Wang Y, Lin H, Tullman R, Jewell CF, Jr, Weetall ML, Tse FL (1999) Absorption and disposition of a tripeptoid and a tetrapeptide in the rat. Biopharm Drug Dispos 20:69–75
Wang W, Goswami S, Lapidus K, Wells AL, Wyckoff JB, Sahai E, Singer RH, Segall JE, Condeelis JS (2004) Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. Cancer Res 64:8585–8594
Warnholtz A, Ostad MA, Velich N, Trautmann C, Schinzel R, Walter U, Munzel T (2007) A single loading dose of clopidogrel causes dose-dependent improvement of endothelial dysfunction in patients with stable coronary artery disease: results of a double-blind, randomized study. Atherosclerosis 196(2):689–695
Wentworth JKT, Pula G, Poole AW (2006) Vasodilator-stimulated phosporprotein (VASP) is phosphorylated on SER157 by protein kinase C-dependent and -independent mechanisms in thrombin-stimulated human platelets. Biochem J 393:555–564
Winocour PD (1992) Platelet abnormalities in diabetes mellitus. Diabetes 92:26–31
Wu C (2005) Migfilin and its binding partners: from cell biology to human diseases. J Cell Sci 118:659–64
Zettl M, Way M (2002) The WH1 and EVH1 domains of WASP and Ena/VASP family members bind distinct sequence motifs. Curr Biol 12:1617–1622
Zhang Y, Tu Y, Gkretsi V, Wu C (2006) Migfilin interacts with vasodilator-stimulated phosphoprotein (VASP) and regulates VASP localization to cell-matrix adhesions and migration. J Biol Chem 281:12397–12407
Zimmermann J, Labudde D, Jarchau T, Walter U, Oschkinat H, Ball LJ (2002) Relaxation, equilibrium oligomerization, and molecular symmetry of the VASP (336–380) EVH2 tetramer. Biochemistry 41:11143–11151
Zimmermann J, Kuhne R, Volkmer-Engert R, Jarchau T, Walter U, Oschkinat H, Ball LJ (2003) Design of N-substituted peptomer ligands for EVH1 domains. J Biol Chem 278:36810–36818

Scaffold/Matrix Attachment Regions (S/MARs): Relevance for Disease and Therapy

A. Gluch, M. Vidakovic, and J. Bode(✉)

Contents

1 Introduction 68
 1.1 Relevance of Non-Coding DNA: "Junk-DNA" and Gene Deserts 69
2 Scaffold/Matrix Attachment Regions (S/MARs): DNA at the Scaffold 73
 2.1 S/MAR Aberrations and Disease 75
 2.2 BURs as Targets for Anticancer Therapy 76
3 Scaffolding: Ubiquitous Fibre-Forming Components and Their Associated Functions 78
 3.1 Lamin Networks 78
 3.2 hnRNPs: SAF-A 80
 3.3 Nuclear Actin 82
 3.4 Nuclear Mitotic Apparatus Protein (NuMA) 82
4 Regulatory Networks: Key Players Qualify by Diverse Interactions with S/MARs or Scaffolds 83
 4.1 PARP 83
 4.2 S/MAR-Dependent Interactions at the IgH Enhancer 92
5 Outlook and Perspectives 95
References 98

Abstract There is increasing awareness that processes, such as development, aging and cancer, are governed, to a considerable extent, by epigenetic processes, such as DNA and histone modifications. The sites of these modifications in turn reflect their position and role in the nuclear architecture. Since epigenetic changes are easier to reverse than mutations, drugs that remove or add the chemical tags are at the forefront of research for the treatment of cancerous and inflammatory diseases. This review will use selected examples to develop a unified view that might assist the systematic development of novel therapeutic regimens.

J. Bode
Both authors contributed equally (A.G.: nuclear structure and function; M.V.: epigenetic properties of PARP)
juergen.bode@helmholtz-hzi.de

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

1 Introduction

Despite the rapid progress in sequencing eukaryotic genomes, our current abilities to interpret sequence information are still limited. Progress is expected from knowledge about the functional links between nuclear architecture and gene expression patterns on one hand and the establishment and maintenance of expression patterns during development on the other. Along these lines, the principles are explored that account for the compartmentalization of replication and transcription machineries within the nuclear compartments. These compartments assemble factors to an extent enabling protein–protein and protein-DNA interactions, and they serve the integration of regulatory signals into specific signal transduction pathways.

During recent years, epigenetic, chromatin-activating principles have entered the stage (review: Hake et al. 2004) together with the elements that delimit differentially regulated domains, so-called genomic insulators and/or boundary elements (Goetze et al. 2005). New evidence has emerged to help understand the role of chromosome territories (CTs), i.e. the structural equivalent of metaphase chromosomes at interphase, together with the interchromatin domain compartment (ICD), originally interpreted as a chromatin-free channel system in between the CTs. Ultimately, such a simplistic model was not consistent with the high frequency of complex chromosomal aberrations, which indicated the presence of inter-chromosome contacts within this space (Bode et al. 2000a; Branco and Pombo 2006).

When Kanda et al. (1998) stained the entire chromatin compartment in living cells, using histone H2b-GFP fusions they could localize putative factor storage sites, such as speckles, Cajal bodies and PML bodies, to extended portions of the interchromatin space, and they demonstrated that chromatin loops can in fact expand into this compartment. This led to the view that active genes interact with the transcriptional machinery only if they are positioned at the surface of CTs or on its looped extensions. Transcriptionally "potentiated" (otherwise called "poised") genes such as the quiescent, but inducible type-I interferon genes were found in a related position (Winkelmann, 2007). Quiescent genes on the other hand were thought to reside within the CTs. This model had to be refined once more when transcription and splicing could not only be observed at the periphery, but also appeared to extend into the territories. This was later ascribed to a highly folded CT structure that still permits access to certain genes that line this interior ICD-channel system (Albiez et al. 2006, Branco and Pombo 2006).

The question if a polymer meshwork, a so-called "nuclear matrix" or "nuclear scaffold", is an essential component of the in vivo nuclear architecture is still a matter of debate (Martelli et al. 2002). While there are arguments that the relative position of CTs may be maintained due to steric hindrance or electrostatic repulsion forces between the apparently highly structured CT surfaces, such an idea has to be reconciled with the following pilot observations:

- When Maniotis and colleagues (1997) "harpooned" nuclei, they could pull out all of the nucleoplasm on a string in interphase and all the chromosomes on a string in metaphase. Depending on the presence of Mg^{++}, they observed unwinding and rewinding of these structures. This effect was lost upon mild DNAse treatment, indicating that the structure of DNA and its associated scaffolds are responsible for this phenomenon;
- Ma et al. (1999) treated cells in situ with the classic extraction procedures that are otherwise used to isolate the nuclear matrix. Chromosome-painting techniques clearly demonstrated that territories remained intact up to the point where a minor subset of acidic nuclear matrix proteins was released – potentially those proteins that governed their association with a nuclear skeleton.

The existence of a nuclear skeleton was first proposed about 60 years ago (Zbarsky and Debov 1948), and methods for the preparation of such an entity have been developed and refined ever since (reviewed by Martelli et al. 1996). We will maintain the idea that a nuclear skeleton acts as a dynamic support for many specialized reactions as the most suggestive model to guide the reader through this review. This concept will rationalize current efforts that are dedicated to inhibitors affecting the interaction of distinct transcription factors with the protein components of such a matrix and its associated DNA elements.

After a brief overview of the architectural principles of eukaryotic genomes, our discussion will deal with the properties of certain DNA regions that can serve as scaffold/matrix attachment regions (S/MARs), which are DNA elements with a well-established spectrum of biological activities. In this context, we will address the dynamic properties of prominent constitutive and facultative fibre-forming protein scaffold constituents and continue with factors that associate with the relevant protein or DNA interaction partners. Regarding the first class, we emphasize the lamins and hnRNPs and their functional interactions. For the second class, we will focus our attention on those examples that hold promise to either assist diagnosis or to lead to administrable pharmaceuticals. Here the ubiquitous poly(ADP-ribosyl) polymerase (PARP-1) and the cell-specific factor SATB1 will serve as the cores within networks of multiple interacting factors with a relation to the scaffold, to S/MARs or to both. It is anticipated that these paradigms will strengthen work at the verge of in vivo and in vitro studies all the more as these projects can now be guided and coordinated by up-to-date system biology approaches (examples are the inserts in Figs. 1 and 2).

1.1 Relevance of Non-Coding DNA: "Junk-DNA" and Gene Deserts

The idea that the amount of DNA per chromosome set might be constant for all cells within individuals appeared more than a century ago. In 1948, Vendrely and Vendrely could confirm this assumption, and they defined the "C-value," the nuclear DNA content per cell, in all the individuals within a given species. These observations provided the first clue that DNA rather than protein is the heritable material.

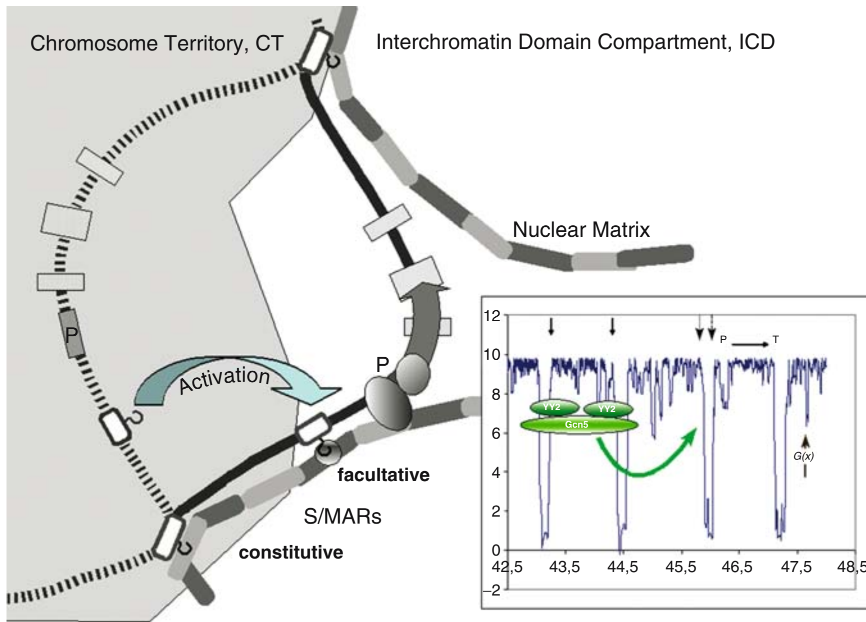

Fig. 1 S/MARs in the framework of the CT-ICD model. The eukaryotic genome is organized into chromatin domains, each of which is delimited by an extended "constitutive" S/MAR, i.e. an element that is permanently attached to components of the nuclear matrix; the matrix itself fills major parts of the ICD compartment. The assembly of activating/remodeling factors at short domain-internal scaffold/matrix attachment regions accompanies gene activation. Thereby these "facultative" S/MARs mediate the factor-induced (reversible) association with the matrix. SIDD profiles can efficiently assist the classification of these elements (reviewed by Winkelmann et al. 2006): while a constitutive S/MAR consists of a series of evenly spaced "unpairing elements" (*UEs*; minima in the SIDD profile), which together form a "base-unpairing region" (*BUR*), the "facultative" class mostly consists of 200–300-bp-long strongly destabilized individual UEs that are separated by >500 bp (see text and Bode et al. 2006). The *insert* exemplifies the latter situation: for the human interferon-β gene domain, all UEs (i.e. the four pronounced minima) coincide with DNAse I hypersensitive sites and do have regulatory potential. Two of these elements associate, each with a molecule of YY1/YY2 (*small elliptic bodies*), which in turn recruit a histone-acetyltransferase molecule (*extended ellipse*) to support activation of the inducible promoter (Klar and Bode 2005). The outline of this figure follows discussions with Thomas Werner (Genomatix Munich) and comprises the concepts by Bode and colleagues (2003a,b)

Soon it was found, however, that genome sizes vary enormously among eukaryotes and that size bears no relationship to the presumed number of genes (the so-called "C-value paradox"; Thomas 1971): while one copy of a human's genome contains about 3.5 pg of DNA packaged into 23 chromosomes, the 5.8-pg equivalent of an aardvark genome is contained in only ten chromosomes and the 140 pg in the genome of some salamanders in only 12 chromosomes. Triggered by the question whether eukaryotes evolved large genomes simply because they can tolerate useless DNA or because they need them for organization or function, the view that transcriptional regulation operates at the level of individual genes had to be continuously extended.

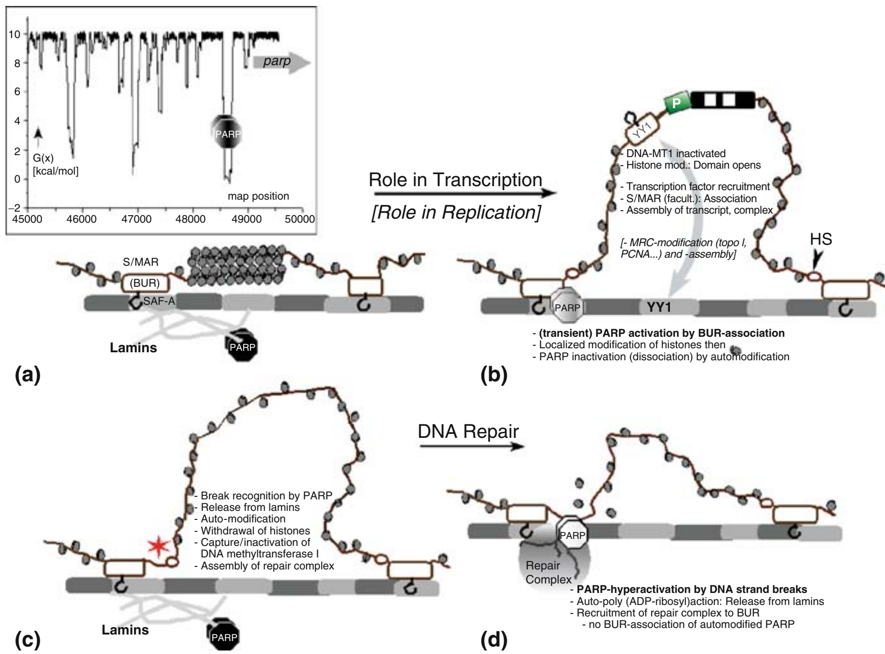

Fig. 2 Functional states of PARP-1. Inactive forms have been drawn in *black*, and increasing activity is indicated in *grey* or *light-grey colours*, respectively. The *insert* (SIDD profile, **a** analyses the PARP promoter for the presence of S/MAR-like elements, mediating the gene's autoregulation (Soldatenkov, 2002). Under certain circumstances, PARP association with an S/MAR may induce activity and enable PARP to transactivate certain genes due to a variety of domain-opening functions (Lonskaya et al. 2006); the figure (**b**) comprises both constitutive elements and a facultative S/MAR element as defined in Fig. 1. PARP-1 has also been shown to be a component of the multiprotein DNA replication complex (*MRC*); it poly(ADP-ribosyl)ates 15 of the ~40 MRC proteins, including DNA pol α, topo I and PCNA. Note the DNAse I hypersensitive site (*HS*), which is a frequent concomitant of constitutive domain borders (Sect. 3)

To account for these developments, we tend not to talk about "genes" anymore, but prefer the terms "chromatin domains" or, more generally, "transcriptional units" for any autonomous regulatory entity in the genome.

For decades, geneticists have focused on just those 2% of mammalian DNA that contain blueprints for proteins, while the remainder was sometimes dismissed as "junk". Actual scans of the mouse genome led to estimates according to which there are between 70,000 and 100,000 transcriptional units, half of which are non-coding. The discovery of these "hidden genes", which work through RNA rather than protein, has initiated a re-thinking, the more so as active forms of RNA are now known to provide an additional level of regulation. Nowadays, long genomic regions without any obvious biological function are referred to as "gene deserts" (Venter et al. 2001). Again, for some of these deserts regulatory sequences could be localized that exert control functions over large distances (Nobrega et al. 2003). Many of these

units have particular evolutionary histories and sequence signatures that make them distinct from the rest of the genome. Other gene-sparse regions, however, may in fact be nonessential to genome function, since they could be deleted without significant phenotypic effects (Nobrega et al. 2004). Information of this kind will be essential for researchers looking for mutations causing disease, because it highlights large areas of the genome that are unlikely to be involved in such a process.

In the context of such a classification, it appears rewarding to consider the genomic distribution of retroelements. Retroelements are involved in shaping the genome and have guided its evolution, extension and organization. Besides endogenous retroviruses, there are populations of truncated retroelements, such as the long, interspersed nuclear elements (LINEs), which constitute about 5% of the total human genome and may encode a functional reverse transcriptase. The short, interspersed nuclear elements (SINEs) represent an even larger proportion, but have many deletions. SINEs can modulate gene expression by movement, amplification and re-insertion into genes and regulatory sequences, but to do so they have to depend on reverse transcriptase from other sources. The human prototype SINE, the Alu repeat, is roughly 300 nucleotides in length. An RNA polymerase III start site is located within some repeats that can direct transcription in response to viral infections or the exposure to carcinogens. This expression may facilitate recombination with other Alus or their flanking regions, and this may be one reason for the fact that Alu sequences are frequent concomitants of chromosomal breakpoints. While LINEs tend to be found in AT-rich DNA, characteristic of intergenic regions, SINEs, and Alus in particular, are more often located in GC-rich regions, where genes tend to reside. This location does not seem to be a function of insertion site preference, but rather appears to be due to differential retention principles. In this respect, it is of note that SINEs participate in the transcriptional regulation of certain genes, suggesting a continuous selection against their random accumulation.

During evolution, retrotransposons have steadily screened mammalian genomes for the most attractive integration sites. For a deeper understanding, we have to consider the nature of these preferred sites. Our studies have clearly demonstrated that, without an exception, all provirus integration sites are associated with a S/MAR (Goetze et al. 2003b; Johnson and Levy 2005 and references therein). The integration of retrotransposons may obey the same rules that govern retroviral integration, and therefore the location of these elements may simply represent a marker for the presence of S/MARs. How then are S/MARs distributed over the genome? A study by Glazko et al. (2003) has localized numerous homologous intergenic tracts (HITs) of largely unknown function in orthologous human-mouse genomic regions. Fifty percent of the hits could be correlated with predicted S/MARs, which suggests that these conserved elements have probably retained their function during the 80–100 million years since the radiation from their common ancestor. The other half of predicted S/MARs turned out to be non-conserved. This group is hence likely to be species-specific and might mediate unique functions. Interestingly, an excess of orthologous S/MARs was observed in spacers between divergently transcribed genes, while there were no conserved S/MARs located between convergent genes. This distribution suggests that the conserved elements are primarily involved in the

regulation (augmentation) of transcription initiation, which would be in accord with a study from this laboratory (Schuebeler et al.1996).

2 Scaffold/Matrix Attachment Regions (S/MARs): DNA at the Scaffold

Obviously, S/MARs map to non-random locations in the genome. They occur at the flanks of transcribed regions, in 5′-introns and telomeres, and also at gene breakpoint cluster regions (review: Bode et al. 1995). S/MARs are association points for common nuclear structural proteins (review: Bode et al. 2000b and below) and proved to be required for authentic and efficient chromosomal replication and transcription, for recombination and chromosome condensation. These are the levels of their firmly established biological activities:

- *Transcriptional level*
    - S/MARs augment gene expression by increasing transcription initiation rates; they are not active in transient expression systems as they require incorporation into authentic (replicated) chromatin structures (review: Bode et al. 1995).
    - They provide long-term stability as they contribute to the assembly of the histone acetylation apparatus (review: Bode et al. 2003).
- *Transcriptional competence*
    - S/MARs either cooperate with genomic insulators or they function as insulators themselves (Antes et al. 2001; Goetze et al. 2005).
    - They enable the topological separation of independently regulated transcription units (Bode et al. 1992; review: Bode et al.1996).
- *Origin-of-replication (ORI) support*
    - Eukaryotic ORIs are consistently associated with S/MAR elements (review: Bode et al. 2001) where these provide ARS-like functions not only in yeast (AK and Benham 2005), but also in mammalia (Nehlsen et al. 2006).
- *Recombination hotspots*
    - S/MAR-associated DNA structures are involved in the generation of breakpoint-cluster regions (BCRs; review Bode et al. 2000a). They also guide the integration of retroviral genomes (Mielke et al. 1996).

There are more recent indications for an additional role in interphase chromatid cohesion and/or separation (Mesner et al. 2003). S/MARs do not have an obvious consensus sequence. Although prototype elements consist of AT-rich regions several hundred base pairs in length, the overall base composition is definitely not the primary determinant of their activity. Instead, binding and (biological) activity appears to require a pattern of “AT patches” that confer the propensity for local strand unpairing under torsional strain (Bode et al. 2006). Both chemical and enzymatic probes have originally been applied to show that this strand separation potential is

utilized in the living cell for anchoring a chromatin domain to the matrix and that DNA accessibility is modulated at times of transcriptional activity (review: Bode 1995). Subsequent bioinformatic approaches support the idea that, by these properties, S/MARs not only topologically separate each domain from its neighbours (Bode et al. 1992), but also provide platforms for the assembly of factors supporting transcriptional events within a given domain (Bode et al. 2003b).

The strand separation potential of a S/MAR is commonly displayed in the form of a stress-induced duplex destabilization (SIDD) profile, which predicts the free energy $G(x)$ needed to effect separation of the base pair at each position x along the DNA sequence, at a certain level of torsional tension (review: Winkelmann et al. 2006). The energy stored in a base-unpaired region (BUR) can serve the formation of nearby cruciforms or slippage structures. These alternate structures, as well as single-stranded bubbles, are recognizable features for DNAses, topoisomerases, poly(ADP-ribosyl) polymerases and related enzymes (see below).

Originally, matrix-attachment elements (MARs) were characterized by their specific (re-)association with the nuclear matrix (i.e. the remnants of a salt-extraction protocol), whereas scaffold-attachment elements (SARs) were mostly characterized by their (re-)association with nuclear scaffolds [i.e. the remnants of a lithium 3,5-diiodosalycilate (LIS, a mild detergent) extraction procedure in the presence of a vast excess of bacterial competitor DNA; Kay and Bode 1995]. The observation that the elements recovered by the reassociation methods are identical or closely related has led to the consensus-term “S/MAR”. Moreover, the outcome of the LIS procedure does not depend on the source of the nuclear scaffolds, as there is cross-competition between S/MARs from plants and mammals (Mielke et al. 1990), and it can be simulated by computer-assisted routines.

The binding of various forms of DNA to the nuclear scaffold has been extensively characterized (Kay and Bode 1994). As a whole, the scaffold has a strong tendency to bind single-stranded (ss) as well as supercoiled (sc) DNA. Recognition of scDNA has been ascribed to topoisomerases, since LIS-extracted scaffolds retain a pronounced nicking-closing activity. This activity appears to occur at a distinct subset of sites, as externally added S/MAR sequences and ssDNA do not interfere with the process. In contrast, there is a competition between ssDNA binding and prototype S/MAR binding on some scaffold-associated proteins, but not on others (Kay and Bode 1994; Mielke et al. 1996). In retrospect, competition patterns have proven valuable as they can be applied to reveal specific binding modes. This criterion, for example, has served to identify a novel class of S/MARs in an extended non-coding region where we detected a striking periodicity of narrow SIDD minima, which obey a periodicity of roughly 2,500 bp (Goetze et al. 2003a). A functional comparison revealed that these elements, in contrast to prototype S/MARs, have transcriptional augmentation, but no insulation activity, hinting at the existence of distinguishable classes of S/MARs (Goetze et al. 2005). While the uniform register of these elements might indicate an involvement in upper levels of chromatin organization, it is also possible that these signals serve regulatory functions, for instance in the expression of transcripts of unknown function (TUFs) that are present across large sections of the human genome.

S/MARs have been classified as either being constitutive (demarcating permanent domain boundaries in all cell types) or facultative (cell type- and activity-related) depending on their dynamic properties (details in Fig. 1 and Sect. 3.2). In the first case, the elements are marked by a constitutive DNAse I hypersensitive site in all tissues (Bode et al. 1995), which typically coincides with the preferred cleavage sites for endogenous topoisomerase II (topo II) in living cells (Iarovaia et al.1995). In the second case, hypersensitivity is correlated with either the potentiated state or active transcription (Heng et al. 2004). S/MARs partition the genome into 50–200-kb regions demarcating chromosomal (sub-)domains and/or replicons, and the number of elements that attach the ends of a (sub-)loop to the scaffold approximates 64,000 (see Fig. 1). There are likely an additional 10,000 S/MARs supporting replication foci. Apart from the technique used to derive such a conclusion, the time of observation and the cell type will also determine what subset of the total number of the estimated 74,000 S/MARs is detected (Linnemann et al. 2007). In 2006 still only a minor fraction of S/MARs (i.e. 559 for all eukaryotes) had met the standard criteria for an annotation in the S/MARt database (Liebich et al. 2000; http://sS/MARtdb.bioinf.med.uni-goettingen.de/).

Figure 1 summarizes the criteria by which the nuclear matrix/scaffold can be functionally integrated into the CT/ICD architecture.

2.1 S/MAR Aberrations and Disease

Changes in nuclear matrix attachment by either the loss of association or by binding to a previously cryptic site have been implicated in the onset of several genetic diseases and disorders. In case of male infertility, the loss of a S/MAR was found to arrest expression (Kramer et al. 1997). On the other hand, interactions between originally "domesticated" elements may trigger genomic instability such as for various forms of cancer where sites of chromosomal fragmentation localize to S/MARs (review: Bode et al. 2000). Although some of the mechanisms remain unknown, S/MARs continue to reveal a multitude of varied roles in pathogenesis that reflect aspects of their complex nature (Linnemann et al. 2007; review: Galande 2002).

Nowadays high-throughput technologies provide the opportunity to directly determine the distribution of scaffold/matrix attachment points in situ across an entire eukaryotic genome. While the first genomic arrays were based on BACs, their resolution could be increased by the use of cosmids and now of oligonucleotides. These tools are being applied to identify endogenous human S/MARs from chromosome 16 in preparations that employ nuclear extraction with either 25 mM or 2 M NaCl, which leaves the nuclear matrix DNA compact, while the non-matrix DNA forms a surrounding halo (Goetze et al. 2003b). Restriction nuclease digestion releases the loop DNA from the matrix, enabling the separation by centrifugation of matrix-associated DNA (pellet) and loop DNA (supernatant). Data for chromosome 16 highlight a variety of differential characteristics for the loop and matrix-bound portions: loop regions are relatively widespread in contrast to the discrete highly

dense regions of matrix attachment. As expected, the tightly grouped matrix-associated regions are mostly observed in gene-rich regions within intronic GC rich segments, whereas a smaller fraction localizes to the borders of extended co-regulated transcription units (Linnemann et al. 2007).

A combination of traditional fractionation protocols, combined with array and PCR technologies, will both refine and accelerate the identification of S/MARs in the human genome. It has been proposed that two of the prevailing strategies, the LIS- and NaCl-based in situ extraction procedures, recover different types of S/MARs: whereas LIS is known to disrupt transcription complexes, NaCl has been shown to disrupt replication *foci*. Accordingly, the procedures recover distinct groups of S/MARs associated with either transcriptionally inactive or active regions (Linnemann et al. 2007). Such a genome-wide identification will enable understanding the mechanistic role of S/MARs in disease and development. An example is the recent report by Petrov et al. (2006), who demonstrate a functional link between the de-localization of a defective chromosome segment from the nuclear matrix and malignant changes in gene expression. These studies underline the predictive value of identifying S/MARs in situ by the “association-approach” (Sect. 3), the more so as the results can be substantiated by a haloFISH method.

2.2 BURs as Targets for Anticancer Therapy

SIDD profiles (i.e. plots of G versus map position) are potent predictive tools for localizing “base-unpairing regions” (BURs), which are the hallmarks of S/MARs. BURs either consist of one dominant $G(x)$ minimum exceeding a threshold extension of 200–300 bp (see inserts to Figs. 1 and 2) or of a succession of multiple, evenly spaced, but moderately destabilized “unpairing elements” (UEs). In the latter case, if spacing between restricted UEs exceeds ≈500 bp, individual elements lose their capacity to communicate with each other, concomitant with a loss of scaffold-binding activity (Bode et al. 2006). In any event, part or all of the BUR would become single stranded at sufficient superhelicity. The relevance of these features is underlined by the success of BUR-affinity chromatographic separation procedures, which served to isolate and identify a considerable number of S/MAR-associated proteins (review: Galande et al. 2002), among these ubiquitous representatives such as PARP-1, Ku autoantigen, HMG-I(Y), nucleolin, mutant p53 and cell-type specific factors such as SATB1 (T-cells) and BRIGHT (B-cells). The observation that prototype BUR binders are early targets for a caspase-mediated apoptotic cleavage is of particular present interest. Among these, PARP-1 and SAF-A/hnRNP-U are cleaved simultaneously by caspase 3, whereas SATB1 is uniquely cleaved by caspase 6.

BURs have recently emerged as general targets for cancer therapy, most likely since distinct BUR-binding proteins are up-regulated in carcinomas (Galande et al. 2002). The ability of small molecules to target regions with BUR potential may therefore provide a general approach for counteracting deleterious effects in

these regions. First of all, the naturally occurring oligopeptide distamycin A prefers A/T-rich DNA sequences for binding to the minor groove of double-stranded DNA, whereby strand unpairing and the association of BUR-specific factors is prevented. This principle can be fortified by using distamycin derivatives to carry cytotoxic alkylating moieties into these regions. On the other hand, there are a number of small molecules available to target BUR-type sequences directly. This group comprises two DNA-specific antitumour agents of the cyclopropylpyrroleindole family, bizelesin and adozelesin (Schwartz et al. 2003), which share a high specificity for AT-patches/BURs. Bizelesin has the highest potential for conferring regiospecific alkylation and is one of the most cytotoxic compounds ever identified: it produces a significantly higher number of lesions in several prominent AT-rich islands within cancer cells than in the bulk of genomic DNA. Strikingly, the SIDD-derived S/MAR potential (Sect. 3 and Winkelmann et al. 2006) of the affected regions correlates well with the total number of bizelesin sites (Woynarowski et al. 2001). Presently the design, chemical synthesis and in vitro testing of BUR-specific polyamides and other cyclopropylpyrroleindole-derived small molecules are underway in order to exploit their highly effective anti-tumor potential.

So far, we have more or less considered the global properties of BUR-binding proteins. If we add, as another criterion, the class of S/MARs they interact with, the following subdivision emerges:

- Constitutive contacts: mostly established by extended S/MARs at the borders of a chromatin domain by association with ubiquitous scaffold proteins, such as scaffold attachment factor A (SAF-A, otherwise known as hnRNP-U), the lamins and possibly NuMA/actin. These "bordering elements" are accompanied by DNAse hypersensitive sites in all cell types
  - Constitutive matrix proteins bind according to the mass-binding phenomenon in a cooperative process. Series of binding sub-sites (i.e. of multiple UEs, which together form a BUR) may correspond to "AT patches," "AT hooks," "SAF boxes" or their equivalents (see Sec. 5.2 and Fackelmayer 2004).
  - Constitutive S/MARs are platforms for the assembly of chromatin-modulating proteins such as topo II and related enzymes, which at these sites initiate apoptotic degradation, histone-acetyltransferases/–deacetylases and methyl-binding proteins such as MeCP2/ARBP (Straetling and Yu 1999).
- Facultative S/MARs: more restricted, domain-internal sites, which in the extreme case consist of a single UE (insert to Fig. 1). These elements preferentially associate with tissue-specific proteins
  - These proteins may be rare transcription factors, which are concentrated at the scaffold owing to a nuclear matrix-targeting sequence (NMTS; Zeng et al. 1997) or due to their association with a central player such as PARP, which exhibits many activity-dependent modes of binding (Sect. 5.1).
  - Cell-type specific factors (such as SATB1 in T-cells, SATB2 and BRIGHT in B-cells) that establish a dynamic equilibrium within the domain (see Sect. 5.2 and the dynamic model depicted in Fig. 1).

The dynamic properties of nuclear matrix functions may explain why this scheme must not be considered a rigid one and why we have to expect factors commuting between both groups.

3 Scaffolding: Ubiquitous Fibre-Forming Components and Their Associated Functions

The backbone of eukaryotic nuclei can be isolated and characterized according to protocols that have been optimized for the removal of soluble proteins, such as histones. For instance, the scaffolds resisting LIS extraction contain components from three nuclear compartments, (1) the lamina (lamins A-C), (2) the nucleolus (nucleolin) and (3) the fibrogranular internal network intermediate filament (IF-type proteins). Ludérus et al. (1992, 1994) have studied the distribution of S/MAR binding centres over the scaffold and found attachment sites distributed equally over the peripheral nuclear lamina and the internal fibrogranular network. The domains observed in nuclear matrix preparations by confocal or electron microscopy are neither collapsed nor floating free. They are rather retained in a precise spatial relationship to other landmarks, demonstrating the function of such a supporting structure. The core of these non-chromatin nuclear structures is formed by the fibrogranular ribonucleoprotein (RNP) network together with two networks of intermediate filaments that stabilize the nuclear envelope from both the outside and the inside. On the outside, a cocoon of filaments connects the nucleus with the cytoskeleton, while the inner face of the nuclear membrane and the nucleus' interior is reinforced by lamins. In combination with these networks, S/MARs impart a wide spectrum of functional properties as they restrict genomic areas to particular nuclear compartments.

3.1 Lamin Networks

Lamin proteins are type-V IF proteins that, unlike other family members, assemble to branched filaments. Besides the cage-like framework at the inner nuclear membrane, lamins are also found throughout the nucleus: fusions of lamins A and B with GFP reveal a homogenous nucleoplasmic "veil" in addition to the intensely fluorescing nuclear lamina. Mobility measurements show that within the veil lamins are more mobile and somewhat less resistant to the conventional extraction steps. Jackson (2005) has described a branched intermediate filament network that ramifies throughout the nucleus of human cells. RNAi techniques were applied to displace each of the nuclear lamin proteins from the filaments with the consequence that the structural changes correlated with profound effects on both RNA and DNA synthesis. An almost complete cessation of transcription by RNA polymerase II and an approximately 70% decrease in the number of S-phase cells suggest that lamin networks contribute to the regulation of chromatin function.

Metazoan cells express A- and B-type lamins, which differ in their length and pI value. While B-type lamins are present in every cell, A-type lamins are only expressed following gastrulation. In humans there are three differentially regulated genes: Lamin A and C are splice variants of the LMNA gene found at 1q21, whereas lamins B1 and B2 are expressed from the LMNB1 and LMNB2 genes on 5q23 and 19q13, respectively. Lamins B (and later A) have been determined as major S/MAR-binding partners within the nuclear scaffold. The relevant binding properties could be reproduced with paracrystal-like lamin polymers revealing two activity-dependent modes that appear to be related to different features of S/MARs. One type involves the regions with single-strand potential and the other the minor groove of the DNA double strand. Both modes of association are interdependent as S/MAR binding is almost completely inhibited by the presence of single-stranded competitors.

3.1.1 Laminopathies

The recent discoveries that mutated lamins and lamin-binding nuclear membrane proteins can be linked to numerous rare human diseases (laminopathies) have changed the cell biologist's view of lamins as mere structural nuclear scaffold proteins (review: Zastrow et al. 2004). So far, however, we can only speculate why mutations in lamin A/C or in the associated emerin or the lamin B receptor genes result in such a wide range of tissue-specific phenotypes and how different mutations in the same gene can give rise to such a diverse set of diseases: Emery-Dreifuss/limb girdle muscular dystrophies, dilated cardiomyopathy (DCM), familial partial lipodystrophy (FPLD), autosomal recessive axonal neuropathy (Charcot-S/MARie-Tooth disorder, CMT2), mandibuloacral dysplasia (MAD), Hutchison Gilford Progeria syndrome (HGS), Greenberg skeletal dysplasia and Pelger-Huet anomaly (PHA). All the mentioned matrix constituents are known to interact with DNA and/or chromosomal proteins, including the core histones, and they provide a complex dynamic link between the peripheral lamina and nucleoskeletal structures. It is anticipated that understanding the cellular dysfunctions that lead to laminopathies will further enhance our insight into the specific roles of the lamina in nuclear organization (review: Burke and Stewart 2002, 2006).

A particular group of LMNA mutations leads to a progeroid disease called "atypical Werner's syndrome" (WS). Fibroblasts from affected patients show a substantially enhanced proportion of nuclei with altered morphology and a disordered lamin structure. So far, there is no molecular explanation for a progeroid disease associated with lamin functions. However, a clue may arise from relating this atypical form to the prototype WS, an inherited disease characterized by sensitivity to DNA-damaging agents, by genomic instability and premature aging: Prototype WS is caused by a missense mutation in the gene of a RecQ family helicase/exonuclease (WRN) for which one of the postulated functions is the participation in a replication complex (Chen et al. 2003). For WS cells the poly(ADP-ribosyl)ation of cellular proteins is severely impaired, suggesting a relation between WRN and PARP-1. Immunoprecipitation studies and protein interaction assays in fact indicate

direct association of PARP-1 with WRN and the assembly of a complex together with Ku70/80. In the presence of DNA and NAD^+, PARP-1 modifies Ku70/80, but not WRN, and it undergoes the typical automodification reaction. These events reduce the DNA binding capacity of Ku70/80 and its potential to stimulate WRN activity, demonstrating that PARP-1 is definitely involved in its regulation (Li et al. 2004). A report by Vidakovíc et al. (2004) proving that the association with lamins modulates the activity of PARP-1 may provide the missing link between the two forms of the syndrome (atypical and prototype; cf. Fig. 2).

Poly(ADP-ribosyl)ation has frequently been linked to longevity, as differences in the catalytic activity of PARP-1 closely correlate with differences in life span. These findings together suggest a functional link between WRN, PARP-1 and Ku70/80, which can consequently be considered as caretakers of genome integrity.

3.2 hnRNPs: SAF-A

Mattern et al. (1997 and references therein) have identified the most abundant proteins that are exclusively present in the internal nuclear network. In line with earlier reports (Nakayasu and Berezney 1991), many of these belong to the group of heterogeneous nuclear ribonucleoproteins (hnRNPs), the sites of nascent transcripts and RNA maturation. These findings support a model in which major matrix protein constituents are involved in RNA metabolism, packaging and transport.

The most abundant component of this group, called “scaffold-attachment factor A” (SAF-A/ hnRNP-U), was first characterized in LIS-extracted scaffolds (Kipp et al. 2000 and references therein). SAF-A associates with multiple S/MARs, and UV-cross-linking experiments show that this established RNA binder is also associated with DNA in vivo. HeLa-cells contain about 2 million molecules per nucleus, half of which associate with the nuclear matrix in a salt-resistant manner. The other half is either bound to hnRNP particles or resides in a DNAseI extractable fraction.

The primary structure of SAF-A reflects its dual function as there are two independent nucleic acid-binding domains, (1) a C-terminal RNA/ssDNA binding domain (RGG box) and (2) a S/MAR-specific 45-amino acid N-terminal domain, called “SAF box”, which is split and inactivated during apoptosis (Kipp et al. 2000). The SAF box is reminiscent of a homeobox lacking the DNA recognition helix and was the first characterized protein domain specifically recognizing S/MARs. SAF boxes are present in organisms as distant as yeast, plants and mammals, but not in prokaryotes, compatible with their specific binding to S/MAR-DNA. Originally unexpected, SAF-box-containing proteins from evolutionarily distant eukaryotes are not orthologs since homologies outside the SAF box are barely detectable. For most of these proteins, the function has remained unknown, with the exception of SAF-B and E1B-AP5, which serve related functions in the nuclear architecture and/or RNA metabolism. Interestingly, a poly(ADP-ribosyl)ase (PARP) from *A. thaliana* contains two SAF boxes in tandem, suggesting that these can substitute for certain functions of, for instance, Zn-finger domains (Kipp et al. 2000). Being a ubiquitous S/MAR-binding

component of the nuclear matrix, a separate chapter Sect. 5.1) will be devoted to PARP-1, the major representative of this class in mammals.

In vitro, SAF-A shows a pronounced propensity to self-polymerize, and this state is required to recognize S/MAR-DNA. Binding follows a cooperative mode, which is also typical for scaffold-S/MAR interactions (Kay and Bode 1994): each individual domain interacts only weakly with a DNA element, i.e. an UE according to Fig. 1. Only the simultaneous binding of SAF boxes to multiple UEs confers a strong and at the same time specific interaction in accord with the "mass-binding mode." This model explains the well-known phenomenon that there are hardly any naturally occurring S/MARs below a critical length of 250 bp. The failure of ssDNA to compete for the interaction of S/MARs with SAF boxes shows that SAF-box proteins alone cannot explain all criteria of scaffold-S/MAR interactions. However, a superimposition of SAF- and lamin-binding characteristics could well account for it.

Fackelmayer and colleagues (2004) have reported results from a defined model system to elucidate the role of scaffolds in DNA replication. They used extracts from *Xenopus laevis* eggs that contain all necessary components to assemble replication-competent nuclei, but do not support interfering reactions such as transcription or RNA maturation. They show, for the first time, that SAF-A provides an architectural framework on which active replication factories are assembled. Even when DNA is removed, SAF-A continues to form a nuclear reticulum. A dominant-negative approach indicates that the same is true in cultured cells. Consistent with its proposed role as a structural component, SAF-A/GFP fusions subjected to "fluorescence recovery after photobleaching" (FRAP) studies showed the protein to be rather immobile. Taken together, the data point to a structuring role of a SAF-A scaffold for DNA replication (congress report by Jackson 2005 and in preparation).

Meanwhile, SAF-A has been implicated in several more functions. Its association with histoneacetyltransferases (Martens et al. 2002) may be taken as an indication that S/MAR effects on histone hyperacetylation (Schlake et al.1994) are actually mediated by SAF-A implementing the protein in transcriptional potentiation (Sect. 3). Its involvement in the maintenance of nonviral episomes (Jencke et al. 2001) emphasizes its function in DNA replication.

3.2.1 SAF-A and Nuclear Hormone Receptor Functions

Nuclear hormone receptors are paradigms of regulated transcription factor systems that integrate signal transduction into nuclear architecture. The family includes receptors for steroid and thyroid hormones, for vitamin A and vitamin D. These receptors have become important pharmacological targets in a wide variety of clinical disorders, such as fertility issues and autoimmune diseases, which are now amenable to treatment with agonists or antagonists. It is anticipated that blocking the scaffold association of hormone receptors should have comparable effects to such an approach, but on a different level of regulation. Synergistic effects may arise when these treatments are used in combination (review: Fackelmayer 2004).

In the absence of a ligand most members of this receptor class localize to the cytoplasm. After hormone binding, they are translocated to the nucleus where they establish a punctuate pattern that is resistant to enzymatic removal of chromatin and thereby suggestive for nuclear matrix binding. It has been hypothesized that matrix-acceptor protein interactions occur in a cell-type-specific fashion. Identifying the acceptor proteins may therefore guide the development of specialized agents to modulate hormone-dependent gene expression patterns.

For the glucocorticoid receptor (GR), a minimal NMTS has been identified. If it was applied to screen for relevant acceptor proteins, SAF-A was recovered. Subsequent assays suggest that SAF-A might serve as the docking site for ligand-bound GR on the matrix. Comparable results are now available, demonstrating the specific interaction of a steroid hormone receptor with SAF-B, the second-most ubiquitous member of the SAF-box family in mammalia. When overexpressed, SAF-B exerts growth inhibition in breast tumor cells. In this context, it is noted that 20% of all breast tumors lack detectable levels of SAF-B, owing to aberrations at its gene locus (Fackelmayer 2004).

3.3 Nuclear Actin

Actin, actin-related proteins and numerous actin-binding proteins (including a nuclear-specific isoform of myosin I) are now known to be present in the nucleus, but their functions are emerging only slowly (Pederson and Aebi 2005). While there is no evidence yet for long actin filaments (F-actin), nuclear actin can form a multitude of dimers, short protofilaments and tubular, flat or branched oligomers. These nuclear actin polymers adopt a unique conformation that is recognized by specific antibodies. Since actins can also bind to two regions in the lamin A/C tail, they may be considered architectural partners of lamin filaments.

The presence of actin and nuclear myosin I (NMI) in the nucleus suggests a role for these motor proteins in nuclear functions. Although a direct participation in the nuclear matrix has remained uncertain, there have been hints for their interaction with nuclear RNAs and with proteins from hnRNP complexes (Percipalle et al. 2002), which, according to Kukalev et al. (2005), are essential for productive pol II transcription. Other studies demonstrate that antibodies against β-actin inhibit pol II transcription in a mammalian system for which actin was detected as a component of pre-initiation complexes and where it played a role in initiation (Hofmann et al. 2004). Since this inhibitory effect could be reproduced on naked DNA templates in vitro, the transcriptional role of actin does not seem to be restricted to chromatin remodelling complexes.

3.4 Nuclear Mitotic Apparatus Protein (NuMA)

NuMA, an abundant 240-kDa protein, binds to microtubules via its carboxyl terminal domain. During interphase, it is found in the nucleus, but during early mitosis it redistributes to the separating centrosomes. As a mitotic component it is essential

for the organization and stabilization of spindle poles up to the onset of anaphase. The cell cycle-dependent distribution and function of NuMA is regulated by phosphorylation (in mitosis) and dephosphorylation.

NuMA has been shown not only to bind S/MARs (Ludérus et al. 1994), but also to be part of nuclear matrix core filaments (Zeng et al. 1994). Its predicted structural features are a globular head, tail domains and a central two-stranded α-helical rod similar to members of IF family, suggesting that NuMA can form filamentous structures via coiled-coil interactions. In line with this expectation an extensive filamentous network of interconnected 5-nm fibers could be demonstrated upon transient over-expression (Harborth et al.1999). Antibodies against NuMA label portions of the nuclear matrix, but not on the 10-nm filaments. Since the actin-related nuclear proteins myosin and structural protein 4.1 bind to NuMA, this interaction may link actin-related and NuMA-related nuclear structures.

4 Regulatory Networks: Key Players Qualify by Diverse Interactions with S/MARs or Scaffolds

Our simplified view (Sect. 3.2) that constitutive S/MAR elements interact with constitutive proteins such as SAF-A or lamins does not exclude the modulation of these contacts by cell-type-specific factors. Besides, there are examples for proteins that gain or lose function by their integration into the matrix via an NMTS or by interaction with specific factors at the matrix. As an example, nuclear receptors have already been mentioned that gain activity by their association with SAF-A (Sect. 4.2.2). The proteins discussed below are involved in more diverse functions. They either interact with a plethora of other factors (PARP functions will be elaborated exemplarily since the scope of its actions is under extensive present investigation) or they are cell specific. A particularly well-studied example from the latter group is SATB1, the first member of a class of cell-type-specific fibre-forming components, which forms a functional link between the central topics of Sects. 4 and 5.

4.1 PARP

More than 40 years ago, Chambon et al. (1963) discovered that the addition of nicotinamide mononucleotide to rat liver nuclear extracts stimulates the synthesis of a polyadenylic acid, later identified as poly(ADP-ribose) (PAR). This discovery initiated research on the enzymes that regulate PAR metabolism, in particular poly (ADP-ribose) polymerase (PARP) and the de-modifying enzyme, poly(ADP- ribose) glycohydrolase (PARG). Since then 18 genes encoding members of the PARP family have been identified, among these the one encoding PARP-1, which accounts for 80% of this post-synthetic modification. PARP-1 forms homodimers, it catalyses the cleavage of NAD^+ into nicotinamide and ADP ribose, and it uses the latter to synthesize and attach nucleic acid-like polymers to Glu-residues in acceptor proteins, including

itself (automodification). The ribose-phosphate backbone of PAR has a higher negative charge even than DNA, enabling it to attract basic proteins with a specificity that is fine-tuned by its branched and helical structure. Nicotinamide, the smaller NAD^+ cleavage product, regulates PARP activity by feedback inhibition.

A still prevailing view is that the enzymatic activity of PARP-1 strictly depends on binding (via its double Zn-finger domain) to free DNA ends. This process triggers the allosteric activation of the enzyme leading to activities 10–500-fold above the so-called "basal state". A damage-induced activation alone, however, does not explain the multiple roles PARP-1 serves in the regulation of cellular functions under normal physiological conditions. Since the role of PARP-1 in DNA repair, apoptosis and necrosis has been reviewed extensively in the past (see Vidakovic et al. 2005b for a recent report from this group), the present article will emphasize PARP functions in transcription regulation. This does not exclude, however, phenomena at the intersection of both topics. As a whole, the chapter will serve to introduce and start to explain the beneficial therapeutic actions of PARP inhibitors in cancer and inflammation-related disease.

4.1.1 The Three as: Activation, Activities, and Actions

Activation: There are several levels at which the activity of PARP-1 can be regulated:

- By the classical pathway, i.e. by binding to single strand- and (even more strongly) to double-strand breaks
- By binding to certain DNA structures with base-unpairing and secondary-structure-forming potential
- By binding to DNA crossovers
- By activating factors that cause allosteric changes and/or prevent an automodification-dependent inactivation of the enzyme (Sect. 5.1.3)
- Other protein partners such as the lamins are storage sites for the inactive form of PARP-1 (Vidakovíc et al. 2004, 2005a,b)
  - PARP-binding sites in its own promoter (Soldatenkov 2002 and insert to Fig. 2), by interacting with lamins, may displace the PARP gene to nuclear periphery (lamina) and prevent its induction.

Recent evidence in fact emphasizes that, besides binding to DNA strand breaks, PARP-1 can also bind, in a cooperative manner, to the following DNA secondary structures: cruciforms, curved or supercoiled forms, crossovers and (either alone or as a complex with Ku antigen) to base-unpaired regions as found in S/MARs (Lonskaya et al. 2006). Some of these structures turned out to be even better activators than damaged DNA. The capacity of PARP-1 to bind to two DNA helices simultaneously (Rolli et al. 2000) indicates that the enzyme might be able to bind to the dyad axis where DNA enters and exits the nucleosome, providing an additional option by which its enzymatic activity can be regulated.

PARP-1 enzymatic activity is not only stimulated by the association with various DNA structures, but also by protein-binding partners. A well-investigated example

is its interaction with the transcription factor YY1, which can stimulate the enzyme as much as ten-fold (Griesenbeck et al. 1999). YY1 is accommodated by PARP-1 at its BRCT (breast cancer susceptibility protein C-terminal) domain. Interestingly, this motif overlaps its automodification domain such that part of the effect may be due to an impaired self-modification that would otherwise lead to PARP-1 inactivation. Actions of this kind might account for local heteromodification processes induced by the recruitment of PARP to particular genes.

Activities: PARP-1's catalytic domain supports several reaction types resulting either in linear or branched PAR polymers that can comprise as many as 200 units (D'Amours et al. 1999). In the absence of DNA damage, the length of the polymer is considerably shorter, ranging from single residues to oligo(ADP-ribose) units. Polymer size and complexity are determined by the relative contribution of the following activities:

- Initiation, i.e. the attachment of ADP-ribose to an acceptor protein
- Elongation
- Branching. The average branching frequency of the polymer is approximately one branch per linear repeat of 20–50 units of ADP-ribose (Alvarez-Gonzalez et al. 1999)

These activities may be regulated independently, at least to a certain extent: while the concentration of activating DNA motifs has been shown to affect the frequency of initiation, the polymer size is determined by the concentration of NAD^+ (Alvarez-Gonzalez et al. 1999). Therefore, it is conceivable that PARP-1 actions cannot only be adapted to the physiological state of the cell (as reflected by energy status and NAD^+ concentration), but also can be fine-tuned by allosteric regulators.

Actions: In line with some other proteins in this chapter (YY1, SATB1), PARP acts in a context-dependent manner (review: Kraus and Lis 2003), which means that the context determines whether it exerts activating or repressing effects:

- Each of the activating processes mentioned above has the potential to result in an opening of the respective chromatin domain, for instance by a local PAR modification of histones and non-histone proteins. Such a process facilitates transcription by RNA polymerases
- As an enhancer/promoter binding factor, PARP can support, *by association*, activators like YY1, p53, AP1, AP2, B-myb, TEF-1/Max, Sp1, Oct-1 and STATs
  - On the other hand, direct interaction with factors such as NFκ-B may inhibit the interaction with an DNA element (Chang and Alvarez-Gonzalez 2001)
- At elevated concentrations of NAD^+, PARP-1 can alternatively inactivate transcription factors such as YY1, p53, fos, SP1, CREB and TBP *by poly (ADP-ribosyl)ation*, which prevents their binding to the cognate sequences. These processes prevent initiation by Pol II, but they do not interfere with ongoing transcription
- Most of the activity-dependent effects are terminated by the PARP-1-specific automodification, which releases the enzyme from DNA or most of its interacting partners (for instance, NFκ-B, which, left alone, regains the capacity to associate with its cognate element)

PARP-1 can also serve structural roles in chromatin since it has a number of properties that are similar (but not identical) to H1, and it competes with H1 for binding to nucleosomes. In detail, PARP-1:

- Protects DNA in the linker region at the exit points of DNA from the nucleosome by binding via its double Zn finger (consistent with reports that PARP-1 can interact simultaneously with two DNA helices)
- Saturates nucleosome binding at a 1:1 molar ratio
- Increases the nucleosomal repeat length when binding to nucleosomal arrays
- In vivo, PARP-1 incorporation occurs into transcriptionally silent chromatin domains that are clearly distinct from histone H1-repressed domains. In this situation, it not only plays a structural role, but it is poised for NAD^+-dependent activation, which in turn leads to various levels of automodification, facilitating chromatin de-condensation and transcription by pol II

During these actions, transcription factors can become inactivated by poly-ADP-ribosylation and released from the DNA, thereby preventing repeated cycles of transcription. Such a process may be accompanied by histone-oligo(ADP-ribosyl)ation, an activity by which PARP-1 modulates chromatin structure. Among the histones H1 and H2B are the main substrates: while modified H1 is found associated with DNAseI hypersensitive sites (HS), core histones are the preferred targets if they are tightly bound to S/MARs. Combinatorial effects have also been described: certain acetylated H4 subspecies become predominantly tri- and tetra-(ADPribosyl)ated (Faraone-Mennella 2005). As usual, the series of events is terminated by extensive PARP automodification triggering its own dissociation from DNA.

The participation of PARP-1 in DNA repair has recently been reviewed (Vidakovíc et al. 2005b) and is briefly summarized in the lower section. The preferred occurrence of DNA strand breaks (*asterix*) at an HS is indicated (review: Bode et al. 2000a)

4.1.2 Autoregulatory Circuits

Only recent studies have suggested that a background PARP-1 activity in normal cells is an integral part of gene regulation during development and in response to specific cellular signals. As mentioned above, PARP-1 can act as a transcriptional regulator that mediates both positive and negative effects in a context-dependent manner. The latter phenomenon includes the suppression of its own promoter (Soldatenkov et al. 2002), for which we could verify the presence of S/MAR elements in several locations that were initially deduced from the BURs in an SIDD diagram (marked in the insert to Fig. 2). We could also show that the strongest (most destabilized) of the UEs mediates PARP binding and is thereby likely to be the responsible element (Vidakovic, in preparation).

Based on these data, we assume that the PARP-1 gene can be switched on upon demand, mostly to replenish the existing cellular pools consisting of non-activated

enzyme. From nuclear fractionation, crosslinking, immunoprecipitation experiments and fluorescence microscopy, we have deduced a functional interaction between non-modified PARP-1 and lamin B (Vidaković et al. 2004, 2005a). These results show that, under physiological conditions, a major part of the enzyme resides at the lamina shell. Since for mammalians the nuclear periphery represents a transcriptionally repressive compartment, it would appear conceivable that gene inactivation follows the association of the PARP promoter with the lamina.

While association with the lamina shell reflects the inactive state of PARP-1, its activation causes a gradual release as expected (Vidaković et al. 2005a). In the framework of our model, therefore, lamins are the major storage site of PARP-1. Both proteins share a high affinity for S/MAR sequences, and they preferentially recognize DNA secondary structures rather than a specific consensus (see Sects. 4.1 and 5.1.1). Because lamins belong to the substrates of PARP-1, these processes might be tuned in a combinatorial way by different degrees of poly(ADP-ribosyl)ation. This suggests that an extensive auto- and heteromodification supports the dissociation of PARP-1 from the lamina, enabling its engagement in other interactions. Dwelling of PARP-1 protein near S/MARs appears as a strategic advantage since S/MAR-associated DNA structures are a common feature of S/MAR-associated breakpoints (Bode et al. 2000a).

There are two reports for functional links between the major sections (transcription and repair) in Fig. 2.

Treatment of nuclei with RNAse or exposure to transcription inhibitors releases a subpopulation of PARP-1, indicating a role in transcription. Vispé et al. (2000) provide evidence for such a previously unrecognized pathway: PARP-1 is found to reduce the rate of transcription elongation by pol II, suggesting regulation at the level of PARP-RNA complexes. In damaged cells, binding of PARP to DNA breaks activates the enzyme in the presence of NAD^+, which promotes extensive automodification and results in its dissociation from DNA. This release allows DNA repair to commence. Since auto-modified PARP is likewise prevented from binding to RNA, mRNA synthesis is up-regulated. After the completion of DNA repair, unmodified PARP-1 will be regenerated by PARG to resume association with RNA.

A second mechanistic connection emerged between components of the DNA repair and transcription machineries: the signal-dependent activation of transcription by nuclear receptors and other classes of DNA binding transcription factors requires DNA topo IIβ-dependent, site-specific dsDNA breaks triggering PARP-1 activity. These transient breaks are in turn a prerequisite for local changes of chromatin architecture (Ju et al. 2006).

4.1.3 PARP and YY1

The function of Yin Yang 1 (YY1) 1 in transcription is context specific and requires interactions with many cellular factors. As a result, YY1 elaborates intracellular networks that allow it to induce multiple functions in transcriptional initiation, activation and repression, ultimately leading to the regulation of normal cell growth

and survival. Even the links to PARP-1 are multifold: YY1 activates the enzymatic activity of PARP-1 in a negative feedback mode as the process is terminated by PARP automodification (Oei and Shi 2001). Moreover, YY1 binding sites are present in the distal region of human PARP-1 promoter, suggesting that this factor participates in PARP-1- gene expression. These examples already suggest multiple functional relationships between both proteins in response to various stimuli.

YY1 was introduced by Guo et al. (1995) as "nuclear matrix protein 1" (NMP-1), i.e. as a DNA-binding factor with sequence-specific recognition of a regulatory element next to a histone H4 gene. Findings of this type suggest that YY1 may mediate gene-matrix interactions and participate in the assembly of multimolecular complexes. Using deletion constructs, McNeil et al. (1998) have demonstrated the role of the C-terminal domain in accord with data by Bushmeyer et al. (1998), who localized a nuclear matrix targeting signal to the Zn-finger region, a domain already known to mediate binding to DNA and to associate with components of the histone-acetylation apparatus (both HAT and HDAC are components of the nuclear matrix). It appears likely that at least some of these interactions are mutually exclusive; therefore, it will be of interest to determine how the association of YY1 with its binding partners is regulated and what role the related factor YY2 (Klar and Bode 2005) plays in this circuitry.

The precise localization of YY1/YY2-binding sites at the flanks of a destabilized region (insert to Fig. 1) deserves particular attention since it indicates the evolutionary conservation of both a potent binding motif and the ability to undergo strand separation. Although YY1 is a factor that requires both DNA strands simultaneously for its binding, this situation may require a flexible DNA backbone, the more so as certain actions of YY1 are ascribed to its DNA bending potential and the direction of the respective bend.

4.1.4 PARP, wt-p53 and mut-p53

Numerous studies have indicated a critical role for PARP-1 and p53 in the maintenance of genome integrity. Both proteins promote base excision repair (BER) via physical interactions with the BER protein complex. While the possible actions are manifold, both the ability of p53 to degrade mis-paired intermediates and the anti-recombinogenic function of PARP-1 may be relevant. PARP-1 exerts this function by protecting strand breaks before the repair complex has correctly been assembled. p53 has a high affinity for auto-modified PARP-1, which in turn is involved in the activation of p53 protein in response to DNA damage and other stimuli. These and numerous additional observations demonstrate that PARP-1 is an essential cofactor in the activation cascade of p53-dependent target genes.

Deppert and co-workers were the first to trace p53 to the nuclear matrix, while Jiang et al. (2001) have demonstrated that this association increases following DNA damage. In an effort to understand p53 functions by their relation to nuclear structures, Okorokov et al. (2002) found that a potential nuclear matrix component for such an association is nuclear actin (Sect. 4.3), the more so as this interaction is strengthened

during DNA damage. The dynamic interaction of p53 with the nuclear matrix is therefore one key to understanding the p53-mediated cellular responses to DNA damage.

Being a tumor suppressor gene, the presence of p53 reduces the occurrence of tumors by promoting apoptosis in cancer cells. A common pathway is the linear one involving bax transactivation, bax translocation from the cytosol to membranes, cytochrome c release from mitochondria and caspase-9 activation, followed by the activation of caspase-3, -6 and -7. p53 is modified by PARP early during apoptosis, leading to its stabilization. Only at later stages is PARP itself cleaved by caspase 3, and PAR is removed from p53 concomitant with the onset of apoptosis. On the

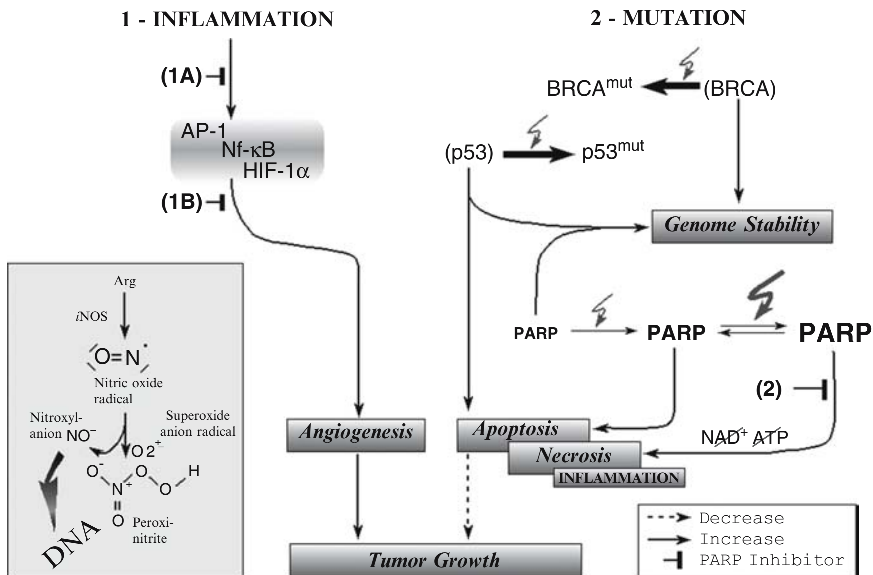

Fig. 3 PARP inhibitors counteract tumor growth and excessive inflammatory responses. Neutrophilic granulocytes mediate the activation of PARP-1 via the products of NO conversion (*insert*), which in turn supports the expression of pro-inflammatory peptides: activator protein 1 (*AP-1*) is a complex from c-jun and c-fos family members out of which the expression of c-jun becomes elevated during inflammatory lesions. NF-κB (p60 × p50) levels are rather constant, but the factor is kept in an inactive state due to association with I-κB. Inflammatory responses resolve the complex and cause NF-κB translocation to the nucleus. After their activation, both AP-1 and NF-κB induce their respective target genes (encoding inflammatory cytokines and matrix-degrading metalloproteinases). HIF-1 adapts leukocyte metabolism to low oxygen pressure as described in the text. PARP inhibitors prevent angiogenesis (and thereby tumor growth) by repressing both the pro-inflammatory (*1A*) and the induced inflammatory factors (*1B*). Regarding the effect of mutations, PARP-1 can, in part, substitute for the action of tumor suppressors (exemplified by p53) and cancer-susceptibility genes (exemplified by BRCA2) if these become inactivated. In this case, PARP-1 inhibitors serve to prevent the consequences from an overshooting activity (i.e. necrosis and subsequent inflammatory responses). If the severe depletion of NAD^+ and ATP pools can be relieved by PARP-1 inhibition, cells may enter the apoptotic pathway even in the absence of p53. If existing damage can be reversed, PARP may aid in approaching genome stability, at least to the extent that might be possible in the presence of p53-mutants

other hand, many apoptosis-related genes become transcriptionally regulated by p53. A full understanding of the mechanisms by which these and other factors induce apoptosis, and the reasons why cell death is bypassed in transformed cells, is of fundamental importance in cancer research and has great implications in the design of novel anticancer therapeutics (see Fig. 3).

While wt-p53 is well known for its tumour suppressor functions and for serving as a “guardian of the genome”, mutation in the p53 gene is the most common event in cancer. This is the case for 80% of all colon tumors, 50% of lung tumors and 40% of breast tumors. A characteristic feature of its mutational spectrum is the frequency of missense point mutations at six hot spots within the region encoding the central DNA-binding domain of the protein. Most of these mutations not only lead to the inactivation of its tumor suppressor activity, but also confer oncogenic properties to its mutant forms (reviews: Deppert 1996; Kim and Deppert 2004). Actually, the transcriptional effects mediated by mut-p53 follow a mechanism totally different from p53 as they arise in the absence of specific responsive elements and affect an entirely different spectrum of genes that are either modulated in the positive or in the negative sense. It is intriguing that the search for *cis*-acting elements, which are targets of mut-, but not wt-p53, led to a variety of S/MARs and that the gain-of-function mutations in p53 could be correlated with the acquisition of a high S/MAR-binding potential ($K_d \sim 10^{-10}$ M). In Sect. 3 we have introduced S/MARs as regulatory DNA elements important for higher-order chromatin organization, long-range enhancer function and the propagation of chromatin modifications. This suggests that mut-p53/S/MAR interactions may serve to activate the expression of genes involved in cell proliferation and tumorigenesis, for instance, by forming a DNA loop that brings *cis*-acting regulatory elements into apposition with the respective promoters. Such a model might involve a p53-mediated sub-looping of chromatin loops for gene activation (Fig. 1) or the disruption of otherwise constitutive gene structures by interfering with the functions of domain borders.

4.1.5 PARP and BRCA1/2

In 1994, two human breast cancer susceptibility genes were identified: BRCA1 on chromosome 17 and BRCA2 on chromosome 13. Individuals with a mutation in either BRCA1 or BRCA2 are at risk of developing breast or ovarian cancer at some point in their lives. It was not clear what the function of these genes is until studies on a related protein in yeast revealed their normal role in the repair of radiation-induced breaks in double-stranded DNA. Now it is thought that mutations in BRCA1 or BRCA2 might disable this mechanism, leading to errors in DNA replication and ultimately to cancerous growth. Studies of *brca1*- and *brca2*-deficient cells in fact indicate that BRCA2 controls the intracellular transport and activity of RAD51, a protein necessary for double-strand break (DSB) repair by homologous recombination (HR). For BRCA1, the mode of action is less clear, but it might involve regulation of the MRE11 exonuclease that is required for creation ssDNA segments at the sites of DSBs.

Sub-cellular fractionation experiments showed a tight interaction with the nuclear matrix for most of BRCA1/2, and this localization was maintained following the treatment with DNA damaging agents that activate homology-mediated DSB repair pathways. Therefore, BRCA1 and -2 add to the list of examples where proteins act from positions that are anchored to the nuclear matrix. These data are consistent with models suggesting that components of specific repair complexes reside at the nuclear matrix to recruit damaged DNA (Huber and Chodosh 2005).

PARP1 activity becomes vital in BRCA2-deficient cells. As a result of their deficiency in HR, these cells are acutely sensitive to PARP inhibitors, presumably because collapsed replication forks are no longer repaired. This requirement can be exploited in order to specifically kill BRCA2-deficient tumours by PARP inhibitors. Such use of a DNA repair inhibitor for the selective killing of a tumour in the absence of an exogenous DNA-damaging agent represents a completely new concept in cancer treatment (Fig. 3).

4.1.6 PARP as a Therapeutic Target

The example of BRCA2 has introduced genes that counteract cancerous growth and for which PARP may play a complementary role. In other words, cells that lose one of these players have to rely on PARP for DNA repair and survival. If PARP is disabled, however, this will lead to the accumulation of DNA damage, causing the shift from repair to apoptosis (review: Vidakovíc et al. 2005b). This in turn may be the reason why PARP inhibition has proven beneficial in antitumour therapy. In a second scenario, damage might lead to the excessive over-activation of PARP. The associated depletion of NAD^+ and ATP would then result in necrosis and, as a consequence, to cell leakage and associated inflammatory responses. If PARP activity is reduced, cells might enter the apoptotic route instead, leading to the desired outcome.

Second-generation PARP inhibitors have now entered the field to support the chemotherapy and radiotherapy of human cancers (review: Virág and Szabo 2002). Their administration together with cytotoxic drugs that cause persistent single- and double-strand DNA has potentiated their activity. These principles are summarized in the right section of Fig. 3 (size of PARP lettering reflects PARP-1 activity).

4.1.6.1 PARP Inhibitors in Inflammation

Inflammation is the first response of the immune system to infection, irritation or other injury. Components of the immune system immediately infiltrate the affected site leading to an increased blood supply and vascular permeability. Neutrophils are at the forefront of cells to appear in the infected area where they trigger a local oxidant burst. This pulse is primarily directed against the invading agent, but it may give rise to pro-inflammatory factors, via DNA lesion/PARP activation, in nearby professional and nonprofessional immune cells. Peroxynitrite, a labile, toxic oxidant arising from the reaction of superoxide anion radical

and nitric oxide (NO), is considered to be the main trigger (Virág and Szabó 2002). These compounds as well as the nitroxyl anion and the hydroxyl radical induce DNA single strand breaks, leading to PARP-1 activity. In this context, it is noted that neutrophilic and eosinophilic granulocytes are the only known mammalian cells that do not contain PARP (Virág and Szábo 2002), probably since its presence would not be compatible with the high levels of local oxidant production they cause.

Different mechanisms have been proposed to explain the fact that an inhibition of PARP-1 improves the outcome of a variety of pathophysiological conditions associated with an irritated tissue and systemic inflammation:

- According to the "suicide hypothesis", the peroxynitrite-induced massive PARP-1 activation leads to a rapid depletion of NAD^+ and ATP. The same agent also induces mitochondrial free-radical generation, which amplifies these effects to trigger necrosis.
- While the extent of PARP-1 activation serves as a molecular switch between necrosis and apoptosis, the associated NAD^+ consumption acts as the metabolic link between DNA damage and cell death.
- Moderate PARP-1 activities promote transcriptional activation in lymphocytes. PARP-1 inhibitors have proven useful in this context and are applied for the treatment of autoimmune disorders, for instance, of the central nervous system.

Inflammatory actions disrupt the vascular structure around the site of injury and thereby lead to decreased oxygen pressure. The responding immune cells have to adapt to these conditions, and it has been found that leukocytes have the unique ability to cause a metabolic switch using HIF-1 (hypoxia-inducible transcription factor-1; Fig. 3). The PI3-kinase/Akt pathway and MAP kinase cascade, respectively, are involved in these pro-inflammatory responses. The adjustment of HIF-1 levels as well as the attenuation of NF-κB- and AP-1 actions by PARP inhibition has proven beneficial in this context, the more so as these measures restrict angiogenesis, which would otherwise support tumor growth (Martin-Oliva et al. 2006).

4.2 S/MAR-Dependent Interactions at the IgH Enhancer

Section 4.2 has shown that most of the ubiquitous proteins at the scaffold comply with BUR-binding characteristics (review: Galande 2002). While these proteins differ widely regarding their DNA-binding domains, it is noteworthy that most of them comprise a structural motif that might confer high affinity towards BURs. Recently, the SAF box could be localized in several DNA-binding proteins and can now be considered as one of the prototype domains. Another motif, the 11-residue "AT hook", was first detected in the HMG-I(Y) protein and associates with multiple A/T tracts that are separated by 6–8 bp, resembling the architecture of BURs. HMG1/2 and related proteins with multiple HMG boxes recognize irregular DNA structures in a sequence-nonspecific manner. One preferred substrate is the cruciform structure that arises by intrastrand

pairing at inverted repeats that are common in S/MARs (Mielke et al. 1996). As base unpairing is a prerequisite for cruciform formation, the latter process directly depends on SIDD properties. While the DNA binding features of PARP-1 and YY1 are governed by Zn-finger domains, the PARP-1/Ku70/86 complex recognizes S/MARs, probably as a consequence of its ss-DNA recognition potential (Galande and Kohwi-Shigematsu 1999). Our own laboratory has detected that YY1 consensus sequences with regulatory potential are consistently found adjacent to UEs, probably in order to utilize the factor's bending potential (see Sect. 5.1.3 and Klar and Bode 2005).

The following section will extend these aspects to cell-specific BUR binders in lymphocytes and discuss the regulatory networks of which they are part. Here, the immunoglobulin heavy-chain (IgH) locus has become a paradigm. Within the locus, transcription is controlled by promoters, located 5' of the individual variable (VH) gene segments, and by a composite downstream enhancer (Eμ). The Eμ region can be subdivided into an enhancer core (220 bp) and two 310–350-bp flanking S/MARs that were first defined by matrix-binding assays in vitro. According to our convention, these S/MARs are classified as being "facultative elements" (Fig. 1) as the associating factors, in striking contrast to the ubiquitous core-enhancer binding counterparts, are cell-type restricted. They have, therefore, the potential to cause dynamic changes of nuclear structures in accord with their suggested function.

4.2.1 SATB1

A factor binding to the 3′ Eμ-associated S/MAR in T-cells, special AT-rich DNA binding protein 1 (SATB1), became the founding father of all BUR binders when it was identified and cloned by virtue of its ability to bind to the core-unpairing element (CUE) located within the 3' S/MAR. SATB1 does not associate with mutated CUEs that lack the unwinding property (Bode et al. 1992). Since it neither binds to nor is competed off by ssDNA, the CUE must be recognized indirectly through an altered sugar phosphate backbone. Thereby it differs from other S/MAR-binding proteins such as mutant p53, which trigger a strand separation. We have anticipated, therefore, that its association would inhibit transcription, and our early data were in accord with this (somewhat simplistic) expectation (Kohwi-Shigematsu et al. 1997).

SATB1 comprises an unusual combination of a S/MAR-binding domain and a homeodomain, both of which are necessary for the recognition of the CUE. In addition, a dimerization motif is needed for BUR binding (Cai et al. 2003). This motif is homologous to PDZ domains, modular protein-binding structures with at least three distinct types of binding:

- Association with specific recognition sequences at the carboxy-termini of proteins
- Association with other PDZ domains to form heterodimers
- Homodimerization, which for SATB1 is one prerequisite for the recognition of BURs

The fact that a BUR-binding protein contains a putative PDZ domain has important biological implications as it is to be expected that SATB1 functions can be strengthened

or modulated by recruiting other PDZ-containing proteins to the S/MARs. This is the likely mechanism by which SATB1 builds up dynamic, cage-like structures within the nucleus (see below).

SATB1 was among the first cell-type-restricted S/MAR binders. It is expressed predominantly (but not exclusively: see Wen et al. 2005) in thymocytes where it represents one of the few gene products that are induced early upon peripheral T cell activation. A biological function emerged from the phenotype of SATB1 knockout mice, where SATB1 was found essential for orchestrating the spatial and temporal expression of a large number of T cell- and stage-specific genes: in the absence of SATB1, T cell development was severely impaired, and immature CD3-/CD4-/CD8- triple negative thymocytes were largely reduced in number. At the same time, SATB1-deficient thymocytes and T-cells in lymph nodes became prone to apoptosis.

The comparison of SATB1 knockout and wild-type mice indicated a role for SATB1 in the dysregulation of about 2% of the genes (Alvarez et al. 2000), also evidenced by the fact that the respective S/MARs were found to be detached from the nuclear matrix in vivo. These observations support the hypothesis that, in their normal context, specific genes are actively anchored to the nuclear matrix and that this association enables an appropriate regulation. Together these data show that there are factors acting as BUR-dependent regulators of cell function. Although the field is still in its infancy, other proteins that are readily detected on Southwestern blots or that are retained on BUR affinity columns may serve related functions at the bases of chromatin loop domains (Galande 2002).

How do SATB1 functions relate to details of the nuclear architecture? Cai et al. (2003) have demonstrated a then unknown nuclear distribution, i.e. a "cage-like" SATB1-containing structure circumscribing heterochromatic areas. They showed that this cage shares properties with a nuclear matrix as it resists the conventional extraction steps. The localization of the SATB1 network outside heterochromatic regions agrees with a model in which the attachment points (BURs) in the network provide landing platforms for chromatin-remodeling complexes (CHRAC, NURD), which constantly rearrange nucleosomes to support both positive and negative transcriptional actions. These pilot findings have widened our view into how a single protein can link the expression of hundreds of genes to nuclear organization.

4.2.2 SATB2

Dobreva et al. (2003) characterized a novel cell type-specific S/MAR-binding protein, SATB2, which is abundantly expressed in pre-B- and B-cells where it serves certain SATB1-like functions. SATB2 differs from its closely related thymocyte-specific relative by sumoylation-dependent modifications. Sumoylation is a recently detected post-translational modification system, biochemically analogous to, but functionally distinct from, ubiquitinylation as it involves the covalent attachment of a SUMO (small ubiquitin-related modifier) sequence to substrate proteins. Mutation of two internal conjugation sites (lysines) clearly enhances its activation potential

in parallel to the association with endogenous S/MARs in vivo, whereas N-terminal fusions with SUMO decrease SATB2-mediated gene activation. Since sumoylation is involved in targeting SATB2 to the nuclear periphery, this may cause modulation of SATB2 activities.

4.2.3 BRIGHT and NF-μNR

BRIGHT (B cell regulator of IgH transcription) is yet another factor transactivating the intronic IgH enhancer by binding to the S/MARs as a tetramer (Kaplan et al. 2001). BRIGHT contains regions homologous to the Drosophila SWI complex, suggesting that it might be involved in chromatin remodelling. A number of experiments suggest that the cell-type specificity of the Eμ enhancer is governed by negative regulatory mechanisms that are dominant to this and other B-cell specific transcriptional activators and that these actions can be ascribed to interference with nuclear matrix attachment. A responsible negative regulatory factor, first called NF-μNR and later found related to Cux/CDP, binds to multiple sites flanking IgH enhancer. Interestingly, the expression of NF-μNR displays a unique developmental pattern, as it is present in most cell lines outside of the B-cell lineage (T cells, macrophages and fibroblasts), but also in B-cells early in development. In contrast, it is absent from more mature cells that express high levels of IgH chains (Wang et al.1999).

5 Outlook and Perspectives

The term “epigenetics” was introduced in the 1940s by the British embryologist and geneticist Conrad Waddington as *the interactions of genes with their environment, which bring the phenotype into being*. Nowadays, the term refers to multiple modifications that influence gene activity without altering the DNA sequence. In the past, research has focused on transcription factors and signal transduction pathways associated with turning genes on and off. Only in the last decade scientists have touched the next layer in the flow of information, the more so as there was awareness of an “epigenetic code” that is central to processes such as development, aging, cancer, mental health and infertility. Since epigenetic changes are much easier to reverse than mutations, drugs that remove or add the chemical tags are at the forefront of cancer therapy.

The first epigenetic layer to be implicated in gene expression was DNA methylation. Drugs inhibiting DNA methyltransferases, such as Vizada (5-azacytidine) and the related Decitabine, are now established agents in the treatment of cancer, as they have the potential to re-activate specific tumour suppressor genes. Probably even closer to the steering centre are the elaborate principles of the “histone code”, which determines how covalent histone modifications such as acetylation, methylation and PAR-addition modify chromatin accessibility to enable or tune gene expression (Hake 2004; Henikoff 2005). This code has become indispensable for

cancer specialists who utilize its principles to develop diagnostic tools and drugs. Virtually every major pharmaceutical company has meanwhile set up a program on histone decacetylases (HDACs) and their inhibitors that mediate chromatin opening by histone (hyper-)acetylation. The acquisition of Acton Pharma by Merck in 2004 provided access to SAHA (suberoylanilide hydroxamic acid), a second-generation drug of this class, and is only one recent example for such a commitment.

From a chemical viewpoint, all these histone modifications change the molecular crowding, electrostatic environment and other push-and-pull actions that are central to the "tensegrity concept" (Maniotis et al. 1997) and the "unified matrix hypothesis" (Scherrer 1989). Awareness is increasing that, ultimately, how chromatin works will turn out to be a scaffolding problem. The fact that histone modifications are mechanistically linked to DNA methylation and nuclear organization will inevitably lead to the merger of fields that, for a long time, existed side by side. Molecular mechanisms controlling gene expression include mechanical and chemical signal transduction pathways. In the framework of this view, it is the "*tensegrity*" (tensional integrity) that couples together the mechanical functions of filamentous structures, providing a balance between tension and compression. It could, in fact, be shown that mechanical stretch imposed upon a cell induces numerous biological responses, including alterations in the cytoskeleton, activation of cell signalling pathways and upregulation of transcription factors. The "unified matrix hypothesis" explains these phenomena by integrating the three skeletal networks, i.e. the extracellular matrix (ECM), the cytoskeleton (CS) and the nuclear matrix (NM), into a global scaffolding system.

Regarding the nuclear scaffold/matrix, one of the seminal findings is its dynamic aspects: although a fixed proportion of the total nuclear matrix proteins (NMPs) is always present, a subset of components, among these rare transcription factors, show variations according to the type and differentiation or transformation status of the cell. Here the structure serves to concentrate these proteins via NMTS signals or related principles and to deliver them to the respective S/MAR-associated control elements. The relevance of knowing about the identity of these specific NMPs became obvious when Fey and Penman (1988) documented a striking cell type specificity based upon a modified procedure for isolating the nuclear matrix-intermediate filament (NM-IF) portion of the nucleus. Soon thereafter, a correlation with cancer became apparent, explaining the observation that very often the cancer cell nuclei are oddly shaped. Antibodies served to detect cancer-specific NMPs that escape into body fluids in a process that is accelerated by the action of cytostatica. Since the late 1980s, these processes have been adapted to cancer diagnosis, and a test for NMP22 could be introduced based on the level of this protein in the urine of patients with bladder cancer (Getzenberg 1994). Subsequently, dedicated proteomics technologies provided access to NMP markers for cervical, breast, prostate and colon cancer. NMP tests of this type obviate any risk of infection, and they are now on the market for one sixth the cost of the cystoscopy-based routines.

Returning to the unified-matrix hypothesis, tumour cells from MLL rat prostate cancer were investigated with respect to their NMP profile. When these cells

were implanted at different organ sites, they developed a novel distinct and organ-specific NMP composition (Replogee-Schwab et al. 1996). Such dependence on the composition of proteins on the nuclear matrix is attributed to a tensegrity-based signal transduction, which initiates in the extracellular matrix microenvironment and terminates within the nucleus. This review has tried to provide a glance into the multiple influences that modulate nuclear matrix structures and functions.

A related aspect, the development of "chromosome-based" vectors, evolved in parallel to these efforts and has been reviewed before (Lipps et al. 2003). This field is dedicated to using chromosomal elements, and S/MARs in particular, to design independent regulatory units, so-called "artificial chromatin domains". After their integration, two bordering elements with insulating capacity will shield such a unit from the generally negative influences at the integration site (Bode et al. 1995; Goetze et al. 2005). An even greater challenge arose when it became possible to reduce the complexity of such a domain to a single S/MAR element and an actively transcribed gene. In the context of a circular (and hence supercoiled) construct, such a vector functions as a self-propagating (replicating) episome in the absence of any viral element. The recent demonstration that the performance of the system could be largely improved by deleting prokaryotic vector parts is considered a breakthrough: being composed of only eukaryotic modules, the resulting "minicircles" successfully escape the defense mechanisms of the host cell and show an extended–maybe unlimited–replication potential in the absence of selection pressure (Nehlsen et al. 2006). Again, it is the S/MAR that provides the link to the nuclear matrix (Jenke et al. 2001). In this position S/MARs not only enable the use of the endogenous transcription factories, but they also counteract silencing. Early observations link these properties to the episome's interaction with the histone acetylation machinery (Jenke et al. 2001).

Acknowledgements This review relies on the firm experimental fundament laid by our (former) coworkers Sandra Broll, Sandra Goetze, Volker Kay, Martin Klar, Dagmar Klehr, Karin Maass, Christian Mielke, Kristina Nehlsen, Thomas Schlake, Hennrik Schröter, Dirk Schuebeler, Achim Pucher, Edgar Wingender and Silke Winkelmann and on many fruitful contacts with the laboratories of Vincent Allfrey (New York), Zoya Avramova/Alexander Tikhonov (then: West Lafayette), Jörg Bartsch (then: Marburg), Craig Benham/Prashanth AK (Davis), Teruhiko Beppu/Minoru Yoshida (Tokyo), Ron Berezney (Buffalo), Ruth Brack-Werner/Thomas Werner (Munich), Morton Bradbury/Peter Yau (Davis), Paola Caiafa (Rome), Howard Cedar/Yehudit Bergman (Jerusalem), Wolfgang Deppert (Hamburg), Manuel Diaz/Pam Strissel (Chicago), Roel van Driel (Amsterdam), Frank Fackelmayer (Hamburg), Dean Jackson (Manchester), Terumi Kohwi-Shigematsu (Stanford), Steve Krawetz (Detroit), Ulli Laemmli (Geneva), Christine Leib-Moesch (Munich), Rolf Marschalek (Frankfurt), Goran Poznanovíc (Belgrade), Eugene Sverdlov/Lev Nikolaev (Moscow), Bernd Puschendorf (Innsbruck), Alla Rynditch (Kiev), Marc Shulman (Toronto), Steve Spiker (Raleigh), Gabor Szabo (Debrecen), Ken Tsutsui (Okayama), Carlo Turano/Anna Ferraro (Rome), Ole Westergard (Aarhus), Lothar Willmitzer/Joerg Stockhaus (then: Berlin), Wolf Straetling (Hamburg), Gad Yagil (Rehovot) – all in alphabetical order. Recent work in the lab of the authors was supported by the Alexander von Humboldt/Hertie Foundation (Roman Herzog stipend to MVi), a grant from the Helmholtz "Wiedereinstiegsprogramm" (AGl) as well as by funding from the FW6 "Epivector" program (JBo) and the CLINIGENE NoE.

References

AK P, Benham C (2005) Susceptibility to superhelically driven DNA duplex destabilization: a highly preserved property of yeast replication origins. PloS Comput Biol 1:41–46

Albiez H, Cremer M, Tiberi C, Vecchio L, Schermelleh L, Dittrich S, Küpper K, Joffe B, Thormeyer T, von Hase J, Yang S, Rohr K, Leonhardt H, Solovei I, Cremer C, Fakan S, Cremer T (2006) Chromatin domains and the interchromatin compartment form structurally defined and functionally interacting nuclear networks. Chromosome Res 14:707–733

Alvarez JD, Yasui DH, Niida H, Joh T, Loh DY, Kohwi-Shigematsu T (2000) The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes during T-cell development. Genes Dev 14:521–535

Alvarez-Gonzalez R, Watkins TA, Gill PK, Reed JL, Mendoza-Alvarez H (1999) Regulatory mechanisms of poly(ADP-ribose) polymerase. Mol Cell Biochem 19319–19322

Antes TJ, Namicu SJ, Fournier REK, Levy-Wilson B (2001) The 5’-boundary of the human apolipoprotein B chromatin domain in intestinal cells. Biochemistry 40:6731–6742

Bode J, Kohwi Y, Dickinson L, Joh T, Klehr D, Mielke C, Kohwi-Shigematsu T (1992) Biological significance of unwinding capability of nuclear matrix associating DNAs. Science 255:195–197

Bode J, Schlake T, Rìos-Ramìrez M, Mielke C, Stengert M, Kay V, Klehr-Wirth D (1995) “Scaffold/matrix-attached regions: structural properties creating transcriptionally active loci”. In: Berezney R, Jeon KW (eds) International review of cytology 162A, on “Structural and Functional Organization of the Nuclear Matrix”, Academic Press, San Diego, pp 389–453

Bode J, Stengert-Iber, M. Schlake T, Kay V, Dietz-Pfeilstetter A (1996) Scaffold/matrix-attached regions: topological switches with multiple regulatory functions. Crit Rev Eukaryot Gene Exp 6:115–138

Bode J, Benham C, Ernst E, Knopp A, Marschalek R, Strick R, Strissel P (2000a) Fatal connections: when DNA ends meet on the nuclear matrix. J Cell Biochem Suppl 35:3–22

Bode J, Benham C, Knopp A, Mielke C (2000b) Transcriptional augmentation: Modulation of gene expression by scaffold/matrix attached regions (S/MAR elements) Crit Rev Eukaryot Gene Exp 10:73–90

Bode J, Fetzer CP, Nehlsen K, Scinteie M, Hinrich BH, Baiker A, Piechazcek C, Benham C, Lipps HJ (2001) The hitchhiking principle: Optimizing episomal vectors for the use in gene therapy and biotechnology. Gene Ther Mol Biol 6:33–46

Bode J, Goetze S, Ernst E, Huesemann Y, Baer A, Seibler J, Mielke C (2003a) Architecture and utilization of highly-expressed genomic sites in “New Comprehensive Biochemistry 38”. In: Bernardi G (ed) Gene transfer and expression in mammalian cells, S. Makrides. Chap 20, Elsevier, Amsterdam, pp 551–572

Bode J, Goetze S, Heng H, Krawetz SA, Benham C (2003b) From DNA structure to gene expression: mediators of nuclear compartmentalization and dynamics. Chromosome Res 11(5):435–445

Bode J, Winkelmann S, Goetze S, Spiker S, Tsutsui K, Bi C, AK P, Benham C (2006) Correlations between scaffold/matrix attachment region (S/MAR) binding activity and DNA duplex destabilization energy. J Mol Biol 358:597–613

Branco MR, Pombo A (2006) Intermingling of chromosome territories in interphase suggests role in translocations and transcription-dependent associations. PLoS Biol May 4(5):e138

Burke B, Stewart CL (2002) Life at the edge: the nuclear envelope and human disease. Nature Rev. Mol Cell Biol 3:575–585

Burke B, Stewart CL (2006) The laminopathies: the functional architecture of the nucleus and its contribution to disease. Ann Rev Genom Hum Genet 7:369–405

Bushmeyer SM, Atchison ML (1998) Identification of YY1 sequences necessary for association with the nuclear matrix and for transcriptional repression functions. J Cell Biochem 68:484–499

Cai S, Han H, Kohwi-Shigematsu T (2003) Tissue-specific nuclear architecture and gene function regulated by a single protein SATB1. Nat Genet 34:42–51

Chambon P, Weill JD, Mandel P (1963) Nicotinamide mononucleotide activation of newDNA-dependent polyadenylic acid synthesizing nuclear enzyme Biochem Biophys Res Commun 11:39–43

Chang WJ, Alvarez-Gonzalez R (2001) The sequence-specific DNA binding of NF-kappa B is reversibly regulated by automodification-reaction of poly (ADP-ribose) polymerase 1. J Biol Chem 276:47664–47670

Chen L, Lee L, Kudlow B, Dos Santos H, Sletvold O, Shafeghati Y, Botha E, Garg A, Hanson N, Martin G (2003) LMNA mutations in atypical Werner's syndrome. Lancet 362:440–445

D'Amours D, Desnoyers S, D'Silva I, Poirier GG (1999) Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem J 342:249–268

Deppert W (1996) Binding of MAR-DNA elements by mutant p53: possible implications for its oncogenic functions. J Cell Biochem 62:172–180

Dobreva G, Chahrour M, Dautzenberg M, Chirivella L, Kanzler B, Fariñas I, Karsenty G, Grosschedl R (2003) SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation. Cell 125:971–986

Fackelmayer FO (2004) Nuclear architecture and gene expression in the quest for novel therapeutics. Curr Pharmaceut Des 10:2851–2860

Faraone-Mennella MR (2005) Chromatin architecture and functions: the role(s) of poly(ADP-ribose) polymerase and poly(ADPribosyl)ation of nuclear proteins. Biochem Cell Biol 83(3):396–404

Fey EG, Penman S (1988) Nuclear matrix proteins reflect cell type of origin in cultured human cells. Proc Natl Acad Sci USA 85:121–125

Galande S (2002) Chromatin (dis)organization and cancer: BUR-binding proteins as biomarkers for cancer. Curr Cancer Drug Targets 2:157–190

Galande S, Kohwi-Shigematsu T (1999) Poly(ADP-ribose) polymerase and Ku autoantigen form a complex and synergistically bind to matrix attachment sequences. J Biol Chem 27:20521–20528

Getzenberg RH (1994) The nuclear matrix and the regulation of gene expression: tissue specificity. J Cell Biochem 55(1):22–31

Glazko GV, Koonin EV, Rogozin IB, Shabalina SA (2003) A significant fraction of conserved noncoding DNA in human and mouse consists of predicted matrix attachment regions. Trends Genet 19:119–124

Goetze S, Gluch A, Benham C, Bode J (2003a) Computational and in vitro analysis of destabilized DNA regions in the interferon gene cluster: the potential of predicting functional gene domains. Biochemistry 42:154–166

Goetze S, Huesemann Y, Baer A, Bode J (2003b) Functional characterization of transgene integration patterns by Halo-FISH: electroporation versus retroviral infection. Biochemistry 42:7035–7043

Goetze S, Baer A, Winkelmann S, Nehlsen K, Seibler J, Maass K, Bode J (2005) Genomic bordering elements: their performance at pre-defined genomic loci. Mol Cell Biol 25:2260–2272

Griesenbeck J, Ziegler M, Tomilin N, Schweiger M, Oei SL (1999) Stimulation of the catalytic activity of poly(ADP-ribosyl) transferase by transcription factor Yin Yang 1. FEBS Lett 443:20–24

Guo B, Odgren PR, van Wijnen AJ, Last TL, Nickerson J, Penman S, Lian JB, Stein JL, Stein GS (1995) The nuclear matrix protein NMP-1 is the transcription factor YY1. Proc Natl Acad Sci USA 92:10526–10530

Hake SB, Xiao A, Allis CD (2004) Linking the epigenetic language of covalent histone modifications to cancer. Brit J Cancer 90:761–769

Harborth J, Wang J, Gueth-Hallonet C, Weber K, Osborn M (1999) Self assembly of NuMA: multiarm oligomers as structural units of a nuclear lattice. EMBO J 18:1689–1700

Heng HHQ, Goetze S, Ye CJ, Lu W, Liu G, Bremer S, Hughes M, Bode J, Krawetz SA (2004) Dynamic features of scaffold/matrix attached regions (S/MARs) in anchoring chromatin loops. J Cell Sci 17:999–1008

Henikoff S (2005) Histone modifications: Combinatorial complexity or cumulative simplicity? Proc Natl Acad Sci USA 102:5308–5309

Hofmann WA, Stojiljkovic L, Fuchsova B, Vargas GM, Mavrommatis E, Philimonenko V, Kysela K, Goodrich JA, Lessard JL, Hope TJ, Hozak P, de Lanerolle P (2004) Actin is part of pre-initiation complexes and is necessary for transcription by RNA polymerase II. Nat Cell Biol 6:1094–1101

Huber LJ, Chodosh LA (2005) Dynamics of DNA repair suggested by the subcellular localization of Brca1 and Brca2 proteins. J Cell Biochem 96:47–55

Iarovaia OV, Bystritskiy A, Ravcheev D, Hancock R, Razin SV (2004) Visualization of individual DNA loops and a map of loop domains in the human dystrophin gene. Nucleic Acids Res 32:2079–2086

Jackson D (2005) Understanding nuclear organization: when information becomes knowledge. EMBO Rep 6:213–217

Jenke BH, Fetzer CP, Jönsson F, Fackelmayer FO, Conradt HC, Bode J, Lipps HJ (2001) An episomally replicating vector binds to the nuclear matrix protein SAF-A in vivo. EMBO Rep 3:349–354

Jiang M, Axea T, Holgate R, Rubbi CP, Okorokov AL, Mee T, Milner J (2001) p53 Binds the nuclear matrix in normal cells: binding involves the proline-rich domain of p53 and increases following genotoxic stress. Oncogene 20:5449–5458

Johnson CN, Levy LS (2005) Matrix attachment regions as targets for retroviral integration. Virol J 2:68 doi:10.1186/1743-422X-2-68

Ju BG, Lunyak VV, Perissi V, Garcia-Bassets I, Rose DW, Glass CK, Rosenfeld MGA (2006) Topoisomerase IIß-mediated dsDNA break required for regulated transcription. Science 312:1798–1802

Kanda T, Sullivan KF, Wahl GM (1998) Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. Curr Biol 8:377–385

Kaplan MH, Zong RT, Herrscher RF, Scheuermann RH, Tucker PW (2001) Transcriptional activation by a matrix associating region-binding protein. J Biol Chem 276:21325–21330

Kay V, Bode J (1994) Binding specificities of a nuclear scaffold: supercoiled, single stranded and SAR-DNA. Biochemistry 33:367–374

Kay V, Bode J (1995) Detection of scaffold attached regions (SARs) by in vitro techniques; activities of these elements in vivo. In: Papavassiliou AG, King SL (eds) Methods in molecular and cellular biology: Methods for studying DNA protein interactions an overview, vol 5, Wiley Liss, Wilmington, pp 186–194

Kim E, Deppert W (2004) Transcriptional activities of mutant p53: when mutations are more than a loss. J Cell Biochem 93:878–886

Kipp M, Schwab BL, Przybylski M, Nicotera P, Fackelmayer FO (2000) Apoptotic cleavage of scaffold attachment factor A (SAF-A) by caspase-3 occurs at a noncanonical cleavage site. J Biol Chem 275:5031–5036

Klar M, Bode J (2005) Enhanceosome formation over the interferon-beta promoter underlies a remote-control mechanism mediated by YY1 and YY2. Mol Cell Biol 25:10159–10170

Kohwi-Shigematsu T, Maass K, Bode J (1997) A thymocyte factor, SATB1, suppresses transcription of stably integrated MAR linked reporter genes. Biochemistry 36:12005–12010

Kramer JA, Zhang S, Yaron Y, Zhao Y, Krawetz SA (1997) Genetic testing for male infertility: a postulated role for mutations in sperm nuclear matrix attachment regions. Genetic Testing 1:125–129

Kraus WLK, Lis JT (2003) PARP goes transcription. Cell 113:677–683

Kukalev A, Nord Y, Palmberg C, Bergman T, Percipalle P (2005) Actin and hnRNP U cooperate for productive transcription by RNA polymerase II. Nat Struct Mol Biol 12:238–244

Li B, Navarro S, Kasahara N, Comai L (2004) Identification and biochemical characterization of a Werner's syndrome protein complex with Ku70/80 and poly(ADP-ribose) polymerase-1. J Biol Chem 279:13659–13667

Liebich I, Bode J, Frisch M, Wingender E (2000) S/MARt DB-A database on scaffold/matrix attached regions. Nucleic Acids Res 30:372–374

Linnemann AK, Platts AE, Doggett N, Gluch A, Bode J, Krawetz SA (2007) Genome-wide identification of nuclear matrix attachment regions: an analysis of methods. Biochem Soc Trans 5:612–617

Lipps HJ, Jenke ACW, Nehlsen K, Scinteie M, Stehle IM, Bode J (2003) Chromosome-based vectors for gene therapy. Gene 304:23–33

Lonskaya I, Potaman VN, Shlyakhtenko LS, Oussatcheva EA, Lyubchenko YL, Soldatenkov VA (2006) Regulation of poly(ADP-ribose) polymerase-1 by DNA structure-specific binding. J Biol Chem 280:17076–17083

Ludérus ME, de Graaf A, Mattia E, den Blaauwen JL, Grande MA, de Jong L, van Driel R (1992) Binding of matrix attachment regions to lamin B1. Cell 70:949–959

Ludérus ME, den Blaauwen JL, de Smit OJ, Compton DA, van Driel R (1994) Binding of matrix attachment regions to lamin polymers involves single-stranded regions and the minor groove. Mol Cell Biol 14:6297–6305

Ma H, Siegel AJ, Berezney R (1999) Association of chromosome territories with the nuclear matrix: disruption of human chromosome territories correlates with the release of a subset of nuclear matrix proteins. J Cell Biol 146:531–541

Maniotis AJ, Bojanowski K, Ingeber DE (1997) Mechanical continuity and reversible chromosome disassembly within intact genomes removed from living cells. J Cell Biochem 65:114–130

Martelli AM, Cocco L, Riederer BM, Neri LM (1996) The nuclear matrix: a critical appraisal. Histol Histopathol 11:1035–1048

Martelli AM, Falcieri EI, Zweyer M, Bortul R, Tabellini G, Capellini A, Cocco L, Moanzoli L (2002) The controversial nuclear matrix: a balanced point of view. Histol. Histopathol 17:1193–1205

Martens JHA, Verlaan M, Kalkhoven E, Dorsman JC, Zantema A (2002) Scaffold/matrix attachment region elements interact with a p300-scaffold attachment factor A complex and are bound by acetylated nucleosomes. Mol Cell Biol 22:2598–2606

Martin-Oliva D, Aguilar-Quesada R, O'Valle F, Muñoz-Gámez JA, Martínez-Romero R, García del Moral R, Ruiz de Almodóvar JM, Villuendas R, Piris MA, Oliver FJ (2006) Inhibition of poly(ADP-ribose) polymerase modulates tumor-related gene expression, including hypoxia-inducible factor-1 activation, during skin carcinogenesis. Cancer Res 66:5744–5756

Mattern KA, van Driel R, De Jong L (1997) Composition and structure of the internal nuclear matrix. In: Bird RC, Stein GS, Lian JB, Stein JL (eds) Nuclear structure and gene expression), Academic, New York, pp 87–110

McNeil S, Guo B, Stein JL, Lian JB, Bushmeyer S, Seto E, Atchinson ML, Penman S, van Wijnen AJ, Stein GS (1998) Targeting of the YY1 transcription factor to the nucleolus and the nuclear matrix in situ: the C-terminus is a principal determinant for nuclear trafficking. J Cell Biochem 68:500–510

Mesner LD, Hamlin JL, Dijkwel PA (2003) The matrix attachment region in the Chinese hamster DHFR origin is dispensable for initiation of local chromatid separation. Proc Natl Acad Sci USA 100:3281–3286

Mielke C, Kohwi Y, Kohwi-Shigematsu T, Bode J (1990) Hierarchical binding of DNA fragments derived from scaffold-attached regions: correlation of properties in vitro and Function in vivo. Biochemistry 29:7475–7485

Mielke C, Maaß K, Tümmler M, Bode J (1996) Anatomy of highly-expressing chromosomal sites targeted by retroviral vectors. Biochemistry 35:2239–2252. Nakayasu H, Berezney R (1991) Nuclear matrins: Identification of the major nuclear matrix proteins. Proc Natl Acad Sci USA 88:10312–10316

Nehlsen K, Broll S, Bode J (2006) Replicating minicircles: Generation of nonviral episomes for the efficient modification of dividing cells. Gene Ther Mol Biol 10:233–244

Nobrega MA, Ovcharenko I, Afzal V, Rubin EM (2003) Scanning human gene deserts for long-range enhancers. Science 302:413

Nobrega MA, Zhu Y, Plajzer-Frick I, Afzal V, Rubin EM (2004) Megabase deletions of gene deserts result in viable mice. Nature 431:988–993

Oei SL, Shi Y (2001) Transcription factor Yin Yang 1 stimulates poly(ADP-ribosyl)ation and DNA repair. Biochem Biophys Res Commun 284:450–454

Okorokov AL, Rubbi CP, Metcalfe S, Milner J (2002) The interaction of p53 with the nuclear matrix is mediated by F-actin and modulated by DNA damage. Oncogene 21:356–367

Pederson T, Aebi U (2005) Nuclear actin extends, with no contraction in sight. Mol Cell Biol 16:5055–5060

Percipalle P, Jonsson A, Nashchekin D, Karlsson C, Bergman T, Guialis A, Daneholt B (2002) Nuclear actin is associated with a specific subset of hnRNP A/B-type proteins. Nucleic Acids Res 30:1725–1734

Petrov A, Pirozhkova I, Carnac G, Laoudj D, Lipinski M, Vassetzky YS (2006) Chromatin loop domain organization within the 4q35 locus in facioscapulohumeral dystrophy patients versus normal human myoblasts. Proc Natl Acad Sci USA 103:6982–6987

Replogle-Schwab TS, Getzenberg RH, Donat TL, Pienta KJ (1996) Effect of organ site on nuclear matrix protein composition. J Cell Biochem 62:132–141

Rolli V, Ruf A, Augustin A, Schulz GE, Ménissier-de Murcia J, de Murcia G (2000) Poly(ADP-ribose) polymerase: structure and function. In: de Murcia G, Shall S (eds) From DNA damage and stress signalling to cell death: poly ADP-ribosylation reactions, Oxford University Press, New York, pp 35–79

Scherrer K (1989) A unified matrix hypothesis of DNA-directed morphogenesis, protodynamism and growth control. Biosci Rep 9:157–188

Schlake T, Klehr-Wirth D, Yoshida M, Beppu T, Bode J (1994) Gene expression within a chromatin domain: the role of core histone hyperacetylation. Biochemistry 33:4197–4206

Schuebeler D, Mielke C, Maaß K, Bode J (1996) Scaffold/matrix-attached regions act upon transcription in a context-dependent manner. Biochemistry 35:11160–11169

Schwartz GH, Patnaik A, Hammond LA, Rizzo J, Berg K, Von Hoff DD, Rowinsky EK (2003) A phase I study of bizelesin, a highly potent and selective DNA-interactive agent, in patients with advanced solid malignancies. Ann Onc 14:775–782

Soldatenkov VA, Chasovskikh S, Potaman VN, Trofimova I, Smulson ME, Dritschilo A (2002) Transcriptional repression by binding of poly(ADP-ribose) polymerase to promoter sequences. J Biol Chem 277:665–670

Straetling WH, Yu F (1999) Origin and roles of nuclear matrix proteins. Specific functions of the MAR-binding protein MeCP2/ARBP. Crit Rev Eukaryot Gene Exp 9:311–318

Thomas CA (1971) The genetic organization of chromosomes. Ann Rev Genet 5:237–256

Vendrely R, Vendrely C (1948) La teneur du noyau cellulaire en acide désoxyribonucléique à travers les organes, les individus et les espèces animales: techniques et premiers résultats. Experientia 4:434–436

Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA et al (2001) The sequence of the human genome. Science 291:1304–1351

Vidaković M, Grdovic, Quesada P, Bode J, Poznanović G (2004) Poly(ADP-ribose) polymerase-1: association with nuclear lamins in rat hepatocytes. J Cell Biochem 93:1155–1168

Vidaković M, Koester M, Goetze S, Winkelmann S, Klar M, Poznanović G, Bode J (2005a). Colocalization of PARP-1 and lamin B in the nuclear architecture: a halo-fluorescence- and confocal microscopy study. J Cell Biochem 96:555–568

Vidaković M, Poznanović G, Bode J (2005b) DNA break repair: Refined rules of an already complicated game. Biochem Cell Biol 83:365–373

Virág L, Szabó C (2002) The therapeutic potential of poly(ADP-Ribose)polymerase inhibitors. Pharm Rev 54:375–429

Vispé S, Yung TM, Ritchot J, Serizawa H, Satoh MS (2000) A cellular defense pathway regulating transcription through poly(ADP-ribosyl)ation in response to DNA damage. Proc Natl Acad Sci USA 97:9886–9891

Wang Z, Goldstein A, Zong RT, Lin D, Neufeld EJ, Scheuermann RH, Tucker PW (1999) Cux/CDP homeoprotein is a component of NF-∝NR and represses the immunoglobulin heavy chain intronic enhancer by antagonizing the Bright transcription activator. Mol Cell Biol 19:284–295

Wen J, Huang S, Rogers, H. Dickinson LA, Kohwi-Shigematsu T, Noguchi CT (2005) SATB1 family protein expressed during early erythroid differentiation modifies globin gene expression. Blood 105:3330–3339

Winkelmann S, Klar M, Benham C, AK P, Goetze S, Gluch A, Bode J (2006) The positive aspects of stress: strain initiates domain decondensation (SIDD). Brief Funct Genomic Proteomic 5:24–31

Winkelmann S (2007) Dynamische Aspekte der Kernarchitektur: S/MARs und ihre Rolle bei der Etablierung aktiver Transkriptionseinheiten. Dissertation Technische Universität Braunschweig. http://www.digibib.tu-bs.de/?docid=00020961

Woynarowski JM, Trevino AV, Rodriguez KA, Hardies SC, Benham CJ (2001) AT-rich islands in genomic DNA as a novel target for AT-specific DNA-reactive antitumor drugs. J Biol Chem 276:40555–40566

Zastrow MS, Vlcek S, Wilson KL (2004) Proteins that bind A-type lamins: integrating isolated clues. J Cell Sci 117:979–987

Zbarsky IB, Debov SS (1948) On the proteins of the cell nuclei. Proc USSR Acad Sci 62:795–798

Zeng C, He D, Brinkley BR (1994) Localization of NuMA protein isoforms in the nuclear matrix of mammalian cells. Cell Motil Cytoskel 29:167–176

Zeng C, van Wijnen AJ, Stein JL, Meyers S, Sun W, Shopland L, Lawrence JB, Penman S, Lian JB, Stein GS, Hiebert SW (1997) Identification of a nuclear matrix targeting signal in the leukemia and bone-related AML/CBF-alpha transcription factors. Proc Natl Acad Sci USA 6746–6751

Clathrin/AP-2-Dependent Endocytosis: A Novel Playground for the Pharmacological Toolbox?

C. Rodemer and V. Haucke(✉)

Contents

1 Introduction . . . 106
2 Steps of Clathrin/AP-2-Mediated Endocytosis . . . 107
3 AP-2: A Member of the AP-Complex Family . . . 108
4 Getting AP-2 to the Plasma Membrane: The Role of Phosphoinositides . . . 111
5 AP-2 Interactions with Cargo . . . 111
6 AP-2 as a Recruiter of Clathrin . . . 113
7 Binding of Accessory Endocytic Proteins to AP-2 Appendage Domains . . . 114
7.1 Interaction Sites Within the α-Appendage Domain . . . 114
7.2 Interaction Sites Within the α2-Appendage Domain . . . 114
8 AP-2-Binding Accessory Proteins Defining Cargo-Selective Pathways . . . 115
9 Pharmacological Inhibition of Clathrin/AP-2-Mediated Endocytosis . . . 116
9.1 Inhibition by Cytosolic Acidification . . . 116
9.2 K^+ Depletion . . . 116
9.3 Chlorpromazine . . . 117
9.4 High Sucrose . . . 117
9.5 Dynasore . . . 117
10 Perspective . . . 118
References . . . 118

Abstract Endocytosis is a vital process for mammalian cells by which they communicate with their environment, internalize nutrients, hormones, or growth factors, or take up extracellular fluids and particles. The best studied among the various pathways to ingest material from the extracellular side is clathrin/AP-2-mediated endocytosis. The past several years have allowed us to gain unprecedented molecular insights into the role of the heterotetrameric AP-2 adaptor complex as a central protein–protein and protein–lipid interaction hub at the plasmalemma. During the initial stages of clathrin-coated pit formation, AP-2 interacts with phosphoinositides and cargo membrane proteins as well as with a variety of accessory proteins and

V. Haucke
Institute of Chemistry and Biochemistry, Department of Membrane Biochemistry,
Freie Universität Berlin, Takustraße 6, 14195 Berlin, Germany
v.haucke@biochemie.fu-berlin.de

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

clathrin to coordinate clathrin coat polymerization with membrane deformation and cargo recruitment. In addition, a growing list of alternative adaptors provides opportunity for clathrin-dependent cargo selective pathways of internalization and endosomal sorting. Many of these interactions are now understood in structural detail and are thus amenable to pharmacological interference. In this review we will summarize our present state of knowledge about AP-2 and its partners in endocytosis and delineate potential strategies for pharmacological manipulations.

1 Introduction

Endocytosis is a collective term summarizing a variety of different processes by which eukaryotic cells take up extracellular material including fluids, particles, hormones and growth factors, or ligand-bound receptors. In mammalian cells a variety of mechanisms of uptake have been described (Conner and Schmid 2003). These include pinocytosis, phagocytosis, as well as clathrin-dependent and -independent pathways of endocytosis. Pinocytosis (cell drinking) refers to the uptake of fluids by invagination of the plasma membrane. This is followed by the formation of vesicles in the cell soma. Phagocytosis ("cell eating") is a process whereby large particles are enveloped by the plasma membrane into a so-called phagocytic cup, which is then internalized to form a phagosome (Mukherjee et al. 1997). Clathrin-independent endocytic pathways may involve lipid microdomains presumably in cooperation with membrane integral scaffolding proteins, which have been postulated to form hairpin-loop structures in the cytosolic leaflet of the membrane bilayer. Prime examples are caveolar uptake of glycosphingolipids and flotillin-1-mediated endocytosis of cholera toxin (Glebov et al. 2006). These membrane-organizing scaffolds have been the subject of excellent recent reviews (Morrow and Parton 2005; Bauer and Pelkmans 2006).

By far the best-characterized internalization route is clathrin- and adaptor-dependent endocytosis (clathrin-mediated endocytosis; CME). In this pathway transmembrane cargo including nutrient or signaling receptors as well as synaptic vesicle proteins (Galli and Haucke 2004; Schweizer and Ryan 2006) are endocytosed into clathrin-coated vesicles (CCVs) that bud from phosphatidylinositol 4,5,-bisphosphate (PIP_2)-enriched sites at the plasma membrane (Krauss and Haucke 2007; Di Paolo and De Camilli 2006) and deliver their cargo to the endosomal system for recycling or degradation. Examples include receptor-mediated uptake of transferrin, epidermal growth factor (EGF), or LDL. As clathrin, itself a heteromeric stable complex comprising three heavy and three regulatory light chains forming a triskelial structure (Kirchhausen 2000), is unable to associate with the plasmalemma, it requires adaptor proteins that recruit clathrin to "endocytic hot spots" and stimulate lattice or coat assembly. Among these the most important adaptor is the hetetrotetrameric AP-2 complex. AP-2 is also crucial to select cargo and to recruit accessory proteins (Robinson and Bonifacino 2001), some of them functioning as cargo and/or clathrin adaptors themselves. Thus, AP-2 and other monomeric adaptors (collectively termed clathrin-coat-associated sorting proteins, CLASPs; Traub 2005) bridge the gap between the outer

clathrin shell and integral membrane cargo proteins (Lewin and Mellman 1998). CLASPs also aid in defining cargo-selective endocytic pathways as further discussed below (Lakadamyali et al. 2006; Haucke 2006). Overwhelming genetic (Mitsunari et al. 2005; Gonzalez-Gaitan and Jackle 1997), biochemical (Praefcke et al. 2004; Edeling et al. 2006b), and cell biological evidence (Motley et al. 2003; Hinrichsen et al. 2003; Huang et al. 2004) supports an important, if not essential role for AP-2 as a central protein–protein interaction hub in clathrin-mediated endocytosis in a variety of model systems ranging from flies to mammals.

2 Steps of Clathrin/AP-2-Mediated Endocytosis

Clathrin-mediated endocytosis can be divided into several distinct steps, including a coat nucleation and assembly phase, maturation of the coated pit, and fission of late coated pits to free vesicles. Finally, coat proteins are shed and disassembled in a process termed "uncoating" that is mechanistically linked to dynamin-mediated scission of the vesicle neck (Newmyer et al. 2003). The clathrin coat is assembled at the cytoplasmic face of the plasma membrane, in part by recruitment of coat proteins from a cytoplasmic reserve, but perhaps also by relocalization of pre-existing clathrin from flat lattices. Recruitment is largely dependent on the AP-2 adaptor complex, which links clathrin to the membrane and coordinates the structural assembly of the coat with the selection of membrane cargo proteins. Many of these display sorting motifs, including tyrosine-based (Yxxø, with ø being a large hydrophobic amino acid) or acidic-cluster di-leucine motifs that are recognized directly by AP-2. In addition, AP-2 serves as a major interaction hub that aids in the organization of the endocytic network by interaction with a large set of so-called accessory proteins. These represent protein factors, including epsins, AP180/CALM, eps15, amphiphysins, AAK1, etc., that aid in membrane deformation and regulation of clathrin assembly, or participate in distinct stages of coated pit maturation and fission, such as auxilin and synaptojanin. Many non-conventional adaptors implicated in cargo-selective pathways of endocytosis, such as β-arrestins, disabled-2 (Dab2), autosomal recessive hypercholesterolemia (ARH), or stonins, also depend on AP-2 for recruitment to sites of vesicle formation. At synapses the unusually rapid exo-endocytosis of neurotransmitter-filled vesicles may involve membrane-associated cargo, such as synaptotagmin 1, a major component of synaptic vesicles in the brain.

Cargo selection and coat assembly are intimately linked processes that are accompanied by dramatic changes in the shape of the underlying plasma membrane. The partitioning of hydrophobic side chains displayed in the context of an amphipathic helix preceding or integrated into a folded lipid-binding domain of accessory proteins may provide the initial driving force for membrane deformation. However, recent studies show that the clathrin scaffold is then needed to stabilize the curved membrane (Hinrichsen et al. 2006). As the coat matures and progressively invaginates, the large GTPase dynamin is recruited, mainly via

interactions with SH3 domain-containing proteins, such as amphiphysins, endophilin, and intersectin, to the coat and the emanating vesicle neck. GTP hydrolysis of assembled dynamin rings then appears to be the driving force for the actual fission reaction. Although the exact mechanism is still somewhat uncertain, it seems likely that the mechanochemical properties of dynamin that correlate with changes in the diameter of the dynamin ring-shaped polymer (Chen et al. 2004; Roux et al. 2006) underlie the scission of the membrane to generate free clathrin-coated vesicles (CCVs). Dynamin-mediated fission is mechanistically linked and temporally coupled to coat dissociation by the chaperone hsc70 and its partner auxilin (Newmyer et al. 2003; Takei and Haucke 2001) and to synaptojanin-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) (Cremona et al. 1999; Harris et al. 2000; Perera et al. 2006). In this review we will focus on the role of AP-2 as a central protein–protein and protein–lipid interaction hub in clathrin-mediated endocytosis. The detailed structural information on AP-2 and its various partners may serve to delineate strategies for pharmacological interference with clathrin/AP-2-based endocytic pathways.

3 AP-2: A Member of the AP-Complex Family

AP-2 is a member of the AP family of heterotetrameric adaptor complexes (termed AP 1–4). APs are found in all nucleated cells from yeast to humans and share a similar overall quaternary structure (Kirchhausen 1999). Two isoforms of the AP-1 complex are components of CCVs originating from the trans-Golgi network (TGN)/recycling endosomal boundary. Of these, the AP-1B complex is only found in polarized epithelial cells where it regulates basolateral delivery of membrane cargo, such as the LDL receptor (Kirchhausen 1999). The AP-3 family member is associated mainly with endosomal membranes, and AP-4 is linked to membranes of the TGN (Boehm and Bonifacino 2001; Kirchhausen 1999). Whether AP-3 and AP-4 associate with clathrin in vivo remains uncertain, and perhaps unlikely.

AP complexes share a common architecture and subunit structure. Each complex contains two large subunits (γ1 and β1 for AP-1, α and β2 for AP-2, δ and β3 for AP3-, ε and β4 for AP-4; each about 100 kD), one medium (μ1, μ2, μ3, μ4; 50 kD each), and one small chain (σ1, σ2, σ3, σ4; 20 kD each) (Boehm and Bonifacino 2001; Kirchhausen 1999; Robinson and Bonifacino 2001). Parts of the two large subunits together with σ and the amino-terminal domain of μ form a compact solenoid core that is connected via flexible hinges to the appendage or ear domains. The ears, which are encoded by the two large adaptin genes, represent flexible protein–protein interaction platforms that bind to simple peptide motifs contained within natively unfolded domains of accessory endocytic factors (discussed in detail below).

The best-characterized member of the family of clathrin adaptor complexes is the AP-2 complex. It was also the first heterotetrameric trafficking adaptor to be characterized in molecular detail. Under the electron microscope, AP adaptors appear as a central core joined by flexible linkers to appendage or ear domains

(Heuser and Keen 1988). Like other adaptors, the AP-2 complex consists of two large subunits of 100–130 kda (α and β2), one medium chain of 50 kDa (μ2), and a small subunit of about 17–20 kDa (σ2) (Kirchhausen 1999). The large subunits form the majority of the trunk (70 kDa), as well as the appendage domains (30 kDa). These are connected by flexible, proteolytically sensitive linkers or hinge regions. σ2 and μ2 are also embedded into the AP-2 core. The structures of the AP-2 core and the ear domains have been solved by X-ray crystallography (Fig. 1).

The AP2-core structure resembles a rectangle, where the trunk domains of α and β2 subunits are on the outside. These represent structurally similar, highly curved α-helical solenoids. The small subunit σ2 and the N-terminal μ2 domain display α/β folds and are nested into the "elbows" of α- and β2-adaptins, respectively. Thus, the AP-2 core can be considered structurally as a dimer of two related α/σ2 and β2/μ2 heteromers. In the closed, presumably inactive conformation of AP-2, the β2/N-μ2 heterodimer forms a shallow dish, in which the μ2-C-terminal domain sits (Collins et al. 2002). The folds of σ2 and N-μ2 are structurally very similar, suggesting that they may fulfill a stabilizing role within the AP-2 complex. The N- and C-terminal domains (C-μ2) of μ2 are connected with each other via a flexible linker (similar to the linker that connects the α and β2 appendage or ear domains to the core) that contains a single phosphorylation site. This site is suggested to be of functional importance for the activation of AP-2 during endocytic cargo recognition and coat assembly (Ricotta et al. 2002), because the C-terminal domain of μ2 harbors the binding site for YxxΘ signals within cargo membrane receptors (Ohno et al. 1995; Owen and Evans 1998). Comparison of the crystal structure of free C-μ2 complexed with Yxxø-cargo peptides and C-μ2 contained within the AP-2 core indicates that the tyrosine binding pocket is preformed, but occluded within non-phosphorylated AP-2 by residues derived from β2 (Collins et al. 2002). If cargo proteins bearing a YxxΘ motif are to associate with C-μ2, a conformational change must occur. This change is presumably triggered by phosphorylation of Thr156, a modification that has been demonstrated to result in a dramatic increase in the affinity for endocytic sorting signals (Ricotta et al. 2002). Cargo recognition is also dependent on phosphatidylinositol 4,5-bisphosphate, which interacts with AP-2 via two independent sites at the amino-terminal end of α and on the surface of C-μ2 (Honing et al. 2005) and is further discussed below. C-μ2 (Owen and Evans 1998) consists of an elongated banana-shaped all β-sheet structure that can be considered as two β-sandwich domains (A and B). Hydrophobic pockets provided by F174 and W421 on the surface of subdomain A accommodate the Y and ø positions of tyrosine motif peptides, which associate with μ2 in an extended conformation (Owen and Evans 1998). In some cases, additional contacts by a third hydrophobic residue upstream of Yxxø may lead to further stabilization and enhanced affinity of tyrosine signals for AP-2μ (Owen et al. 2001).

The structure of the α and β2 appendage domains has also been solved (Fig. 1). The α-appendage can be divided into two subdomains. The N-terminal subdomain is a nine-stranded β-sandwich. The C-terminal subdomain is made of a five-stranded beta sheet flanked by helices (Owen et al. 1999). The two domains are joined by a short linker. The β2 appendage domain (Owen and Luzio 2000) shows

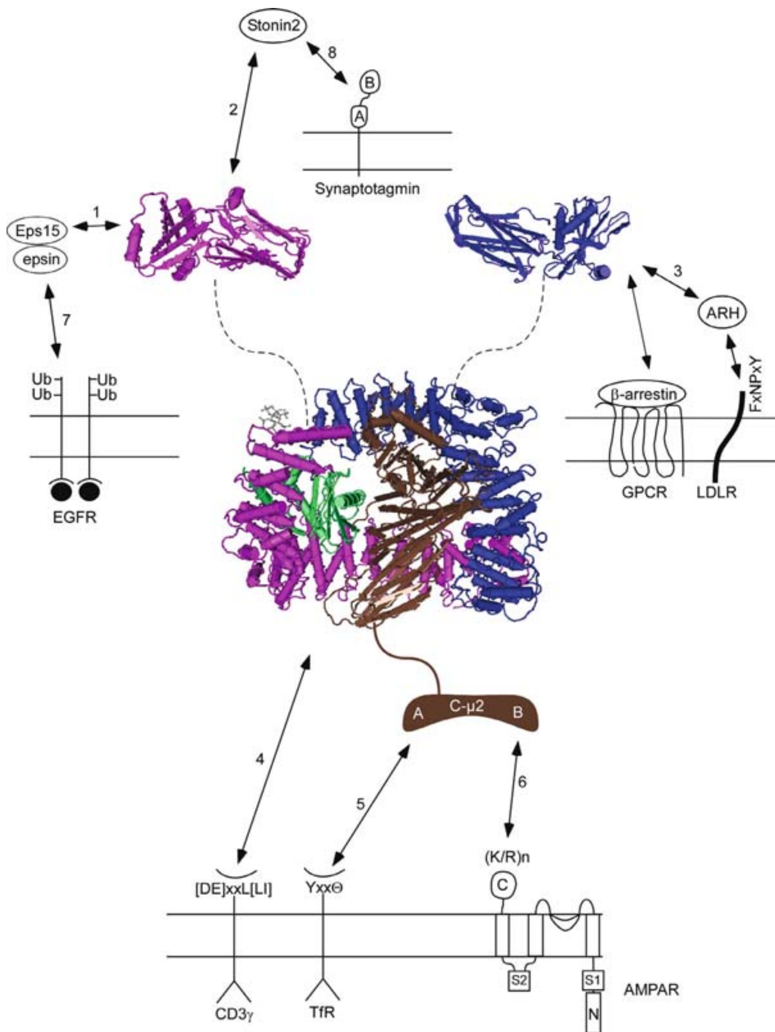

Fig. 1 Three-dimensional structure of the AP-2 adaptor complex and schematic representation of select binding partners. The α subunit is shown in *pink*, β2 in *blue*, σ2 in *green* and μ2 in *brown* (PDB:1GW5). During recognition of Yxxø-based endocytic sorting signals, AP-2 is predicted to undergo a conformational change such that the banana-shaped C-terminal domain of μ2 (schematically depicted in *brown*) undergoes a rotational movement towards the membrane for interaction with PIP_2 (not shown) and cargo. *Dotted lines* represent the hinge regions of α and β2 subunits. Appendage domains (PDB:2G30 and PDB:1QTP) are colored identically to the corresponding trunk domains. Protein–protein interactions are assigned with numbers referred to in the text. *CD3* , γ subunit of T cell antigen CD3; *TfR*, transferrin receptor; *EGFR*, epidermal growth factor receptor; *AMPAR*, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate-receptor with segments S1 and S2, cytoplasmic domain (*C*), and an amino-terminal ligand binding domain (*N*); *GPCR*, G-protein-coupled receptor; *LDLR*, low-density lipoprotein receptor; *ARH*, autosomal recessive hypercholesterolemia protein; *UB*, ubiquitin. *Yxxø*, tyrosine-based endocytic sorting signal; *[DE]xxL[LI]*, di-leucine-based endocytic sorting signal; $[KR]_n$, basic cluster-type endocytic sorting signal

a structure overall very similar to that of α-appendage, although the sequence identity is relatively low. The two subdomains of the β2 appendage contact each other at a tight interface leaving little space for motion.

Both appendage domains serve as important platforms for the recruitment of accessory proteins. These interactions involve short peptide motifs within natively unfolded domains and will be discussed in detail below (see Fig. 1 and Table 1).

4 Getting AP-2 to the Plasma Membrane: The Role of Phosphoinositides

Overwhelming genetic, cell biological, and biochemical evidence implicates phosphoinositides, specifically phosphatidylinositol 4,5-bisphosphate (PIP_2), in clathrin-mediated endocytosis (Di Paolo and De Camilli 2006; Krauss and Haucke 2007). The AP-2 complex binds to PIP_2 and perhaps also to phosphatidyl-inositol (3,4,5) trisphosphate (PIP_3) (Beck and Keen 1991; Haucke 2005). PIP_3 can be synthesized at sites of endocytosis from PIP_2 by a clathrin activated PI3 kinase C2α (Gaidarov et al. 2001) or downstream of receptor activation (Lefkowitz and Shenoy 2005). The α-chain of AP-2 interacts with high affinity with PIP_2 via a positively charged site, including R11, N39, K43, K57, Y58, and K61 (Collins et al. 2002; Honing et al. 2005). Mutations in the putative binding motif of the α chain lead to AP-2 mislocalization (Gaidarov and Keen 1999), suggesting that this site serves as the primary determinant for targeting AP-2 to sites of endocytosis. Consistent with this, masking or depleting PIP_2 in living cells has been shown to result in AP-2 mislocalization and inhibition of endocytosis (Jost et al. 1998; Krauss et al. 2003; Varnai et al. 2006).

The μ2-chain of the AP-2 complex was also found to interact with PIP_2. The interaction of PIP_2 with μ2 chain is again mediated by a number of basic residues (Lys341, Lys343, Lys345, Lys354, and Lys356) (Collins et al. 2002). This lysine cluster is a surface-exposed positively charged patch within subdomain B of the cargo recognition domain of μ2 (Owen and Evans 1998; Nesterov et al. 1999). Changing residues K345, K354, and K356 to glutamates reduced the μ2 affinity to PIP_2. Mutant μ2 lacking this cluster of conserved lysine residues fails to bind $PI(4,5)P_2$ and to compete the recruitment of native clathrin/AP-2 to PI(4,5)P2-containing liposomes or to presynaptic membranes (Rohde et al. 2002). Moreover, it was shown that expression of mutant μ2 inhibits receptor-mediated endocytosis in living cells (Rohde et al. 2002). However, mutating residues K341, K343, and K345 lead to fully functional μ2 following siRNA-mediated knockdown and rescue (Motley et al. 2006).

5 AP-2 Interactions with Cargo

One of the best understood interactions in endocytosis is the recognition of cargo membrane proteins bearing Yxxø-based (where x is any amino acid and ø is a bulky hydrophobic residue) endocytic sorting motifs within their cytoplasmic domains by

Table 1 AP-2 binding partners and the corresponding recognition domains or sites within AP-2

AP-2 binding partners	AP-2 subunit/ domain involved	Motif in AP-2 binding partner	Site within AP-2	Approximate K_d
Membrane cargo (i.e., TfR, EGFR)	μ2	Yxxϕ	Subdomain A (F174, W421, R423)	1–10 μM [increased by $PI(4,5)P_2$]
Membrane cargo (i.e., LDLR, megalin)	μ2	FxNPxY	Unknown site within μ2	ND
Membrane cargo (i.e., CD4, MPR46)	μ2? β2 trunk?	[DE]xxxL[LI]	alpha/ sigma2 interface within AP-2 core	>90 μM [increased by $PI(4,5)P_2$]
Membrane cargo (i.e., GluR2, $GABA_A$R-β3, synaptotagmin 1)	μ2	Basic cluster	Subdomain B	0.2–10 μM
Clathrin	β2-hinge	β-propeller (N-terminal domain)	LLNLD (clathrin box)	20 μM
$PI(4,5)P_2$	α-trunk μ2		R11, N39, K43, K57, Y58, K61 K341,K343,K345,K354,K356	3–8 μM
Stonin2	α-ear	Wxx[FW]ac	Sandwich domain (F740, G742)	1–10 μM
Synaptojanin1(170)	μ2		subdomain A (F174, W421, R423)	
AAK1				
NECAP1/2				
Eps15	α-/β2-ear	DP[FW]	Platform domain (i.e., W840 of α-ear)	120 μM
Epsin1				
Dab2				
Amphiphysin1	α-ear	FxDxF	Platform domain (W840)	ND
AP180				
Synaptojanin1 (170)				
ARH	β2-ear	[ED]xxFxx[FL]xxxR	Platform domain (Y888)	1–5 μM
β-arrestin				
Eps15	β2-ear	[FL]xxGFxDF or [FL](x)$_n$GFxDF (n>3)	Sandwich domain (Y815)	10–100 μM
EpsinR				

Shown are also the consensus motifs for interaction with AP-2 and their approximate binding constants (K_d). *GluR2*, a subunit of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamate-receptors; *$GABA_A$R- 3*, β3 subunit of type A γ-aminobutyric acid receptors; *TfR*, transferrin receptor; *EGFR*, epidermal growth factor receptor; *CD4*, cluster of differentiation antigen 4; *LDLR*, low-density lipoprotein receptor; *AAK1*, adaptor-associated kinase 1; *Dab2*, disabled-2; *AP180*, adaptor protein of 180 kDa; *ARH*, autosomal recessive hypercholesterolemia protein; *NECAP1/2*, adaptin-ear-binding coat-associated proteins 1 and 2; *eps15*, epidermal growth factor receptor protein substrate 15

AP-2μ (Fig. 1, no. 5). As described in detail above, peptides with this motif fit into a hydrophobic pocket on the surface of subdomain A of C-μ2 (Owen et al. 1999). A good example is represented by the sequence [20]YTRF within the cytosolic domain of the transferrin receptor, which functions as an autonomous and transplantable signal for internalization (Traub 2003).

AP-2 has also been postulated to recognize FxNPxY motifs present in the cytoplasmic tail of low-density-lipoprotein (LDL) receptor family members (Boll et al. 2002). It does not require the Yxxø binding pocket of μ2, but depends on other, unidentified residues. Given that specialized endocytic adaptors for FxNPxY motifs, such as Dab2 and ARH, have been identified, the physiological significance of this interaction is uncertain. Dileucine-based motifs [DE]xxxL[LI] (Bonifacino and Lippincott-Schwartz 2003) serve as internalization signals in many single and multi-spanning transmembrane proteins (Bonifacino and Traub 2003). Again, conflicting data exist regarding the exact site of interaction within AP-2, but recent results suggest that the alpha/sigma2 hemicomplex of the AP-2 core is involved. Association with AP-2 is dependent on LL or LI pairs preceded by acidic residues at the -4 and -5 position with respect to the first leucine (Fig. 1, no. 4). So far, attempts to co-crystallize AP-2 with [DE]xxxL[LI] peptides have failed (Collins et al. 2002).

AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate receptors (AMPARs) are the main class of receptors involved in rapid excitatory neurotransmission in the mammalian CNS (Derkach et al. 2007). AMPA receptors undergo constitutive and ligand-induced internalization that requires dynamin and the AP-2 complex. Recently, it was reported that an atypical basic motif within the cytoplasmic tails of AMPA-type glutamate receptors directly associates with nanomolar affinity with μ2-adaptin. Recognition by AP-2μ involves a basic motif within the cytolasmic tails of AMPA receptor subunits GluR1-3, $GABA_A$ receptors, and in the C2 domain of synaptotagmin 1 (Kastning et al. 2007) (Fig. 1, no. 6). Unlike constitutively internalized cargo proteins, these synaptic membrane proteins interact with subdomain B of C-μ2. Why such non-conventional endocytosis signals are used is unknown, but may be related to the fact that pre- and postsynaptic membrane proteins must undergo sorting to a specific endosomal subpopulation (Lee et al. 2004) that may be capable of segregating synaptic from constitutively internalized proteins. This is similar to what has been reported recently for other ligand-activated receptors (Lakadamyali et al. 2006).

6 AP-2 as a Recruiter of Clathrin

Clathrin plays a key role in endocytosis by functioning as a molecular scaffold that drives formation of transport vesicles, in part by stabilizing curved membrane domains (Hinrichsen et al. 2006). In solution clathrin forms a stable heterohexameric complex consisting of three heavy and three light chains organized into a three-legged “triskelion.” The triskelia are thought to multimerize into polyhedral cages that provide the mechanical force to form vesicles from a membrane. Although purified clathrin can

self-assemble into cages, its recruitment to membranes and assembly in vivo is regulated by a number of clathrin-binding proteins including AP-2 well as a variety of AP-2-associated accessory factors, such as amphiphysin, epsins, AP180/CALM, etc.

Interactions with clathrin are generally accomplished through simple linear arrangements of peptide motifs harboring hydrophobic and usually acidic residues (Dell'Angelica 2001) within natively unfolded domains. Analysis of clathrin-binding motifs in a variety of endocytic proteins has uncovered two classes of motifs: type-I and type-II clathrin boxes. Type-I motifs conform to the consensus sequence LΘxΘ (with Θ representing a bulky hydrophobic residue); type-II motifs are represented by the motif LΘDLL. Both types of motifs interact with the N-terminal β-propeller domain of the clathrin heavy chain and promote clathrin cage assembly in vitro and presumably also in vivo (Maldonado-Baez and Wendland 2006).

Recent studies have revealed that AP-2 in addition to the known type-I clathrin box within the hinge domain of its β2 subunit (i.e., LLNLD) harbors a second clathrin binding site within the β2 appendage domain (Edeling et al. 2006a). Mutations in this site reduced AP-2 binding to clathrin, suggesting that both sites need to cooperate for stable association with clathrin. The relative importance of AP-2 as opposed to other endocytic proteins in getting clathrin to membranes is somewhat uncertain. RNAi-mediated knockdown of AP-2 leads to a strong reduction of clathrin-coated pits at the plasma membrane, suggesting that AP-2 may be a key factor in clathrin recruitment (Hinrichsen et al. 2003; Motley et al. 2003).

7 Binding of Accessory Endocytic Proteins to AP-2 Appendage Domains

7.1 Interaction Sites Within the α-Appendage Domain

The α-appendage domain serves as a major interaction hub for various accessory proteins. The platform subdomain can bind to eps15, epsins, and Dab2 via DP[FW] motifs. It also interacts with amphiphysin, AP180, and synaptojanin 1-SJ170 via FxDxF sequences that bind to an overlapping site within the platform subdomain. The sandwich subdomain of α-ears bears associates with synaptojanin1 (Jha et al. 2004), stonin2 (Walther et al. 2004), AAK1 and NECAP1/2 (Ritter et al. 2003) by association with WXX[FW]x[DE] consensus motifs (Table 1) (Praefcke et al. 2004).

7.2 Interaction Sites Within the α2-Appendage Domain

The β2 appendage domain binds to a subset of proteins that also interact with the α-appendage domain, such as AP180/ CALM, epsins, Dab2, and eps15. The latter harbors DP[FW] motifs that target the platform subdomain of AP-2β-appendages. Eps 15 and

its close cousin eps15R, in addition, are capable of binding to the β2 sandwich using a non-conventional [FL]xxGFxDF or [FL]$(x)_n$GFxDF (n>3) peptide sequence.

The cargo-specific adaptors ARH and β-arrestins (see also below) associate with the platform subdomain of β2-appendages via a helical motif with the consensus [ED]xxFxx[FL]xxxR (Table 1) (Edeling et al. 2006a; Praefcke et al. 2004).

8 AP-2-Binding Accessory Proteins Defining Cargo-Selective Pathways

Although the role of AP-2 as an important protein–protein and protein–lipid interaction is undisputed, a simplistic model according to which all cargo is sorted via physical association with AP-2 seems unlikely. Instead, several AP-2 binding accessory proteins function as clathrin-associated sorting proteins (CLASPs), alternate adaptors for cargo-specific routes of internalization (Haucke 2006). For instance, heptahelical G-protein coupled receptors (GPCRs) following ligand binding and activation associate with β-arrestins, atypical adaptors that rapidly translocate to phosphorylated GPCRs and promote their internalization via clathrin/ AP-2-dependent endocytosis (Ahn et al. 2003). Upon engagement of activated GPCRs, conformational changes within β-arrestin lead to its association with clathrin and AP-2β via mechanisms described above (Fig. 1, no. 3). Thus, the task of facilitating internalization of the largest family of receptors known is not left to AP-2, but is in fact assigned to β-arrestins as dedicated monomeric sorting adaptors. Recent data suggest that β-arrestin-bound GPCRs via PDZ-mediated linkage to the actin cytoskeleton are sorted into a distinct subset of clathrin-coated pits characterized by delayed dynamin-mediated fission (Puthenveedu and von Zastrow 2006).

Other AP-2 appendage domain-associated proteins that function as CLASPs for specific cargo are ARHs (autosomal recessive hypercholesterolemias), an adaptor targeting hepatic LDL receptors for internalization via recognition of its FxNPxY motif. ARH deficiency is genetically associated with early onset hypercholesterolemia in mice and men (Jones et al. 2007). A similar function is fulfilled by the DPF motif-containing CLASP disabled-2 (Dab2) in HeLa cells.

In neurons, clathrin/AP-2-mediated endocytosis plays an important, if not essential role in the recycling of small synaptic vesicles (SVs) (Takamori et al. 2006) to sustain continued neurotransmitter release. During exocytosis SV proteins become incorporated into the presynaptic plasma membrane from where they need to be sorted directly into a subset of clathrin-coated vesicles dedicated to SV biogenesis (Maycox et al. 1992). The calcium-sensing protein synaptotagmin 1 appears to function in coupling the exo- and endocytic limbs of the SV cycle, at least in part by interacting with AP-2μ (discussed above) and the monomeric CLASP stonin 2/stoned B (Fig. 1, no. 2). In the mammalian brain stonin 2 is closely associated with AP-2 via α-appendage domain binding Wxx[F/W]ac motifs (Fig. 1, no. 8) and with the endocytic accessory proteins eps15 and intersectin (Walther et al. 2004; Martina et al. 2001). Stonin2 targets synaptotagmin 1 and perhaps other associated SV proteins to the recycling vesicle pool and

thereby serves as a CLASP specifically involved in SV endocytosis (Diril et al. 2006). Similar observations have been made for its ortholog stoned B in *Drosophila melanogaster* (Fergestad and Broadie 2001; Stimson et al. 2001).

In recent years, it has become clear that covalent modification of cargo by mono- or multi-ubiquitination, usually following ligand-induced receptor activation and perhaps phosphorylation, can also function as an endocytosis signal. As with the examples described above, ubiquitinated cargo is not recognized by AP-2 directly, but instead associates with ubiquitin-binding accessory proteins including the UIM (ubiquitin-interacting motif)-containing factors eps15 and epsin. Examples of cargo undergoing regulated ubiquitination and clathrin-dependent internalization include the EGF and growth hormone receptors, epithelial sodium channels (ENaC), and Notch (Stang et al. 2004; Gupta-Rossi et al. 2004; Wang et al. 2006). Other accessory factors may also function in cargo-selective clathrin-dependent internalization pathways. These include HIP1/1R, dishevelled (Dvl), and numb.

9 Pharmacological Inhibition of Clathrin/AP-2-Mediated Endocytosis

In spite of the extensive knowledge about clathrin/AP-2-mediated endocytosis and the mechanics of the underlying protein–protein interaction network, the development of pharmacological tools to interfere with this process is still in its infancy. Most inhibitors known to interfere with the clathrin pathway are ill characterized and non-specific, and thus display other negative side effects on cells. These are summarized below.

9.1 Inhibition by Cytosolic Acidification

Acidification of the cytosol to a pH below 6.5 strongly reduces the endocytic uptake of transferrin or EGF. However, the number of binding sites for transferrin is not reduced, nor is the number of coated pits. The apparent molecular reason for the observed inhibition of internalization is that coated vesicles are unable to pinch off from the plasma membrane, although they are assembled correctly (Heuser 1989; Sandvig et al. 1987). Thus, cytosolic acidification prevents endocytosis by paralyzing clathrin lattices that remain attached to the plasma membrane. Under these conditions, adaptors including AP-2 remain aggregated at the plasma membrane and associated with receptors (Sorkin and Carpenter 1993; Hansen et al. 1993). Negative side effects of cytosol acidification on cell physiology are to be expected, but have not been investigated in detail.

9.2 K^+ Depletion

Fibroblasts incubated in potassium-free medium lose about 80% of their intracellular potassium within 10 min and show disrupted clathrin-coated pits. When the K^+ level

reaches a critical threshold of 40% below normal, the number of coated pits declines by more than 80%, and the rate of endocytosis is decreased by 70–95%, despite normal receptor binding. However, the morphology of cells changes rapidly, suggesting that other important cellular functions may be affected (Altankov and Grinnell 1993; Larkin et al. 1983). Why K^+ depletion affects endocytosis is unknown. Also, it is unclear to which extent other clathrin-independent internalization pathways are affected.

9.3 Chlorpromazine

Chlorpromazine is a cationic amphiphilic drug that prevents recycling of receptors for transferrin, LDL, and EGF (Wang et al. 1993). Chlorpromazine and related drugs decrease the amount of clathrin-coated pits on the cell surface. They also cause coated pits to assemble on endosomes and relocation of the AP-2 complex from the plasma membrane to the cytoplasm and to cytoplasmic vesicles of unknown origin or identity. As with other endocytosis inhibitors, the effects appear to be reversible. Given that cationic amphiphilic drugs affect the activity of many different enzymes like phosphatadic acid phosphorylase, protein kinase C, or Ca^+/calmodulin-dependent enzymes (Wang et al. 1993), inhibition of clathrin-mediated endocytosis will almost certainly be accompanied by perturbation of other metabolic pathways.

9.4 High Sucrose

When fibroblasts are incubated in hypertonic media containing high levels of sucrose (0.45 M), normal clathrin lattices largely disappear. Instead, microcages, unusual polymers that are sharply curved and devoid of membrane, accumulate. Concomitantly, LDL receptors are dispersed all over the cell surface. However, when returning to normal medium at 37°C, the changes are reversed within 10 min (Heuser and Anderson 1989). How such treatments affect cell physiology again is unknown.

9.5 Dynasore

Recent advances in large-scale screening approaches have led to the identification and characterization of the first chemical inhibitor of the large GTPase dynamin, an essential factor in fission of clathrin/AP-2-coated vesicles (Macia et al. 2006). Dynasore selectively and dose-dependently inhibits the GTPase activity of dynamins 1 and 2, and at higher doses presumably other dynamin family members. Dynasore-mediated blockade of clathrin-coated vesicle fission takes place within seconds and results in the accumulation of U-shaped, half-formed pits, as well as O-shaped intermediates stalled at the fission stage.

Based on its mechanism of action, dynasore acts as an inhibitor of all endocytic pathways known to be dependent on dynamin-mediated fission. These include, besides clathrin/AP-2-mediated endocytosis, fission of TGN-derived vesicles (McNiven 1998), caveolar endocytosis (Henley et al. 1998), podosome (Ochoa et al. 2000) and centrosome function (Thompson et al. 2004), and cell migration (Macia et al. 2006).

10 Perspective

The structural era of endocytosis that we have witnessed for the past decade or so has provided us with a detailed molecular framework of the mechanics of cargo selection, membrane deformation, and vesicle fission in the clathrin/AP-2 pathway. We have detailed knowledge of the elaborate network of protein–protein and protein–lipid interactions within the endocytic mesh. AP-2, clathrin, and phosphoinositides appear to correspond to central hubs in the organization of the endocytic network. Based on the detailed biochemical and structural knowledge, we now seem to be in a position to use this information for the design of chemical approaches to rapidly and selectively interfere with clathrin/ AP-2 function in general or with cargo-selective pathways of internalization.

Automated screening paired with sensitive fluorescence-based functional assays in living cells and in vivo may pave the way to identify compounds that selectively target interaction hubs or specific interaction sites within a hub protein. We know now that intracellular signaling events are interrelated with receptor endocytosis. Moreover, gradients of morphogens are generated by fine-tuning of clathrin/AP-2-mediated endocytic events in complex cellular systems or developing polarized tissues (Berdnik et al. 2002; Dudu et al. 2004). The availability of selective pharmacological inhibitors targeting hubs within the endocytic network may provide important tools to acutely interfere with clathrin/AP-2-dependent endocytic mechanisms.

Perhaps, as importantly, the functional and molecular characterization of endocytic AP-2-binding accessory proteins as cargo-selective adaptors identifies these as potential targets for the design of selective inhibitors targeting subsets of clathrin-coated vesicles without perturbing endocytic mechanisms in general. If paired with powerful genetics including RNA interference-mediated knockdown of endocytic proteins, we are also in a position to ascertain that the pharmacological tools identified exhibit specific effects without affecting other cell physiological functions.

Acknowledgements Work in the authors' laboratory is supported by grants from the Deutsche Forschungsgemeinschaft (DFG; FOR806), the German Ministry of Science and Technology (BMBF, RENTRAFF), and the European Molecular Biology Organization (EMBO YIP award to V.H.).

References

Ahn S, Nelson CD, Garrison TR, Miller WE, Lefkowitz RJ (2003) Desensitization, internalization, and signaling functions of beta-arrestins demonstrated by RNA interference. Proc Natl Acad Sci USA 100:1740–1744

Altankov G, Grinnell F (1993) Depletion of intracellular potassium disrupts coated pits and reversibly inhibits cell polarization during fibroblast spreading. J Cell Biol 120:1449–1459
Bauer M, Pelkmans L (2006) A new paradigm for membrane-organizing and -shaping scaffolds. FEBS Lett 580:5559–5564
Beck KA, Keen JH (1991) Interaction of phosphoinositide cycle intermediates with the plasma membrane-associated clathrin assembly protein AP-2. J Biol Chem 266:4442–4447
Berdnik D, Torok T, Gonzalez-Gaitan M, Knoblich JA (2002) The endocytic protein alpha-adaptin is required for numb-mediated asymmetric cell division in Drosophila. Dev Cell 3:221–231
Boehm M, Bonifacino JS (2001) Adaptins: the final recount. Mol Biol Cell 12:2907–2920
Boll W, Rapoport I, Brunner C, Modis Y, Prehn S, Kirchhausen T (2002) The mu2 subunit of the clathrin adaptor AP-2 binds to FDNPVY and YppO sorting signals at distinct sites. Traffic 3: 590–600
Bonifacino JS, Traub LM (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 72:395–447
Bonifacino JS, Lippincott-Schwartz J (2003) Coat proteins: shaping membrane transport. Nat Rev Mol Cell Biol 4:409–414
Chen YJ, Zhang P, Egelman EH, Hinshaw JE (2004) The stalk region of dynamin drives the constriction of dynamin tubes. Nat Struct Mol Biol 11:574–557
Collins BM, McCoy AJ, Kent HM, Evans PR, Owen DJ (2002) Molecular architecture and functional model of the endocytic AP2 complex. Cell 109:523–535
Conner SD, Schmid SL (2003) Regulated portals of entry into the cell. Nature 422:37–44
Cremona O, Di Paolo G, Wenk MR, Luthi A, Kim WT, Takei K, Daniell L, Nemoto Y, Shears SB, Flavell RA, McCormick DA, De Camilli P (1999) Essential role of phosphoinositide metabolism in synaptic vesicle recycling. Cell 99:179–188
Dell'Angelica EC (2001) Clathrin-binding proteins: got a motif? Join the network! Trends Cell Biol 11: 315–318
Derkach VA, Oh MC, Guire ES, Soderling TR (2007) Regulatory mechanisms of AMPA receptors in synaptic plasticity. Nat Rev Neurosci 8:101–113
Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. Nature 443:651–657
Diril MK, Wienisch M, Jung N, Klingauf J, Haucke V (2006) Stonin 2 is an AP-2-dependent endocytic sorting adaptor for synaptotagmin internalization and recycling. Dev Cell 10:233–244
Dudu V, Pantazis P, Gonzalez-Gaitan M (2004) Membrane traffic during embryonic development: epithelial formation, cell fate decisions and differentiation. Curr Opin Cell Biol 16: 407–414
Edeling MA, Mishra SK, Keyel PA, Steinhauser AL, Collins BM, Roth R, Heuser JE, Owen DJ, Traub LM (2006a) Molecular switches involving the AP-2 beta2 appendage regulate endocytic cargo selection and clathrin coat assembly. Dev Cell 10:329–342
Edeling MA, Smith C, Owen D (2006b) Life of a clathrin coat: insights from clathrin and AP structures. Nat Rev Mol Cell Biol 7:32–44
Fergestad T, Broadie K (2001) Interaction of stoned and synaptotagmin in synaptic vesicle endocytosis. J Neurosci 21:1218–1227
Gaidarov I, Keen JH (1999) Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits. J Cell Biol 146:755–764
Gaidarov I, Smith ME, Domin J, Keen JH (2001) The class II phosphoinositide 3-kinase C2alpha is activated by clathrin and regulates clathrin-mediated membrane trafficking. Mol Cell 7:443–449
Galli T, Haucke V (2004) Cycling of synaptic vesicles: how far? How fast! Sci STKE 2004: re19
Glebov OO, Bright NA, Nichols BJ (2006) Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nat Cell Biol 8:46–54
Gonzalez-Gaitan M, Jackle H (1997) Role of Drosophila alpha-adaptin in presynaptic vesicle recycling. Cell 88:767–776
Gupta-Rossi N, Six E, LeBail O, Logeat F, Chastagner P, Olry A, Israel A, Brou C (2004) Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor. J Cell Biol 166:73–83

Hansen SH, Sandvig K, van Deurs B (1993) Clathrin and HA2 adaptors: effects of potassium depletion, hypertonic medium, and cytosol acidification. J Cell Biol 121:61–72
Harris TW, Hartwieg E, Horvitz HR, Jorgensen EM (2000) Mutations in synaptojanin disrupt synaptic vesicle recycling. J Cell Biol 150:589–600
Haucke V (2005) Phosphoinositide regulation of clathrin-mediated endocytosis. Biochem Soc Trans 33:1285–1289
Haucke V (2006) Cargo takes control of endocytosis. Cell 127:35–37
Henley JR, Krueger EW, Oswald BJ, McNiven MA (1998) Dynamin-mediated internalization of caveolae. J Cell Biol 141:85–99
Heuser J (1989) Effects of cytoplasmic acidification on clathrin lattice morphology. J Cell Biol 108:401–411
Heuser JE, Anderson RG (1989) Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. J Cell Biol 108:389–400
Heuser JE, Keen J (1988) Deep-etch visualization of proteins involved in clathrin assembly. J Cell Biol 107:877–886
Hinrichsen L, Harborth J, Andrees L, Weber K, Ungewickell EJ (2003) Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells. J Biol Chem 278:45160–45170
Hinrichsen L, Meyerholz A, Groos S, Ungewickell EJ (2006) Bending a membrane: how clathrin affects budding. Proc Natl Acad Sci USA 103:8715–8720
Honing S, Ricotta D, Krauss M, Spate K, Spolaore B, Motley A, Robinson M, Robinson C, Haucke V, Owen DJ (2005) Phosphatidylinositol-(4,5)-bisphosphate regulates sorting signal recognition by the clathrin-associated adaptor complex AP2. Mol Cell 18:519–531
Huang F, Khvorova A, Marshall W, Sorkin A (2004) Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. J Biol Chem 279:16657–16661
Jha A, Agostinelli NR, Mishra SK, Keyel PA, Hawryluk MJ, Traub LM (2004) A novel AP-2 adaptor interaction motif initially identified in the long-splice isoform of synaptojanin 1, SJ170. J Biol Chem 279:2281–2290
Jones C, Garuti R, Michaely P, Li WP, Maeda N, Cohen JC, Herz J, Hobbs HH (2007) Disruption of LDL but not VLDL clearance in autosomal recessive hypercholesterolemia. J Clin Invest 117:165–1674
Jost M, Simpson F, Kavran JM, Lemmon MA, Schmid SL (1998) Phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. Curr Biol 8:1399–102
Kastning K, Kukhtina V, Kittler JT, Chen G, Pechstein A, Enders S, Lee SH, Sheng M, Yan Z, Haucke V (2007) Molecular determinants for the interaction between AMPA receptors and the clathrin adaptor complex AP-2. Proc Natl Acad Sci USA 104:2991–2996
Kirchhausen T (1999) Adaptors for clathrin-mediated traffic. Annu Rev Cell Dev Biol 15: 705–732
Kirchhausen T (2000) Clathrin. Annu Rev Biochem 69:699–727
Krauss M, Haucke V (2007) Phosphoinositides: Regulators of membrane traffic and protein function. FEBS Lett 581: 2015–2011
Krauss M, Kinuta M, Wenk MR, De Camilli P, Takei K, Haucke V (2003) ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type Igamma. J Cell Biol 162:113–124
Lakadamyali M, Rust MJ, Zhuang X (2006) Ligands for clathrin-mediated endocytosis are differentially sorted into distinct populations of early endosomes. Cell 124:997–1009
Larkin JM, Brown MS, Goldstein JL, Anderson RG (1983) Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. Cell 33: 273–285
Lee SH, Simonetta A, Sheng M (2004) Subunit rules governing the sorting of internalized AMPA receptors in hippocampal neurons. Neuron 43:221–236
Lefkowitz RJ, Shenoy SK (2005) Transduction of receptor signals by beta-arrestins. Science 308: 512–517
Lewin DA, Mellman I (1998) Sorting out adaptors. Biochim Biophys Acta 1401:129–145

Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T (2006) Dynasore, a cell-permeable inhibitor of dynamin. Dev Cell 10:839–850
Maldonado-Baez L, Wendland B (2006) Endocytic adaptors: recruiters, coordinators and regulators. Trends Cell Biol 16:505–513
Martina JA, Bonangelino CJ, Aguilar RC, Bonifacino JS (2001) Stonin 2: an adaptor-like protein that interacts with components of the endocytic machinery. J Cell Biol 153:1111–1120
Maycox PR, Link E, Reetz A, Morris SA, Jahn R (1992) Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling. J Cell Biol 118:1379–1388
McNiven MA (1998) Dynamin: a molecular motor with pinchase action. Cell 94:151–14
Mitsunari T, Nakatsu F, Shioda N, Love PE, Grinberg A, Bonifacino JS, Ohno H (2005) Clathrin adaptor AP-2 is essential for early embryonal development. Mol Cell Biol 25:9318–9323
Morrow IC, Parton RG (2005) Flotillins and the PHB domain protein family: rafts, worms and anaesthetics. Traffic 6:725–740
Motley A, Bright NA, Seaman MN, Robinson MS (2003) Clathrin-mediated endocytosis in AP-2-depleted cells. J Cell Biol 162:909–918
Motley AM, Berg N, Taylor MJ, Sahlender DA, Hirst J, Owen DJ, Robinson MS (2006) Functional analysis of AP-2 alpha and mu2 subunits. Mol Biol Cell 17:5298–5308
Mukherjee S, Ghosh RN, Maxfield FR (1997) Endocytosis. Physiol Rev 77:759–803
Nesterov A, Carter RE, Sorkina T, Gill GN, Sorkin A (1999) Inhibition of the receptor-binding function of clathrin adaptor protein AP-2 by dominant-negative mutant mu2 subunit and its effects on endocytosis. EMBO J 18:2489–2499
Newmyer SL, Christensen A, Sever S (2003) Auxilin-dynamin interactions link the uncoating ATPase chaperone machinery with vesicle formation. Dev Cell 4:929–940
Ochoa GC, Slepnev VI, Neff L, Ringstad N, Takei K, Daniell L, Kim W, Cao H, McNiven M, Baron R, De Camilli P (2000) A functional link between dynamin and the actin cytoskeleton at podosomes. J Cell Biol 150:377–389
Ohno H, Stewart J, Fournier MC, Bosshart H, Rhee I, Miyatake S, Saito T, Gallusser A, Kirchhausen T, Bonifacino JS (1995) Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science 269:1872–1875
Owen DJ, Evans PR (1998) A structural explanation for the recognition of tyrosine-based endocytotic signals. Science 282:1327–1332
Owen DJ, Luzio JP (2000) Structural insights into clathrin-mediated endocytosis. Curr Opin Cell Biol 12:467–474
Owen DJ, Vallis Y, Noble ME, Hunter JB, Dafforn TR, Evans PR, McMahon HT (1999) A structural explanation for the binding of multiple ligands by the alpha-adaptin appendage domain. Cell 97:805–815
Owen DJ, Setiadi H, Evans PR, McEver RP, Green SA (2001) A third specificity-determining site in mu 2 adaptin for sequences upstream of Yxx phi sorting motifs. Traffic 2:105–110
Perera RM, Zoncu R, Lucast L, De Camilli P, Toomre D (2006) Two synaptojanin 1 isoforms are recruited to clathrin-coated pits at different stages. Proc Natl Acad Sci USA 103: 19332–19337
Praefcke GJ, Ford MG, Schmid EM, Olesen LE, Gallop JL, Peak-Chew SY, Vallis Y, Babu MM, Mills IG, McMahon HT (2004) Evolving nature of the AP2 alpha-appendage hub during clathrin-coated vesicle endocytosis. EMBO J 23:4371–4383
Puthenveedu MA, von Zastrow M (2006) Cargo regulates clathrin-coated pit dynamics. Cell 127: 113–124
Ricotta D, Conner SD, Schmid SL, von Figura K, Honing S (2002) Phosphorylation of the AP2 mu subunit by AAK1 mediates high affinity binding to membrane protein sorting signals. J Cell Biol 156:791–795
Ritter B, Philie J, Girard M, Tung EC, Blondeau F, McPherson PS (2003) Identification of a family of endocytic proteins that define a new alpha-adaptin ear-binding motif. EMBO Rep 4: 1089–1095
Robinson MS, Bonifacino JS (2001) Adaptor-related proteins. Curr Opin Cell Biol 13:444–453

Rohde G, Wenzel D, Haucke V (2002) A phosphatidylinositol (4,5)-bisphosphate binding site within mu2-adaptin regulates clathrin-mediated endocytosis. J Cell Biol 158:209–214

Roux A, Uyhazi K, Frost A, De Camilli P (2006) GTP-dependent twisting of dynamin implicates constriction and tension in membrane fission. Nature 441:528–531

Sandvig K, Olsnes S, Petersen OW, van Deurs B (1987) Acidification of the cytosol inhibits endocytosis from coated pits. J Cell Biol 105:679–689

Schweizer FE, Ryan TA (2006) The synaptic vesicle: cycle of exocytosis and endocytosis. Curr Opin Neurobiol 16:298–304

Sorkin A, Carpenter G (1993) Interaction of activated EGF receptors with coated pit adaptins. Science 261:612–615

Stang E, Blystad FD, Kazazic M, Bertelsen V, Brodahl T, Raiborg C, Stenmark H, Madshus IH (2004) Cbl-dependent ubiquitination is required for progression of EGF receptors into clathrin-coated pits. Mol Biol Cell 15:3591–3604

Stimson DT, Estes PS, Rao S, Krishnan KS, Kelly LE, Ramaswami M (2001) Drosophila stoned proteins regulate the rate and fidelity of synaptic vesicle internalization. J Neurosci 21: 3034–3044

Takamori S, Holt M, Stenius K, Lemke EA, Gronborg M, Riedel D, Urlaub H, Schenck S, Brugger B, Ringler P, Muller SA, Rammner B, Grater F, Hub JS, De Groot BL, Mieskes G, Moriyama Y, Klingauf J, Grubmuller H, Heuser J, Wieland F, Jahn R (2006) Molecular anatomy of a trafficking organelle. Cell 127:831–846

Takei K, Haucke V (2001) Clathrin-mediated endocytosis: membrane factors pull the trigger. Trends Cell Biol 11:385–391

Thompson HM, Cao H, Chen J, Euteneuer U, McNiven MA (2004) Dynamin 2 binds gamma-tubulin and participates in centrosome cohesion. Nat Cell Biol 6:335–342

Traub LM (2003) Sorting it out: AP-2 and alternate clathrin adaptors in endocytic cargo selection. J Cell Biol 163:203–208

Traub LM (2005) Common principles in clathrin-mediated sorting at the Golgi and the plasma membrane. Biochim Biophys Acta 1744:415–437

Varnai P, Thyagarajan B, Rohacs T, Balla T (2006) Rapidly inducible changes in phosphatidylinositol 4,5-bisphosphate levels influence multiple regulatory functions of the lipid in intact living cells. J Cell Biol 175:377–382

Walther K, Diril MK, Jung N, Haucke V (2004) Functional dissection of the interactions of stonin 2 with the adaptor complex AP-2 and synaptotagmin. Proc Natl Acad Sci USA 101:964–969

Wang LH, Rothberg KG, Anderson RG (1993) Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. J Cell Biol 123:1107–1117

Wang H, Traub LM, Weixel KM, Hawryluk MJ, Shah N, Edinger RS, Perry CJ, Kester L, Butterworth MB, Peters KW, Kleyman TR, Frizzell RA, Johnson JP (2006) Clathrin-mediated endocytosis of the epithelial sodium channel. Role of epsin. J Biol Chem 281:14129–14135

PDE4 Associates with Different Scaffolding Proteins: Modulating Interactions as Treatment for Certain Diseases

A.C. McCahill, E. Huston, X. Li, and M.D. Houslay(✉)

Contents

1 Introduction 126
2 PDE4 128
 2.1 Structure of PDE4 129
 2.2 Regulation of PDE4 130
 2.3 Therapeutic Uses and Limitations of PDE4 Inhibitors 133
 2.4 Targeting/Compartmentalization of PDE4 135
 2.5 Intracellular Targeting via Protein–Protein Interactions 137
3 Alternatives to PDE4 Catalytic Site Inhibitors 150
 3.1 Peptide Array Analyses to Identify Protein–Protein Interaction Sites 151
 3.2 Disruptor Therapeutics 153
 3.3 Dominant Negative Approach to Inhibition by Displacement 153
4 Conclusions 155
References 156

Abstract cAMP is an ubiquitous second messenger that is crucial to many cellular processes. The sole means of terminating the cAMP signal is degradation by cAMP phosphodiesterases (PDEs). The PDE4 family is of particular interest because PDE4 inhibitors have therapeutic potential for the treatment of various inflammatory and auto-immune diseases and also have anti-depressant and memory-enhancing effects. The subcellular targeting of PDE4 isoforms is fundamental to the compartmentalization of cAMP signaling pathways and is largely achieved via protein–protein interactions. Increased knowledge of these protein–protein interactions and their regulatory properties could aid in the design of novel isoform-specific inhibitors with improved efficacy and fewer prohibitive side effects.

M.D. Houslay
Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G128QQ, Scotland, UK
m.houslay@bio.gla.ac.uk

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,

© Springer-Verlag Berlin Heidelberg 2008

1 Introduction

Cyclic adenosine 3′, 5′-monophosphate (cAMP) is an ubiquitous second messenger used in all cells to control key processes including metabolism, cytoskeletal function, gene expression, proliferation and the cell cycle (Beavo and Brunton 2002; Tasken and Aandahl 2004; Wong and Scott, 2004; Baillie and Houslay 2005; Dumaz and Marais 2005; Taylor et al. 2005; Bos, 2006). cAMP is generated from ATP by the action of adenylyl cyclases at the plasma membrane (Cooper 2003). These adenylyl cyclases are primarily activated via the stimulation of transmembrane G-protein coupled receptors (GPCRs), which, upon agonist stimulation, couple to stimulatory G-protein, Gs, to activate adenylyl cyclase and produce cAMP (Pierce et al. 2002; Hill 2006). Increased levels of cAMP are translated into cellular responses by cAMP effectors, the best known of which is protein kinase A (PKA) (Wong and Scott, 2004; Taylor et al. 2005) but which also include EPACs (exchange proteins activated by cAMP) and cAMP-gated ion channels (CNGCs) (Karpen and Rich 2001; DiPilato et al. 2004; Kooistra et al. 2005; Li et al. 2006; Bos, 2006). These three effectors of cAMP mediate a myriad of cAMP signaling pathways in many cells and tissues (Baillie and Houslay 2005) (Fig. 1).

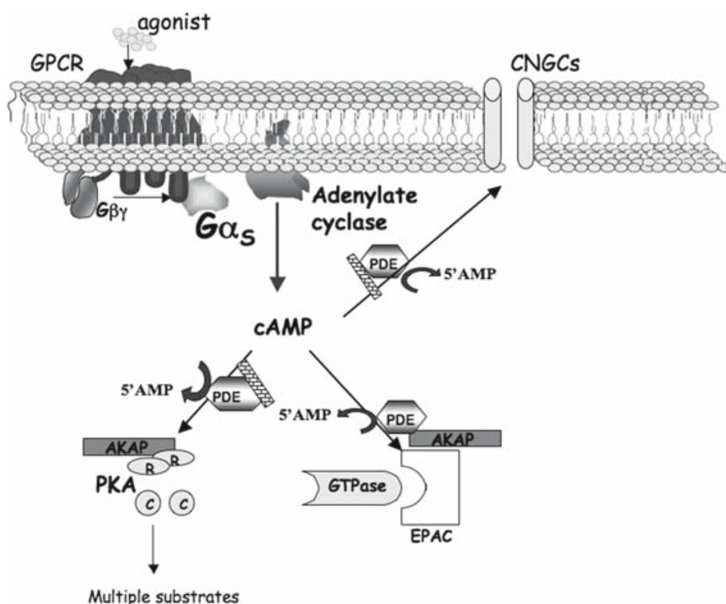

Fig. 1 Compartmentalization of the cAMP signaling pathway. Specific Gs-protein-coupled receptors are activated by binding of agonist; this allows the production of cAMP at specific regions of the plasma membrane by appropriately localized adenylyl cyclases. Propagation of the cAMP signal is then shaped by the action of differentially targeted PDEs. This compartmentalized system allows the differential activation of spatially distinct pools of cAMP effectors, such as AKAP-tethered PKA, EPAC or CGNCs

The concept of cAMP signaling compartmentalization (compartmentation) was first introduced in 1981 by Brunton et al. In this pioneering work, they demonstrated that the cAMP generated by stimulation of different Gs-coupled receptors in cardiac myocytes led to differential activation of the type-I and type-II isoforms of PKA. This groundbreaking work has led to a wide body of evidence for the spatial and temporal control of cAMP signaling pathways (Houslay and Adams 2003; Baillie and Houslay 2005; Cooper 2005; Cooper and Crossthwaite 2006). A number of studies have now demonstrated the presence of cAMP microdomains in cardiac myocytes (Zaccolo and Pozzan 2002; Brunton 2003; Vandecasteele et al. 2006) and other cell types (Karpen and Rich 2001; Cooper 2003), and it is now well recognized that cAMP signaling responses are compartmentalized (Houslay and Adams 2003; Baillie and Houslay 2005; Cooper 2005). The recent advances in the development of fluorescence resonance energy transfer (FRET) probes based on either PKA or EPAC and fluorescent CNGC probes have consolidated this hypothesis by allowing the visualization of these spatial and temporal gradients of cAMP in living cells (Zhang et al. 2001; Zaccolo and Pozzan 2002; Bos 2003; Nikolaev et al. 2004; Cooper 2005).

The extent of the complexity of the molecular mechanisms that exist in order for the cell to be able to compartmentalize cAMP signals is only now becoming understood. The formation of cAMP gradients is partially achieved through the restricted localization of the GPCRs and adenylyl cyclases that generate cAMP to specific regions of the plasma membrane. The activation of distinct adenylyl cyclases by specific Gs-coupled receptors is suggested to generate spatially segregated “clouds” of cAMP from different plasma membrane locales (Houslay and Milligan 1997; Cooper 2003). Once cAMP is generated, the sole means of its degradation to 5′ AMP to terminate the signal is through the action of cAMP phosphodiesterases (PDEs). A large family of cAMP-specific PDEs have been shown to be differentially targeted to distinct subcellular compartments in a cell-type-specific manner and are therefore in a position to play a pivotal role in shaping cAMP gradients (Houslay and Adams 2003). This non-uniform distribution of adenylyl cyclases and PDEs allows cAMP gradients to form within the 3-D matrix of the cell, whereby the cAMP concentration is highest nearest the membrane and least in areas that contain the highest levels of PDEs. Thus, the activation of certain GPCRs can increase the cAMP levels within discrete compartments in the cell and cause unique physiological responses (Hayes and Brunton 1982). Compartmentalization allows spatially distinct pools of cAMP effectors to be differentially activated. A further contributory mechanism to this is the tethering of various PKA isoforms to specific intracellular sites by their interaction with AKAPs (A-kinase anchoring proteins) (Colledge and Scott 1999). The ability of PKA to be activated by gradients of cAMP will therefore depend on the cell-type-specific expression and distribution of AKAPs. In addition to anchoring PKA isoenzymes, AKAPs are also shown to be able to assemble multi-protein signaling complexes that include other proteins such as PDEs, PKC and protein phosphatases (Baillie et al. 2005; Higashida et al. 2005). In this way, AKAPs and PDEs facilitate signal transduction, signal termination and crosstalk with other signaling pathways and so organize cAMP signaling in both space and time.

PDEs exist as a superfamily of enzymes with the ability to hydrolyze the cyclic nucleotides cAMP and cGMP into their 5′ monophosphate derivatives (Houslay and Milligan 1997; Beavo and Brunton 2002; Lugnier 2006). They have attracted attention as therapeutic targets with PDE inhibitors appearing to have a potentially broad application in treating various diseases including asthma, chronic obstructive pulmonary disease (COPD), heart failure, atherosclerotic peripheral arterial disease and neurological disorders (Huang et al. 2001; O'Donnell and Zhang 2004; Houslay et al. 2005; Boswell-Smith et al. 2006; Fan Chung 2006; Stehlik and Movsesian 2006; Ravipati et al. 2007). The best-known example of therapeutic use for a PDE inhibitor is the inhibition of PDE5 by sildenafil (Viagra™) for the treatment of erectile dysfunction (Carson and Lue 2005). The PDE4 family is of particular interest as it has been shown to have links with asthma, COPD, depression and cognition (Barnette and Underwood 2000; Giembycz 2000; Souness et al. 2000; Ghavami et al. 2006). PDE4 inhibitors have proven therapeutic effects in these areas; however their usefulness has been marred by unwanted side-effects such as nausea and vomiting (Barnette 1999; Robichaud et al. 1999). PDE4 is also one of the most extensively studied PDE families, and much is now known about its regulation and targeting via protein–protein interactions. This review will focus on these interactions and explore the possibility of exploiting the wealth of knowledge on PDE4 targeting by designing new molecules that can disrupt specific interactions for therapeutic benefit.

2 PDE4

PDE4 is a cAMP-specific member of the cyclic nucleotide PDE superfamily, which consists of 11 different family members classified on the basis of their sequence identities, substrate specificities, allosteric regulatory characteristics and pharmacological properties (Bolger 1994; Conti and Beavo, 2007; Houslay 1998, 2001, 2005, 2006; Conti and Jin 1999; Conti et al. 2003; Houslay and Adams 2003; Houslay et al. 2005). Eight PDE families generate over 30 different isoforms that are able to hydrolyze cAMP, achieved by a combination of multiple genes and alternate splicing. The existence of multiple PDE isoforms with the same catalytic action implies that such diversity must be of functional importance. This is exemplified by the PDE4 family where a plethora of isoforms have been shown to be differentially involved in intracellular targeting or crosstalk with other signaling pathways. Evidence from RNA silencing, transgenic knockouts in mice, selective inhibitors and dominant negative approaches have further confirmed the lack of redundancy between PDE4 isoforms (Ariga et al. 2004; Jin et al. 2005; Lynch et al. 2005; McCahill et al. 2005; Xiang et al. 2005; Lugnier 2006).

The mammalian PDE4 is a homologue of the Drosophila *dunce* gene – so called as mutations in this gene give rise to flies with memory and learning defects (Davis et al. 1995). PDE4s have also been linked to memory and cognition by virtue of the memory-enhancing effects of PDE4 inhibitors. The archetypal PDE4 inhibitor is a

compound named rolipram, and PDE4s are, in part, classified on their ability to be inhibited by this drug, although the product of the Drosophila *dunce* gene is insensitive to inhibition by rolipram, despite being a PDE4 species. As well as cognition effects, rolipram and other drugs of this class are also involved in regulating inflammation. Indeed, the potent anti-inflammatory effects of PDE4 inhibitors have made them of great interest to drug companies for development of novel therapies for disease states such as COPD, asthma and rheumatoid arthritis (Houslay et al. 2005). More recently, PDE4 variants have been linked with ischemic stroke (Gretarsdottir et al. 2003; Meschia et al. 2005) and schizophrenia (Millar et al. 2005), which has only served to increase the interest in PDE4 as a therapeutic target.

2.1 Structure of PDE4

The mammalian PDE4 family comprises around 20 different isoforms encoded by four genes, PDE4A, PDE4B, PDE4C and PDE4D (Conti et al. 2003; Houslay and Adams 2003; Houslay et al. 2005) (Fig. 2). Each gene is large and complex, spanning around 50 kb and having around 20 exons, which gives rise to multiple isoforms by alternative mRNA splicing (Monaco et al. 1994; Bolger et al. 1997). Each isoform has a modular structure consisting of an isoform-specific N-terminal region, all or part of

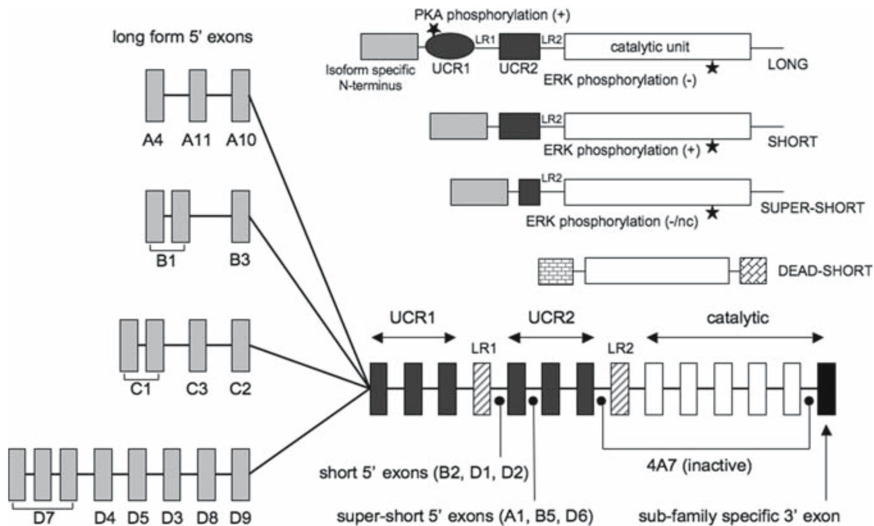

Fig. 2 The PDE4 enzyme family. Schematic diagram of the gene organization of the four PDE4 sub-families showing the exons that encode the core UCR1/2 and catalytic regions, the exons that encode the long form 5′ unique regions and the positions of other unique exons. Also shown are the four isoform subcategories based upon presence or absence of UCR1/2 regions and generated by alternative mRNA splicing

two regulatory sequences termed upstream conserved region 1 (UCR1) and UCR2, a highly conserved catalytic domain and a sub-family-specific C-terminal region (Bolger et al. 1993) (Fig. 2). The unique N-terminal region is generally encoded by a single exon, except for the first or most 5′ isoform of each gene, which seems to be encoded by two or more 5′ exons (Sullivan et al. 1999; Rena et al. 2001; D'Sa et al. 2002; Wang et al. 2003), and which is immediately downstream of the promoter that drives its expression (Rena et al. 2001; Wallace et al. 2005). This unique N-terminal region characterizes each isoform and confers the ability to interact with specific binding partners and thus be targeted to distinct intracellular regions (see Sects. 2.4 and 2.5). UCR1 and UCR2 are each encoded by three separate exons and are highly conserved between the four PDE4 sub-families (Bolger 1994). These regulatory regions are unique to isoforms of the PDE4 family, and their presence, in conjunction with susceptibility to rolipram inhibition, distinguishes PDE4s from all other classes of PDE. The various PDE4 isoforms can be further categorized into four groups, namely the long PDE4 forms that contain both UCR1 and UCR2, the short forms that lack UCR1, but have an intact UCR2, the super-short forms that not only lack UCR1, but have an N-terminally truncated UCR2 (Houslay 2001), and the "dead-short" isoforms that are both N- and C-terminally truncated so as to be catalytically inactive (Johnston et al. 2004; Houslay et al. 2007). UCR1 and UCR2 function as a regulatory domain that controls the catalytic unit and confers regulatory functions on PDE4 by orchestrating the functional outcome of phosphorylation by PKA and ERK (Houslay and Adams 2003). UCR2 is joined to UCR1 by linker region 1 (LR1) and to the catalytic region by LR2. These short linker regions vary greatly between PDE4 sub-families for reasons that are not understood at present. The catalytic unit is encoded by six exons and, as might be expected, is highly conserved between members of the PDE4 family. Solving of the crystal structure of PDE4B2 catalytic unit revealed that it comprises three sub-domains formed by 17 α-helices (Xu et al. 2000; Ke and Wang 2007). The cAMP-binding active site is created as these three sub-domains come together to form a deep pocket containing two metal ions essential for catalytic activity and lined with hydrophobic and negatively charged residues, The structure, proposed catalytic mechanism and conformational changes of the catalytic unit have been discussed in detail elsewhere (Houslay and Adams 2003; Houslay et al. 2005).

The final exon encodes part of the catalytic unit together with the sub-family-specific C-terminal region, which is of unknown function, but which has been used advantageously to generate sub-family-specific antisera (Houslay et al. 1998).

2.2 Regulation of PDE4

PDE4 activity appears to be regulated at a number of levels, including post-translationally by multi-site phosphorylation (Lim et al. 1999; MacKenzie et al. 2000; MacKenzie et al. 2002), at the transcriptional level (Vicini and Conti 1997; Rena et al. 2001; Wallace et al. 2005) and through regulation of mRNA stability (Liu et al. 2000). Central to coordinating the functional output of regulation by phosphorylation are the UCR1

and UCR2 domains. These interact to, presumably, alter the conformation of the catalytic unit and mediate the consequences of phosphorylation by PKA and ERK (Houslay 2001). Their role in regulation of enzymatic activity first came to light when truncation studies revealed that removal of UCR2 led to an increase in catalytic activity (Jin et al. 1992; Lim et al. 1999). The constitutive inhibitory effect of UCR2 on PDE activity can be relieved by PKA phosphorylation at a site in the N-terminal region of UCR1 (Sette and Conti 1996; Hoffmann et al. 1998; MacKenzie et al. 2002). This site is present in all long PDE4 isoforms, but absent in short forms, giving insight into the importance of alternative splice variants (MacKenzie et al. 2002). The phosphorylation of UCR1 by PKA has also been shown to disrupt its intramolecular interaction with UCR2 (Beard et al. 2000). Interaction between UCR1 and UCR2 has been demonstrated by a variety of methods and is thought to occur via electrostatic interactions between the hydrophobic C-terminal portion of UCR1 and the hydrophilic N-terminal region of UCR2 (Beard et al. 2000). That PKA phosphorylation can activate all long PDE4 isoforms accounts for the early observation that elevation of intracellular cAMP leads to increased PDE4 activity (Marchmont and Houslay 1980) and also provides a feedback mechanism to reset the cellular cAMP levels after stimulation. PKA phosphorylation of UCR1 is thought to cause a conformational change, which activates PDE4 long forms by attenuating the interaction of UCR1 with UCR2 (Beard et al., 2000). In PDE4D3, the PKA target residue is serine 54 (S54), and mutation of this residue to a negatively charged aspartate or glutamate residue can mimic PKA activation (Hoffmann et al. 1998). Mutation of the conserved neighboring glutamate residue (E53) also mimics PKA activation, leading to the hypothesis that this negatively charged residue is required for an ion pair interaction, which holds the enzyme in a low activity state. Disruption of this ion pair interaction by E53 mutation or PKA phosphorylation of S54 shifts the PDE to an active conformation. The increase in the activity of PDE4 long forms observed on addition of the negatively charged phospholipid, phosphatidic acid (PA) is thought to work via the same mechanism (Nemoz et al. 1997). In PDE4D3 alone, the PKA phosphorylation of S54 also leads to an increase in sensitivity to rolipram inhibition (Alvarez et al. 1995; Hoffmann et al. 1998). Mutations in S54 and E53 lead to a range of effects on PKA activation and rolipram inhibition, showing that small changes in configuration or charge of UCR1 can lead to measurable activity changes via conformational changes to the catalytic unit (Hoffmann et al. 1998).

On the basis of studies done with PDE4D3, it has recently been proposed that a further means by which the UCR1 and UCR2 regions can regulate PDE4 enzymes is by mediating PDE4 homo-dimerization. Long forms of PDE4 have been proposed to exist as dimers within cells, while short forms have been proposed to exist as monomers, again highlighting the differential regulation of short and long forms. Deletion analysis indicated that dimerization requires the C-terminal half of UCR1 and the N-terminal half of UCR2, but does not involve the same charged residues reported to mediate the intramolecular interaction (Richter and Conti 2002). Mutations that prevent the dimerization of PDE4D3 also ablated its activation by PKA or PA, demonstrating the significance of dimerization for enzyme regulation (Richter and Conti 2004). Mutations that abolished PDE4D3 dimerization also reduced the sensitivity of this isoform to rolipram inhibition.

PDE4s are generally accepted to exist in a number of different states that have different affinities for the inhibitor, rolipram (Souness and Rao 1997). It now appears that dimerization via UCR1 and UCR2 may be involved in the generation and stabilization of certain of these different rolipram binding conformers (Bolger et al. 2007; Richter and Conti 2004). Other means of generating such conformers include protein–protein interactions, where changes in rolipram affinity have been seen with RACK1 binding to PDE4D5 (Yarwood et al. 1999), and SH3-domain-containing proteins with PDE4A4 (McPhee et al. 1999).

In addition to PKA phosphorylation, PDE4s can also be regulated via phosphorylation by the MAP kinase, ERK (Hoffmann et al. 1999; MacKenzie et al. 2000; Baillie et al. 2001; Hill et al. 2006). All PDE4 subfamilies, except for PDE4A, contain a single ERK consensus motif (P-X-S-P) within the third subdomain of their catalytic unit. This serine residue is subject to phosphorylation by ERK both in vitro and in vivo. In order to phosphorylate PDE4 isoforms, ERK must bind to the PDE4 via two docking sites (see Sect. 2.5.8), which flank the phosphorylation site (MacKenzie et al. 2000). The functional consequences of ERK phosphorylation are dependent on the presence of UCR1 and UCR2, such that long forms are profoundly inhibited, short forms are activated, and super-short forms are weakly inhibited (Hoffmann et al. 1999; MacKenzie et al. 2000). However, ERK inhibition of long isoforms can be negated by PKA phosphorylation (Hoffmann et al. 1999) and switched to activation by additional phosphorylation of a site within the catalytic unit through activation of reactive oxygen (ROS) signalling cascades (Hill et al. 2006). The complement of long or short isoforms generated by alternative RNA splicing will therefore determine cellular response to crosstalk between cAMP signaling and the ERK pathway. The importance of this is emphasized in U937 cells as they undergo remodeling of their PDE4 profile upon differentiation from a monocyte-like to macrophage-like phenotype. In monocytic U937 cells, long PDE4D isoforms predominate, so activation of ERK has an overall inhibitory effect, but upon differentiation to macrophages, the short form PDE4B2 is up-regulated to become predominant, and so activation of ERK elicits an overall increase in PDE4 activity (Shepherd et al. 2004). In the case of PDE4 long forms, a novel feedback system is in operation, whereby the inhibition induced by ERK phosphorylation causes a localized increase in cAMP levels, which activates PKA that, in turn, phosphorylates UCR1 to activate the enzyme and abolish ERK inhibition. Long forms of PDE4B, PDE4C and PDE4D can therefore affect a transient programmed rise in cAMP levels in response to activation of the ERK pathway (Hoffmann et al. 1999; Houslay and Kolch 2000).

Regulation of PDE4 also occurs at the level of transcription and translation (Conti 2002). As discussed above, the cell-type-specific expression pattern of different PDE4 isoforms is of crucial importance in determining response to their phosphorylation by PKA and ERK; however, the mechanisms behind this differential expression are only beginning to be elucidated. As would be expected for such large and complex genes, multiple mechanisms of regulation appear to be in operation. Intronic promoters have been identified for a number of specific PDE4 isoforms (Vicini and Conti 1997; Olsen and Bolger 2000; Rena et al. 2001; Le Jeune et al. 2002; Wallace et al. 2005). The presence of multiple promoters allows different combinations of long and short

isoforms to be expressed as required. Expression of a number of PDE4 isoforms was observed to be sensitive to cAMP, such that chronic stimulation with the adenylyl cyclase activator forskolin, or cAMP analogues, results in their up-regulation (Swinnen et al. 1991; Vicini and Conti 1997; Seybold et al. 1998; Le Jeune et al. 2002). This effect is now known to be mediated by cAMP response elements (CREs) in the promoter regions of PDE genes (D'Sa et al. 2002; Le Jeune et al. 2002). After phosphorylation by PKA, the transcription factor, CREB (CRE binding protein), can bind to the CRE regions and modulate gene transcription (Mayr and Montminy 2001). This feedback mechanism, whereby cAMP modulates the expression of the enzyme that degrades it, is though to be a long-term adaptive desensitization response that complements the short-term desensitization response accomplished by increased PDE4 activity on the PKA phosphorylation of long isoforms. A number of other agents have been reported to regulate PDE4 expression by mechanisms that remain undetermined (Houslay 2001). Of these, the most significant is the pro-inflammatory agent LPS (lipopolysaccharide), which specifically up-regulates PDE4B in monocytes and macrophages (Ma et al. 1999; Wang et al. 1999). This suggests that PDE4B, and in particular PDE4B2, may play a key role in inflammatory responses and is therefore the most appropriate target for the development of anti-inflammatory drugs (Ma et al. 1999). Finally, the expression of PDE4 isoforms is also likely to be regulated at the level of mRNA stability (Swinnen et al. 1991), as has been demonstrated to be the case for a number of PDE4D isoforms in vascular smooth muscle cells (Liu et al. 2000). In these cells, activation of the cAMP-PKA pathway results in the induction of the PDE4D1 and PDE4D2 isoforms; however, simultaneous activation of the cAMP-PKA and the ERK MAPK pathways attenuates induction of these two short forms by a mechanism involving altered mRNA stability.

2.3 Therapeutic Uses and Limitations of PDE4 Inhibitors

PDE4 inhibitors are of great interest to the pharmaceutical industry because their action in increasing intracellular cAMP can produce a wide range of desirable effects. Selective inhibitors of PDE4 form the largest group of inhibitors for any PDE family and have been studied as anti-inflammatory drugs targeting asthma and chronic obstructive pulmonary disease (COPD) and also as therapeutic agents for rheumatoid arthritis, multiple sclerosis, type II diabetes, septic shock and atopic dermatitis (Barnette and Underwood 2000; Giembycz 2000; Souness et al. 2000; Huang et al. 2001; Sturton and Fitzgerald 2002). PDE4 inhibitors have also been demonstrated to exhibit anti-depressant and memory-enhancing properties (O'Donnell and Zhang 2004), so are also of potential therapeutic use for the treatment of depression, Alzheimer's disease, Parkinson's disease and schizophrenia (Houslay et al. 2005; Zhang and O'Donnel 2007). They also have potential for treating sciatic nerve injury by promoting axonal growth (Gao et al. 2003; Pearse et al. 2004).

The prototypical PDE4 selective inhibitor is rolipram, a highly selective first generation PDE4 inhibitor, which has been extensively used for many years as a research

tool to chemically ablate PDE4 activity in order to elucidate its cellular role. Rolipram was clinically investigated for use as an anti-depressant (Zeller et al. 1984) and to treat Parkinson's disease (Parkes et al. 1984), but had to be withdrawn because of unacceptable gastro-intestinal side effects such as nausea and vomiting. Despite these problems, the excellent anti-inflammatory properties of PDE4 inhibitors meant they remained prime candidates for therapeutic development. A large number of second-generation PDE4 inhibitors have now been developed with the hope of maximizing the therapeutic benefit while minimizing side effects (Burnouf and Pruniaux 2002). These inhibitors have been slow to realize their early potential, with many plagued by side effects similar to that of rolipram, and none have yet reached the marketplace. However, Altana's roflumilast and GSK's cilomilast, the two most advanced PDE4 inhibitors in clinical trials for the treatment of asthma and COPD, have reached the late phase-III clinical trial/pre-registration stage, but have not yet been considered to show sufficient efficacy for approval to be granted (Lipworth 2005; Fan Chung 2006). These compounds clearly have potential, but side effects due to their general inhibition of all PDE4s continue to constrain their therapeutic window.

One theory postulated to explain the emetic activity of rolipram was centered on the fact the PDE4 isoforms can adopt two distinct conformational states with very different affinities for rolipram. These are known as the low-affinity rolipram binding state (LARBS) and the high-affinity rolipram binding state (HARBS) (Houslay 1998). While PDE4 LARBS was reported to be associated with anti-inflammatory activity, HARBS, which is generally expressed in the central nervous system, was linked to the high levels of adverse effects, such as nausea and vomiting, triggered by rolipram treatment (Fan Chung 2006). Consequently, efforts were made to design PDE4 inhibitors with improved LARBS and HARBS ratios; however, this hypothesis is now regarded as overly simplistic. At one time, HARBS and LARBS were thought to represent two distinct binding sites; however, it has now been unequivocally demonstrated that rolipram binds at a single site in the catalytic region of PDE4s, where it acts as a competitive inhibitor (Card et al. 2004; Xu et al. 2004; Wang et al. 2007). Current evidence suggests that HARBS and LARBS represent different conformers of PDE4, which may arise as a result of phosphorylation or the interaction of distinct PDE4 isoforms with other proteins such as XAP2, RACK1 and certain SH3 domain-containing proteins (Bolger et al. 2007). Indeed, a variety of studies have been performed suggesting that certain compounds may show selectivity to certain PDE4 sub-families. However, such evaluations are fraught with difficulties as the conformation of the enzyme preparations for each sub-family may be influenced by the nature of the isoform chosen, any post-translational modification and any interacting proteins. To date, no "Gold-standard" preparations with isoforms in each sub-family locked in either "low" or "high" affinity states for rolipram inhibition have been generated and then thoroughly analyzed against a range of PDE4-selective inhibitors.

A more recent proposal is that it is the specific inhibition of the PDE4D sub-family that is responsible for the emetic effects of PDE4 inhibitors by stimulation of neurons in the vomiting centre of the brain (Zhang and O'Donnell 2007). This hypothesis is based on inferences from PDE4D knockout mice and remains to be proven in conditional knockouts, in which developmental effects can be eliminated,

and with isoform-specific knockouts. It also depends upon inferences made from studying a model system in mice, a species that does not in fact exhibit emesis. Thus, such inferences require caution until further work is done. It seems clear, however, that one way forward is to develop inhibitors with sub-family or isoform specificity. In order to differentiate between enzymes with identical or almost identical catalytic sites, it will be necessary to focus on the cellular interactions of particular PDE4 isoforms that are integral to their compartmentalization and function. The last decade has seen significant advances in the understanding of these, mainly protein–protein, interactions (see Sect. 2.4 and 2.5), but presumably many more await discovery. Full characterization of these interactions will facilitate the development of inhibitors that either (1) act at the catalytic site, but are targeted to different isoforms as a consequence of conformational changes due to protein–protein interactions or (2) alter the function of a particular isoform by targeting the protein–protein interactions themselves so as to remove the target isoform from its functionally relevant compartment in the cell (see Sect. 3).

2.4 Targeting/Compartmentalization of PDE4

The existence of a myriad of PDE4 isoforms that are targeted to discrete sub-cellular locations and that have distinct regulatory properties means that PDE4s are key players in compartmentalizing cAMP signaling and regulating input from other signaling pathways (Baillie and Houslay 2005). The ability of PDE4s to be strategically targeted and anchored throughout the cell can be largely attributed to associations with other proteins. These protein–protein interactions are mediated mainly, but not exclusively, by the unique N-terminal regions of PDE4 isoforms (Houslay and Adams 2003; Huston et al. 2006b).

2.4.1 Role of the N-Terminal Region of PDE4s in Intracellular Targeting and cAMP Compartmentalization

As discussed in Sect. 2.1, a unique N-terminal region defines each PDE4 isoform. Evidence from our laboratory has shown unequivocally that this N-terminal region is essential in the intracellular targeting of PDE4 isoforms. Pivotal in this work has been the study of the super-short PDE4A, PDE4A1. PDE4A1 is unique in the PDE4 family in that it is entirely membrane bound and found, in a number of cell types, to be predominantly localized to the Golgi and to vesicles underlying the plasma membrane (Shakur et al. 1993, 1995; Pooley et al. 1997). However, removal of its isoform-unique 25-amino-acid N-terminal region generates a fully active and entirely soluble cytosolic form (Shakur et al. 1993). This discovery led to the proposal that intracellular targeting was pivotal to the PDE4 family and so formed an inherent part of the observed isoform multiplicity. As such, PDE4A1 has provided a particularly useful model in underpinning the notion of PDE4 intracellular targeting.

NMR analysis has revealed the N-terminal region of PDE4A1 is composed of 25 amino acids that form two distinct helical structures bound by a flexible and mobile hinge region (Smith et al. 1996). It was observed in the original work by Shakur et al. (1993) that engineered constructs lacking this N-terminal region formed entirely soluble, fully active species. In addition, it was shown that when the N-terminal region of PDE4A1 was bound to an entirely cytosolic protein, such as CAT or GFP, the resultant chimeric protein was entirely membrane bound (Scotland and Houslay 1995; Smith et al. 1996; Baillie et al. 2002). These studies formed the paradigm for the importance of the N-terminal regions of the PDE4 enzymes in directing intracellular targeting and thereby allowing exquisite control of the shape and formation of gradients of cAMP within the cell, the basis of cAMP compartmentalization. The identification of the role of the N-terminal region of PDE4s in targeting and the knowledge that this region provides the signature of each individual PDE4 isoform led to the realization that this region could allow tailored compartmentalization of the cAMP signal through selective expression of PDE4 isoforms. Indeed, further studies identified the N-terminal regions of PDE4A5, PDE4A4 and PDE4D4 to interact with members of the SRC tyrosyl kinase family (O'Connell et al. 1996; Beard et al. 1999; McPhee et al. 1999), PDE4A5 to interact with the immunophilin XAP2 (Bolger et al. 2003b), PDE4D5 to interact with both RACK1 and β-arrestin (Yarwood et al. 1999; Steele et al. 2001; Bolger et al. 2002; Bolger et al. 2003a), PDE4B1 to interact with DISC1 (Millar et al. 2005) and PDE4D3 to interact with myomegalin, mAKAP and AKAP450 (Dodge et al. 2001; Tasken et al. 2001; Verde et al. 2001; McCahill et al. 2005). These interactions are discussed in Sect. 2.5 (and see Fig. 3).

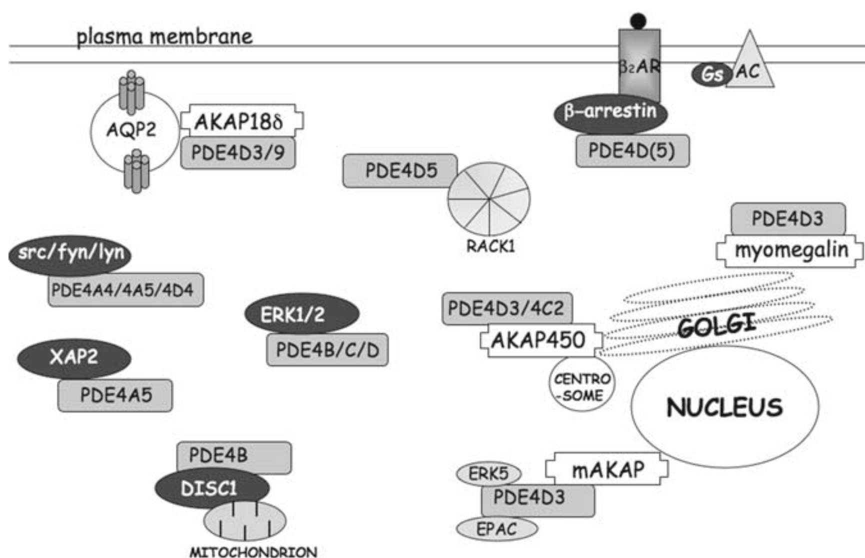

Fig. 3 Summary of the known protein–protein interactions of various PDE4 isoforms shown in their appropriate locations within a hypothetical cell

The interactions of PDE4 N-terminal regions are not confined to protein–protein interactions. Indeed, within PDE4A1 it is a microdomain within the second helical structure named TAPAS1 that confers Ca^{2+}-gated membrane insertion and shows a preferential binding for net −2 charge phosphatidic acid (Baillie et al. 2002). More recently, it has been shown that this TAPAS1/PA interaction is not sufficient for the targeting of PDE4A1 in intact cells. Indeed, while TAPAS1 provides the core insertion domain, a separate site located within helix 1 is responsible for efficiency of the insertion and also for the retention of PDE4A1 to the Golgi (Huston et al. 2006a). It is suggested that this second site within helix1 may interact with another protein, as this site is not a lipid interaction site. The presence of more than one binding site for efficient targeting of proteins is synonymous with a number of other PDE4 protein–protein interactions where a second site of interaction within the PDE enzyme has also been identified. Binding of PDE4A4 to LYN SH3 has been shown to be dependent on the interaction of LYN-SH3 with sites located within both the N-terminus of PDE4A4 and within the LR2 region of the protein (McPhee et al. 1999). Likewise, binding sites for both β-arrestin and RACK1 have recently been found within both the N-terminal regions and catalytic region of PDE4D5 (Bolger et al. 2006). That such a level of complexity exists within the interactions of PDE4 isoforms with their myriad of binding partners indicates that absolute definition of the molecular basis of these interactions will be of great value in the development of powerfully specific drug therapies.

2.5 Intracellular Targeting via Protein–Protein Interactions

2.5.1 SH3-Domain-Containing Proteins

The N-terminal region of the long-form human isoform, PDE4A4, has been shown to be important in directing localization of this protein and also to play a role in determining the inherent sensitivity of the enzyme to the archetypical PDE4 inhibitor, rolipram (Huston et al. 1996). PDE4A4 is distributed within both the particulate and cytosolic fractions of cultured COS7 cells. This localization was shown to have a profound effect on the inhibition of the enzyme by rolipram, with the particulate form of the enzyme showing a dramatic increase in its sensitivity to rolipram and also showing striking changes in the kinetics of enzyme inhibition, which indicated a change in conformation of PDE4A4 when localized at the membrane. These differential characteristics were shown to be, at least in part, due to the N-terminal region of the enzyme (Huston et al. 1996). When the N-terminal region of both this isoform and its rat homologue PDE4A5 were examined in more detail, it was found that both of these enzymes contained a number of conserved Pro-X_{aa}-X_{aa}-Pro- X_{aa}- X_{aa}-Arg domains in their N-termini, indicative of proteins that bind SH3-domain-containing proteins (O’Connell et al. 1996; McPhee et al. 1999). SH3 domains are found in various

families of proteins, including adaptor proteins, cytoskeletal proteins and various signal transduction proteins, such as the SRC family tyrosyl kinases (Pawson and Gish 1992; Terasawa et al. 1994). These proteins are distinct self-folding proteins that are generally thought to confer protein–protein interactions (Cowan-Jacob 2006). The N-terminal region of PDE4A5 was shown to be responsible for its binding to a number of members of the SRC-tyrosyl kinase family (O'Connell et al. 1996). In contrast, however, the binding of PDE4A4 to these same SH3-domain-containing proteins could not be explained purely by an interaction with the N-terminal region; instead, an additional proline-rich region located between UCR2 and the catalytic region, namely LR2, was found to be necessary both for the correct localization of the enzyme and also for its interaction with LYN-SH3 (McPhee et al. 1999). In addition, the interaction of PDE4A4 with LYN-SH3 was shown to be pivotal to the change in conformation of PDE4A4 and the resultant increase in sensitivity to rolipram seen in the particulate fraction of the enzyme in previous studies (McPhee et al. 1999). In the case of PDE4A5, deletion of the defined SH3-binding region was, in itself, insufficient to cause complete loss of intracellular targeting of the enzyme. Indeed, it was shown that multiple regions within the N-terminal non-catalytic region of PDE4A5 contribute to its intracellular targeting (Beard et al. 2002). Further studies examining the interaction of PDE4s to SH3-domain-containing proteins in brain revealed a third isoform able to bind SRC-family tyrosyl kinases. This study revealed PDE4D4 to bind with equal affinity to LYN, FYN and SRC, suggesting a subfamily specificity in the ability of PDE4s to bind SRC-family tyrosyl kinases (Beard et al. 1999).

2.5.2 Interaction Between PDE4A5 and Caspase3: Consequences for SH3 Binding

Further information as to the functional role of protein–protein interactions in controlling subcellular localizations of PDE4s and thus regulation of cAMP microdomains is demonstrated by the action of caspase3 on PDE4A5.

In mammals, various stimuli of apoptosis lead to the activation of a family of cysteine proteases with specificity for aspartic acid residues, referred to as caspases (Thornberry et al. 1992). Caspases exist as zygomens until proteolytically activated, either auto-catalytically or by other caspases (reviewed in Nagata 1997). Caspase activation is induced by a wide array of death signals and leads to precipitous cleavage of protein substrates, thereby disabling important cellular processes and breaking down structural components of the cell. The ubiquitously expressed caspase 3 (CPP32/Yama/apopain) is implicated as a downstream effector protease of this cascade (Fernandes-Alnemri et al. 1994). Our laboratory has identified the rat homologue of long-form PDE4A, PDE4A5, as a target of caspase-3 cleavage (Huston et al. 2000). This cleavage occurs at the motif, DAVD, which is unique to the N-terminal region of PDE4A5, and is induced both in rat-1 fibroblast cells, by the apoptotic agent staurosporine, and in rat neuronal-like PC12 cells, by withdrawal of NGF (Huston et al. 2000). The cleavage of PDE4A5 by caspase-3 leads to a loss in the ability of PDE4A5 to bind to SH3-domain-containing proteins. Cleavage also leads to a change in the intracellular

localization of PDE4A5. The perinuclear localization of the full-length protein that has been shown to co-localize with the SH3-domain-containing protein, LYN, is replaced, in the cleaved product, by a distribution throughout the cytosol of the cell (Huston et al. 2000). Thus, cleavage of PDE4A5 by caspase-3 removes the ability of the protein to bind to SH3-domain-containing proteins and causes relocalization of PDE4A5; this could potentially lead to changes in the local concentration of cAMP. The significance of this is profound when the reported importance of cAMP in the control of apoptosis is considered. The second messenger has been found to mediate apoptotic signals in a wide variety of cells, augmenting or inhibiting apoptosis depending on the cell type examined (McConkey et al. 1990; Lomo et al. 1995; Kim and Lerner 1998). Indeed, in a number of studies PDE4 inhibition itself has been implicated in the induction of apoptosis (for review, see Lerner and Epstein 2006). That over-expression of PDE4A5 protects RAT1 fibroblasts against staurosporine-induced apoptosis, whereas over-expression of PDE4A8, which exhibits a subcellular distribution similar to the cleaved PDE4A5 in both COS cells and rat-1 fibroblasts, does not (Huston et al. 2000), would suggest the importance of the localization of PDE4A isoforms in modulation of the apoptotic pathway.

2.5.3 XAP2

A further example of the potential of PDE4s to interact with signaling complexes is illustrated by the ability of PDE4A5 to interact with XAP2. XAP2 (hepatitis-B virus x-associated, also known as ARA9 or AIP) was identified in 1996 as part of the FKB52 class of immunophilins due to its structural similarity to these proteins (Carver and Bradfield 1997; Ma and Whitlock 1997; Petrulis and Perdew 2002). However, despite sharing some sequence homology in its tetratricopeptide repeat (TPR) motif, a motif typical of proteins that interact with hsp90 proteins, XAP2 does not bind immunosuppressive drugs such as cyclosporin, FK506 or rapamycin, another typical characteristic of immunophilins (Carver and Bradfield 1997). XAP2 was first identified as part of the aryl hydrocarbon receptor (AhR) complex via yeast-2-hybrid and direct immunoprecipitation experiments in mouse (Kuzhandaivelu et al. 1996). Its function within this complex has been much studied, with many suggesting a role in the subcellular localization of AhR. Others suggest that overexpression of XAP2 leads to an increase in the cellular levels of AhR and also an increase in AhR-mediated gene induction (Meyer et al. 2000), and this has recently been suggested to occur as a consequence of XAP2 acting to stabilize the AhR by protecting it from ubiquitination by the carboxyl terminus of hsc70-interacting protein (CHIP) ubiquitin ligase (Lees et al. 2003; Morales and Perdew 2007). The AhR is of great therapeutic interest due to its activation by tobacco smoke, which has been shown to lead to the generation of pro-inflammatory mediators from fibroblasts (Martey et al. 2005). In addition, the AhR has been shown to bind to the carcinogen TCDD, exposure to which has been implicated in COPD and lung cancer (Martinez et al. 2002).

The work of Bolger et al. (2003b) identified a novel binding partner of XAP2 in PDE4A5. Immunophillins closely related to XAP2, the AIPL1 protein, FKBP51 or FKBP52 show no binding to PDE4A5, suggesting the binding of XAP2 to PDE4A5 to

be exclusive. Within the sequence of XAP2, it was found that it was a TPR domain located within the carboxy half of the protein that was important for PDE4A5 binding (Bolger et al. 2003b). The TPR regions of many proteins have been shown to mediate their interaction with other proteins; indeed, hsp90 has been shown to bind the TPR regions of a number of proteins, including XAP2 (Sikorski et al. 1990; Uzawa et al. 1990). The authors (Bolger et al. 2003b) suggest that, as it is only PDE4A5 within the vast array of members of the PDE4 family that can bind XAP2, the unique N-terminal region of PDE4A5 must be involved in the interaction. However, this study also implicated a second site located within the UCR2 region of PDE4A5. Here, an EELD motif similar to that in the XAP2 binding region of hsp90 was shown by mutational analysis to bind XAP2 (Bolger et al. 2003b). In addition to the binding of XAP2, this region has been shown to be important in the interaction of UCR1 and UCR2 regions of PDE4D3, and the region has also been implicated in the ability of PDE4D isoforms to dimerize (Beard et al. 2000; Richter and Conti 2002), which suggests that when proteins interact with PDE4 isoforms, they stabilize the monomeric forms. This would presumably prevent complex oligomers forming thereby ensuring the fidelity of interaction between a particular PDE4 molecule and a single scaffold species. This UCR2 region is conserved within all PDE4 isoforms and so cannot account for the specificity of XAP2 binding to the PDE4A5 isoform, implying a role for the isoform-specific N-terminal region.

The binding of PDE4A5 to XAP2 was found to have a number of functional consequences (Bolger et al. 2003b). Following binding of XAP2, the activity of PDE4A5 was severely inhibited; this would presumably modulate the cAMP concentration around the locale of XAP2. In addition, binding of the two proteins was shown to greatly increase the sensitivity of PDE4A5 to its cognitive inhibitor rolipram. Both of these functional consequences suggest that a conformational change in the catalytic region of PDE4A5 takes place as a result of XAP2 binding. Binding of XAP2 to PDE4A5 also culminates in the attenuation of the ability of PDE4A5 to be phosphorylated by PKA. Phosphorylation of PDE4A5 by PKA acts to increase the activity of the enzyme (MacKenzie et al. 2002). However, it appears that this action is independent of the ability of the inhibition of the enzymatic activity of PDE4A5 by binding of XAP2 (Bolger et al. 2003b).

Although binding of PDE4A5 to XAP2 was shown to have a number of functional outcomes for the PDE4A5 enzyme, it remains to be seen whether reciprocal functional outcomes can be identified for XAP2. It is interesting to note that recently it has been suggested that an environment of high cAMP causes the nuclear translocation of the AhR receptor (Oesch-Bartlomowicz and Oesch 1990; Bock and Kohle 2006), itself a PKA target (Dolwick et al. 1993), with consequences for its protein–protein interactions. It will be of interest to investigate whether binding of PDE4A5 to XAP2, which is a well-defined binding partner of the AhR, has further functional consequences.

2.5.4 DISC1

A number of genetic and neuropsychiatric studies have suggested that mutations with Disrupted in schizophrenia 1 (*DISC1*) gene lead to a predisposition to schizophrenia and related psychological disorders (reviewed in Carter 2006; Hennah et al.

2006; Porteous and Millar 2006). DISC1 is expressed exclusively in brain and has a structure that is consistent with structural scaffolding proteins. As such, the search for interacting partners of this protein has been wide. One such study identified various partners of DISC1, including the neurodevelopmental proteins, FEZ1 and NDEL1, and also identified an interaction between DISC1 and PDE4B (Millar et al. 2003). PDE4s have long been implicated in functions of the CNS (Zhang and O'Donnell 2007). Mutations in the *dunce* gene of Drosophila, the homologue of human PDE4, were shown to effect learning and memory (Davis et al. 1995). In addition, early studies on the prototypical PDE4 inhibitor, rolipram, showed its potential for treating depression and diseases of cognitive impairment (Zhu et al. 2001). However, severe challenges in the development of therapeutic agents have become apparent due to an incomplete understanding of the function and regulation of PDE4s in the brain. The identification of specific interactions of PDE4 family members with neuronally functional proteins allows an increase in the understanding of PDE4 function within the CNS.

Co-immunoprecipitation and co-localization studies in various cell types verified the original yeast-2-hybrid data showing interaction of DISC1 with PDE4B. Further analysis revealed that interaction of the two proteins was achieved via the amino terminal end of DISC1 and the UCR2 region of PDE4B. The UCR2 region of PDE4s is highly conserved between all family members, and as such it was revealed that DISC1 was able to bind to members of all four of the PDE4 gene families (Millar et al. 2005). More recently, however, further binding sites for DISC1 within PDE4s have been found that may modulate and regulate the interaction of specific PDE4s to DISC1 (Murdoch et al. 2007). Phosphorylation of PDE4B1 by PKA in response to increases in local cAMP levels causes release of PDE4B1 from DISC1, suggesting a dynamic interrelationship between the two proteins (Millar et al. 2005).

In addition to biochemical data, genetic relevance to this interaction was implied by the finding that cousins suffering from schizophrenia and psychotic illness presented a disruption in the gene encoding PDE4B. When patient-derived lymphoblastomal cells were examined, it was shown that this translocation led to a dramatic decrease in the protein expression of PDE4B1 (Millar et al. 2005). The discovery that chromosomal disruptions in both the DISC1 and PDE4B genes are independent risk factors in schizophrenia and that these two proteins interact to modulate cAMP signaling is of potential therapeutic significance. Further definition of the molecular basis of the DISC1/PDE4B relationship will be of great value in developing this therapeutic potential.

2.5.5 β-Arrestins

β-arrestins are signaling scaffold proteins that have a number of cellular functions, including the desensitization of GPCRs such as the β_2-adrenergic receptor (β_2AR). In the classic paradigm of receptor desensitization, agonist-occupied and activated β_2AR couples to Gs, leading to the activation of membrane-bound adenylyl cyclases (ACs) and generation of cAMP (Hall 2004). This process is

rapidly desensitized when G-protein-coupled receptor kinases (GRKs) phosphorylate the activated β_2AR, which promotes the recruitment of cytosolic β-arrestins to the plasma membrane and thus uncouples the further interaction between β_2AR and Gs (Luttrell and Lefkowitz 2002). Recent studies add a new facet of β-arrestin function to this β_2AR desensitization process, as β-arrestin has been shown to form a complex with PDE4 enzymes (Perry et al. 2002). This provides a means of delivering a cAMP-degrading enzyme to the site of cAMP synthesis and quenching the existing cAMP at the plasma membrane in an agonist-dependent manner. β-Arrestins therefore play a dual role in resetting cAMP levels after agonist stimulation by preventing further AC stimulation and, additionally, recruiting PDE4 to mop up excess cAMP. The transient rise in cAMP affected by receptor stimulation results in activation of locally tethered PKA that in turn leads to phosphorylation of PKA substrates in the local environment, one of which is the β_2AR itself. PKA phosphorylation of the β_2AR allows it to switch its coupling from Gs to the inhibitory guanine nucleotide regulatory protein, Gi. This switch inhibits AC activity and instead couples the β_2AR to activation of ERK1 and ERK2 via a pathway involving SRC, Ras and Raf (Daaka et al. 1997). Thus, by controlling the levels of cAMP at the plasma membrane, β-arrestin-recruited PDE4 regulates the PKA-mediated switching of the β_2AR between Gs-mediated activation of AC and Gi-mediated activation of the ERK signaling pathway (Baillie et al. 2003).

All PDE4 isoenzymes are capable of binding β-arrestin1 and 2, independent of agonist treatment, through a common site located within their conserved catalytic domain (Perry et al. 2002; Bolger et al. 2003a). However, studies in a number of cell types indicated that the PDE4D5 was the isoform predominantly or exclusively found in association with β-arrestin and recruited to the β_2AR on agonist stimulation, even when it was expressed at a much lower level than other PDE4 isoforms (Bolger et al. 2003a). This was found to be due to an additional β-arrestin binding site in the unique N-terminal region of PDE4D5, which conferred it with a higher affinity for β-arrestin than other PDE4s (Bolger et al. 2003a). Subsequent peptide array analyses (see Sect. 3.1) defined the β-arrestin2 binding sites on PDE4D5 in more detail (Fig. 4) (Bolger et al. 2006). Efforts to map the PDE4D5 binding sites on β-arrestin2 defined two regions that are necessary for the interaction, one located at the extreme N-terminal end of the N-domain of β-arrestin2 and the other within the C-terminal C-domain (Bolger et al. 2006). Again, peptide array analyses were utilized to confirm and expand these data, revealing that the binding site in the N-domain of β-arrestin2 interacts with the common PDE4 catalytic unit, while two distinct sites in the N-domain confer specificity for PDE4D5 (Baillie et al. 2007). Interaction of PDE4D5 with both the N- and C-domains of β-arrestin2 was demonstrated to be necessary for regulation of the β_2AR (see Sect. 3.1).

RNA silencing of specific PDE4 isoforms in HEK293B2 cells confirmed that PDE4D5, and not any other PDE species, is the isoform required to control the PKA-mediated switching of the β_2AR from Gs to Gi (Lynch et al. 2005). The

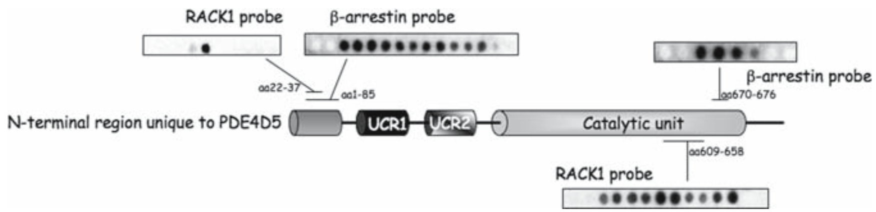

Fig. 4 A schematic diagram showing the respective binding sites of β-arrestin and RACK1 on PDE4D5, illustrating the powerful advance of peptide array technology. Within the N-terminal region, which is unique to PDE4D5, both β-arrestin and RACK1 compete for the same binding site. Within the C-terminal region, which is common to all PDE4D isoforms, distinct binding sites for β-arrestin and RACK1 have been identified. Peptide array scans, which were used to identify these binding regions, are shown. In these the sequence of PDE4D5 was used to generate a library of overlapping 25-mer peptides, each shifted by five amino acids, immobilized on nitrocellulose. The peptide blots were then probed with tagged, recombinant β-arrestin or RACK1 as indicated. *Dark spots* indicate peptides that may contribute to the binding site. These sites are then verified by each of the positive spots being used as a template to design peptide arrays in which each of the amino acids is sequentially mutated, usually to alanine. These are then probed with the corresponding tagged, recombinant proteins, and substitutions that lead to loss of binding indicate critical interacting residues

importance of β-arrestin in recruiting and tethering PDE4D5 to the β_2AR was demonstrated by a dominant negative approach whereby catalytically inactive PDE4D5 was over-expressed in both HEK293B2 cells (Lynch et al. 2005) and cardiac myocytes (Baillie et al. 2003). As shown in Fig. 5, this species exerted a dominant negative effect by binding to β-arrestin and displacing the active endogenous PDE4D5 from the β_2AR signaling complex and potentiating agonist-stimulated ERK activation by its failure to degrade local cAMP. However, this dominant negative effect was abolished when a point mutation was made in the unique N-terminal region of catalytically inactive PDE4D5 that ablated its ability to interact with β-arrestin, thereby implying that recruitment by β-arrestin is critical to the function of PDE4D5 in these cells (Lynch et al. 2005; Bolger et al. 2006). Taken together, these studies demonstrate that a particular PDE4 isoform can exert a specific functional role within a cell and that function is critically dependent on targeting by protein–protein interaction. The interaction between PDE4D5 and β-arrestin has recently been reviewed in detail elsewhere (Baillie and Houslay 2005; Baillie et al. 2005; Bolger et al. 2007; Houslay 2007).

2.5.6 RACK1

RACK1 (Receptor for Activated C Kinase 1) was originally identified as a scaffolding protein for activated PKC (Ron et al. 1994), but its role has been expanded through identification of an array of novel binding partners (McCahill et al. 2002). RACK1 is a 36-kDa protein that contains seven tryptophan/aspartate-rich

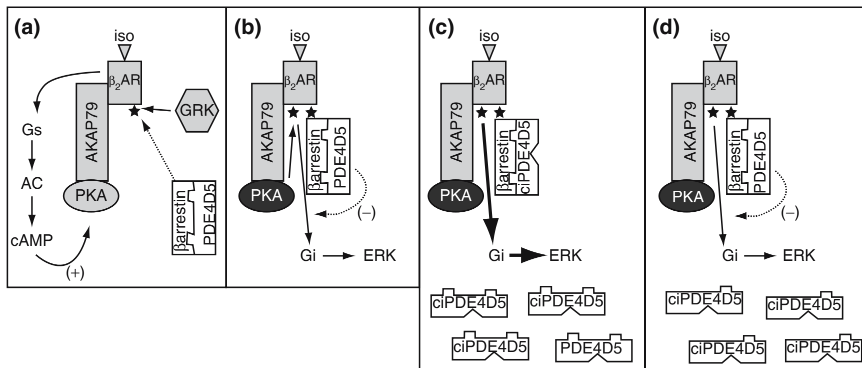

Fig. 5 Schematic of the dominant negative strategy used to show the importance of β-arrestin-targeted PDE4D5 in the regulation of β_2AR signaling. (**a**) Isoprenaline-stimulated β_2AR couples to Gs, leading to the activation of ACs, the generation of cAMP and the activation of AKAP79-tethered PKA. This process is rapidly desensitized when GRKs phosphorylate the activated β_2AR, promoting the recruitment of cytosolic β-arrestin, and its associated PDE4D5, to the plasma membrane, preventing further interaction between the β_2AR and Gs. (**b**) Activated PKA phosphorylates the β_2AR, allowing it to switch its coupling to Gi and ERK activation. β-arrestin-recruited PDE4D5 attenuates Gi-coupling and ERK activation by degrading local cAMP and so diminishing PKA phosphorylation of the β_2AR. (**c**) Over-expressed, catalytically inactive (ci) PDE4D5 exerts a dominant negative effect by displacing active endogenous PDE4D5 and enhancing iso-mediated ERK activation. (**d**) Over-expressed, catalytically inactive PDE4D5 that has a mutation in its N-terminal β-arrestin binding site is not recruited to the signaling complex and so fails to enhance ERK activation

(WD) repeat motifs, which are proposed to form a seven-bladed, β-sheet propeller structure (for modeling details, see Bolger et al. 2002), similar to the G-protein β-subunit (Sondek and Siderovski 2001). This structural design provides multiple protein interaction surfaces for RACK1 to scaffold such signal transduction proteins as PKC, SRC, integrin β-subunits, the NMDA receptor and the IGF1 receptor (Ron et al. 1994; Liliental and Chang 1998; Chang et al. 2001; Hermanto et al. 2002; Yaka et al. 2002). RACK1 has been shown to be involved in various biological functions, such as the regulation of heart contraction by noradrenaline (Johnson et al. 1996), glucose-stimulated insulin secretion in pancreatic β cells (Yedovitzky et al. 1997), NMDA channel function (Thornton et al. 2004) and integration of adhesion and insulin-like growth-factor-I signaling and cell migration (Kiely et al. 2005).

RACK1 was first demonstrated to interact with PDE4D5 when it was pulled out by a yeast two-hybrid screen with PDE4D5 as the bait (Yarwood et al. 1999). This interaction, which was confirmed by pull downs and immunoprecipitations, was proved to be highly specific as RACK1 did not interact with any other PDE4 isoforms, while PDE4D5 did not interact with any other WD-repeat proteins (Yarwood et al. 1999). As would be predicted from this specificity, truncation analysis showed that the unique N-terminal region of PDE4D5 was essential for its interaction with

RACK1. A combination of yeast-two-hybrid, mutagenesis and modeling approaches demonstrated that WD-repeats 5–7 of RACK1 were sufficient for the interaction with PDE4D5 and that the important residues for interaction were all clustered on a single surface of RACK1 (Steele et al. 2001). Subsequent studies delineated the *RA*CK1 *I*nteraction *D*omain in the N-terminal region of PDE4D5, termed RAID1, which is proposed to form an amphipathic helical structure that could interact with the β-propeller blades of RACK1 in a manner analogous to the interaction of the helical Gγ protein with the β-propeller protein, Gβ (Bolger et al. 2002). A synthetic peptide of the RAID1 region bound to RACK1 with a similar affinity to that of PDE4D5 and could also inhibit the interaction between PDE 4D5 and RACK1 in a concentration-dependent manner (Bolger et al. 2002; Smith et al. 2007). Binding of PDE4D5 to RACK1 was found not to affect its activity, but did slightly alter its sensitivity to rolipram (Yarwood et al. 1999), which may be due to the fact that peptide array analysis identified a novel binding site for RACK1 in the PDE4D catalytic unit that abuts the active site for cAMP binding (Bolger et al. 2006). The functional consequence of the RACK1/PDE4D5 interaction was not apparent until it was discovered that β-arrestin could compete with RACK1 for binding to PDE4D5 (Bolger et al. 2006; Smith et al. 2007). A combination of yeast three-hybrid and peptide array approaches (see Sect. 3.1) showed that RACK1 and β-arrestin have overlapping binding sites on the N-terminal region of PDE4D5, and they therefore interact with PDE4D5 in a mutually exclusive manner (Fig. 4). Peptide array analysis also uncovered an additional binding site for RACK1 in the catalytic region of PDE4D5, which had not been appreciated by previous approaches. Knockdown of RACK1 using siRNA (small interfering RNA) markedly increased the proportion of PDE4D5 that can associate with β-arrestin. This, in turn, increased the recruitment of PDE4D5 to the β_2AR upon isoproterenol stimulation, which significantly attenuated PKA phosphorylation of the β_2AR and activation of ERK (Bolger et al. 2006; Smith et al. 2007). The interaction of RACK1 and PDE4D5 can therefore impact on β-adrenergic signaling by sequestering distinct pools of PDE4D5 such that it is unavailable to modulate the function of the β_2AR. This study eloquently demonstrated that different pools of the same isoform might themselves be compartmentalized within the cell by virtue of their existence within different functional complexes. Furthermore, it underlines the importance of defining in detail the molecular basis of the protein–protein interactions of PDE4 isoforms and demonstrates the potential to manipulate their roles within various functional complexes by disruption of their interacting domains for the development of novel drug therapies.

2.5.7 AKAPs

AKAPs are a diverse family of proteins that bind to PKA regulatory (R) subunits in order to constrain PKA at various subcellular locations, where it can detect local cAMP gradients and confer the appropriate response on PKA substrates that are either within the same complex or in their close proximity. In addition to PKA and its substrates, AKAPs also scaffold an ever-growing number of other

signaling proteins including other kinases such as PKC (Klauck et al. 1996), phosphodiesterases (Houslay and Adams 2003), phosphatases (Smith et al. 2006), GPCRs (Malbon et al. 2004) and G proteins (Diviani et al. 2006). AKAPs are therefore essential elements of compartmentalized cAMP signaling that orchestrate efficient signal transduction, termination and crosstalk with other signaling pathways. The recruitment of PDE4 into these AKAP-mediated signaling complexes allows the control of local cAMP gradients, and thus the control of PKA activity within the complex. A number of PDE4 isoforms are recognized to be targeted to AKAPs, and the functional consequences of these interactions are now becoming clear (Bolger et al. 2007).

PDE4D3 has been shown to specifically interact with mAKAP, an AKAP primarily found in skeletal and cardiac muscle (Dodge et al. 2001). The inclusion of a PDE4 long isoform into the mAKAP-PKA complex allows for self-regulation via negative feedback. As cAMP levels rise and mAKAP-associated PKA is activated, PDE4D3 within the same complex is consequently activated by PKA phosphorylation of UCR1. This PDE4D3 activation upregulates the degradation of cAMP, which facilitates the deactivation of mAKAP-associated PKA and the subsequent dephosphorylation of PDE4D3. In such a manner, cAMP levels are reset to basal levels (Dodge et al. 2001).

Truncation studies demonstrated that the mAKAP-PDE4D3 interaction is dependent upon the 15-amino-acid, unique N-terminal region of PDE4D3, which accounts for the fact that mAKAP interacts poorly with other PDE4 isoforms (Dodge et al. 2001). This region of PDE4D3 contains a PKA consensus site at serine 13 (S13) in addition to the activating PKA site within UCR1, which is found in all PDE4 long forms. Subsequent studies showed that PKA phosphorylation of S13 in the unique region of PDE4D3 dramatically increases its interaction with mAKAP (Carlisle Michel et al. 2004). This study was the first to ascribe a function for S13 phosphorylation of PDE4D3 and went on to propose that activation of mAKAP-anchored PKA facilitates the recruitment of PDE4D3 in order to achieve faster signal termination. The mAKAP-PDE4D3 interaction also involves a central region of the mAKAP (amino acids 1286–1401), which is clearly distinct from the C-terminal PKA anchoring site (Dodge et al. 2001). mAKAP is known to be induced and to accumulate in the perinuclear region in rat neonatal ventriculocytes in response to hypertrophy (Kapiloff et al. 1999). That PDE4D3 undergoes redistribution in order to colocalize with the induced mAKAP at the perinuclear region suggests that this complex may be required as cells adapt to the requirement for increased contractile power in the failing heart (Dodge et al. 2001). Scott and colleagues have recently demonstrated that ERK5 and EPAC1 are also complexed with mAKAP, PKA and PDE4D3 in rat neonatal ventriculocytes (Dodge-Kafka et al. 2005; see Sect. 2.5.8).

PDE4D3 has been shown to be targeted to another AKAP, AKAP450, which allows for its tethering to PKA at the centrosomal region of testicular Sertoli cells (Tasken et al. 2001). Here, the tight regulation of PKA activity by an associated PDE might be predicted to play a role in cell cycle control.

The functional significance of AKAP/PDE4 tethering has been approached using a novel dominant negative strategy (McCahill et al. 2005). In this, single point mutations in the active site of various PDE4 isoforms were made in order to render them catalytically inactive (see Sect. 3.3). These were then over-expressed in cells so as to displace the cognate endogenous active PDE4 isoforms from their cellular anchor sites, thereby raising cAMP levels in that specific locale. Such an approach has no need for knowledge of the mode of targeting of that specific isoform, but only yields a phenotype upon displacement. Doing this in COS1 cells, PDE4D3 and PDE4C2, but not PDE4A4 and PDE4B1, were proposed to gate the activation of AKAP450-tethered PKA type-II in the centrosomal region of unstimulated cells (McCahill et al. 2005). Additionally, these recombinant long PDE4 isoforms are able themselves to act as spatially constrained reporters for localized PKA activity by virtue of the PKA site in their UCR1 region, providing a means of gauging local cAMP action. In COS1 cells, only catalytically inactive PDE4D3 and PDE4C2 were phosphorylated by PKA in resting cells, suggesting that these species are anchored close to PKA. Catalytically inactive PDE4D3 and PDE4C2 were found to co-immunoprecipitate with AKAP450 and, additionally, phosphorylated catalytically inactive PDE4D3 and PDE4C2 colocalized with AKAP450 and PKA type-II in the centrosomal region. Taken together, these data suggest that specific endogenous PDE4 isoforms are targeted to AKAP450 at the centrosome where they tightly regulate local cAMP levels to prevent the inappropriate activation of PKA in resting cells (McCahill et al. 2005).

The most recent report of a protein–protein interaction between an AKAP and a PDE4 is that of AKAP18δ with PDE4D3 and/or PDE4D9 in the aquaporin-2 (AQP2)-bearing vesicles of renal principle cells (Stefan et al. 2007). In these cells, fusion of vesicles containing the AQP2 water channel with the plasma membrane increases the water permeability of the cells, which facilitates water reabsorption from the collecting duct (King et al. 2004). Shuttling of AQP2-bearing vesicles to the plasma membrane occurs upon PKA phosphorylation of AQP2, a process that is triggered by binding of antidiuretic hormone [arginine vasopressin (AVP)] to its receptor and the consequential rise in cAMP levels and PKA activity (Klussmann et al. 1999). The recent study by Stefan et al. (2007) demonstrates that AKAP18δ tethers PDE4D and PKA to AQP2-bearing vesicles where the PDE4D tightly controls the basal PKA activity by hydrolysis of local cAMP. Furthermore, they show that AQP2 and PDE4D translocate to the plasma membrane in response to AVP, where PDE4D is involved in terminating cAMP-dependent water reabsorption. The AQP2-associated PDE4D migrated at 98 kDa on SDS-PAGE, which is consistent with the migration of PDE4D3, PDE4D8 and PDE4D9, but unfortunately no isoform-specific antisera were available to distinguish between these species. RT-PCR identified transcripts for PDE4D3 and PDE4D9, indicating that one or both of these isoforms is the PDE4D associated with AQP2-bearing vesicles. Peptide array analysis of a library of 25-mer peptides scanning the entire sequence of PDE4D3 with an AKAP18δ-GST probe revealed two regions of PDE4D3 that bind directly to AKAP18δ. These regions were amino acids 341–365 at the extreme N-terminal of the PDE4D catalytic unit, and 571–635 in the PDE4D sub-family-specific C-terminal region. Interaction at these

two sites would suggest that all PDE4D isoforms should, potentially, be capable of interacting with AKAP18δ. Subsequent substitution analysis of peptides revealed that residues 353–355 (DLE) and 598–600 (FQF) are critically important for the interaction. Interestingly, the FQF motif at 598–600 also represents the ERK-specific docking site on PDE4D3 (MacKenzie et al. 2000), implying that when PDE4D3 is bound to AKAP18δ, it will be unable to bind ERK and so will not be subject to any down-regulation of its activity via ERK phosphorylation.

Overlay of an AKAP18δ peptide array with a PDE4D3-GST probe revealed four sites of interaction. Truncation analysis of AKAP18δ revealed that only the most C-terminal of these sites is, seemingly, essential for the PDE4D3 binding and additionally that the N-terminal region negatively regulates the interaction.

The novel compartmentalized cAMP-signaling module involving PDE4D3/9, AKAP18δ and PKA offers the potential for therapeutic intervention to treat imbalances of body water homeostasis. PDE4 inhibitors have been found to increase water permeability (Stefan et al. 2007) and so could be used in instances when the receptor for AVP is non-functional, whereas disruption of the AKAP-PKA interaction may be useful to treat water retention.

2.5.8 ERK and EPAC

The ERK pathway regulates critical cellular processes including survival, proliferation and differentiation by transducing extracellular signals such as growth factors, hormones and stress (Houslay and Kolch 2000). As discussed in Sect. 2.2 above, ERK can bind to, phosphorylate and thus regulate PDE4B, PDE4C and PDE4D isoforms. In common with other authentic in vivo MAPK substrates (Sharrocks et al. 2000), the ERK phosphorylation site in the catalytic region of PDE4 isoforms is flanked by two MAPK docking sites. One of these sites, the "KIM" domain, which can be utilized by both ERK and JNK, is situated 120–150 residues N-terminal to the target serine. ERK specificity is provided by the FQF motif, situated 5–30 residues C-terminal to the target serine (MacKenzie et al. 2000; Sharrocks et al. 2000). In the PDE4 catalytic unit the KIM domain is of the sequence *V*ET*KK*VTSSGV*L*LL and is located on an exposed β-hairpin loop, while the FQF motif resides on an exposed α-helix (Houslay and Adams 2003). Thus, both sites are well presented for interaction with ERK. Mutation of these docking sites to alanine prevented the phosphorylation of PDE4D3 by ERK both in vitro and in vivo, and also blocked the co-immunoprecipitation of ERK and PDE4D3 from cell lysates (MacKenzie et al. 2000). The functional outcome of ERK phosphorylation of PDE4 isoforms is determined by their complement of UCR modules in that long forms are drastically inhibited, short forms are activated, and super-short forms are weakly inhibited (Hoffmann et al. 1999; MacKenzie et al. 2000). PDE4 isoforms then represent a point of crosstalk between cAMP signaling and the ERK pathway. This PDE4-centered crosstalk has been demonstrated to be of physiological importance to the processes of

memory and learning in the rat brain (Zhang et al. 2004) and in a model of NGF-mediated neuronal regeneration (Gao et al. 2003).

ERK5 was recently found to be in a complex with mAKAP, PKA and PDE4D3 in rat neonatal ventriculocytes (see Sect. 2.5.7; Dodge-Kafka et al. 2005). Binding studies indicated that PDE4D3 is the adapter protein that connects ERK5 to the mAKAP complex. This was confirmed when an ERK binding mutant of PDE4D3, in which the KIM and FQF domains were ablated, failed to recruit ERK5 to the complex. Treatment of these cells with serum to activate ERK resulted in a 50% drop in the PDE activity of the complex, an effect that could be blocked using a MEK inhibitor, indicating that anchored ERK5 activity can inhibit anchored PDE4D3 by phosphorylation of the ERK site at serine 579 (Hoffmann et al. 1999). The cells were treated with combinations of ERK and cAMP activators and inhibitors in order to evaluate the nature of the crosstalk between cAMP and ERK signaling. Pretreatment of cells with forskolin to increase intracellular cAMP prevented activation of mAKAP-associated ERK5 by serum. However, several different PKA inhibitors failed to reverse the suppression of ERK5 by cAMP, indicating that a cAMP effector other than PKA must be involved. EPAC1, the cAMP-dependent guanine nucleotide exchange factor specific for the small G-protein Rap1 (de Rooij et al. 1998), was subsequently found to co-immunoprecipitate with the mAKAP complex, while a specific activator of EPAC was found to result in inhibition of the ERK5 activity within the complex. PDE4D3 was shown to be required to recruit EPAC1 to the mAKAP complex in HEK293 cells and was shown to interact directly with EPAC1 in vitro (Dodge-Kafka et al. 2005). The mechanism of this PDE4D3-EPAC1 interaction was not discussed in this paper; however, recent observations in our laboratory suggest that EPAC interaction is not specific to PDE4D3 because there is a common binding site on PDE4 for EPAC (M Lynch, G Baillie, M.Houslay, H Bos, unpublished data). Recent work shows that mAKAP organizes two integrated cAMP effector pathways in rat neonatal ventriculocytes, comprising PDE4D3, PKA, EPAC1 and ERK5. mAKAP determines the sub-cellular localization of the complex and tethers PDE4D3 and PKA, while PDE4D3 recruits EPAC1 and ERK5 to the complex and also plays a key regulatory role (Dodge-Kafka et al. 2005). ERK5 phosphorylation of PDE4D3 inhibits tethered PDE activity leading to a local increase in cAMP levels and subsequent activation of PKA and EPAC1. Activation of EPAC1 leads to ERK5 inhibition via a Rap1 signaling pathway. Meanwhile, activated PKA phosphorylates PDE4D3 to increases cAMP metabolism that ultimately represses the EPAC1-mediated block of ERK5. As well as being organized spatially, these signaling events appear to be regulated in a temporal fashion because PKA, PDE4D3 and EPAC1 react to different threshold levels of cAMP (Beavo and Brunton 2002). In rat neonatal ventriculocytes, this intricate mAKAP-mediated signaling complex was demonstrated to be involved in cytokine-induced cardiac hypertrophy (Dodge-Kafka et al. 2005), but similar complexes have the potential to regulate crosstalk between cAMP and ERK signaling in a wide variety of cell types.

2.5.9 Myomegalin

A yeast two-hybrid screen to identify new PDE4D binding partners pulled out a very large, novel protein that was subsequently named myomegalin (Verde et al. 2001). Myomegalin is a 2,324 amino acid protein that contains extensive regions of α-helix and coiled-coil structures. Other structural features include a region homologous to the ARP1-binding domain of the microtubule-associated protein, dynactin, and a leucine zipper identical to that in the centrosomin protein of Drosophila. Myomegalin exists as multiple isoforms with a 230–250-kDa protein found enriched in rat skeletal and cardiac muscle, while a 62-kDa isoform was found only in rat testis. Myomegalin transcripts have also been detected at low levels in all cells studied including brain, lung and liver. PDE4D3 was demonstrated to colocalize with myomegalin in the Z band of the sarcomere and in the Golgi/centrosomal region of the testes (Verde et al. 2001). In cells co-transfected with myomegalin and PDE4D3, the two proteins were found to co-immunoprecipitate, co-localize at the golgi/centrosomal region, and myomegalin could also target PDE4D3 to the particulate fraction (Verde et al. 2001). The authors propose that myomegalin acts as a scaffold protein that tethers PDE4D3 in order to control local PKA activity (Verde et al. 2001). Yeast two hybrid analysis indicated that myomegalin interacts with the UCR2 region of PDE4D. Consistent with this, PDE4D3 and PDE4D2 could co-immunoprecipitate with myomegalin from the lysates of transfected COS7 cells, whereas the PDE4D1 short form failed to interact. It appears then that myomegalin interacts with certain PDE4D isoforms and targets them to specific subcellular locations. The fact that myomegalin interacts with the UCR2 regulatory domain means that myomegalin may interact with isoforms from other PDE4 families, which is an avenue of investigation that would be interesting to pursue.

3 Alternatives to PDE4 Catalytic Site Inhibitors

PDE4 enzymes have been the subject of extensive research because of the therapeutic potential of PDE4 inhibitors to treat a number of major disease areas. However, PDE4 inhibitors have been slow to fulfill this therapeutic potential because their use is associated with dose-limiting side effects such as nausea and vomiting. Despite concerted efforts to refine PDE4 inhibitors in order to improve efficacy and minimize side effects, current inhibitors are still largely non-selective. The universal inhibition of all PDE4 activity by non-selective inhibitors is undoubtedly a major factor in the generation of unpleasant side effects. The development of subtype and isoform selective inhibitors is therefore desirable in an effort to overcome the clinical limitations of non-selective inhibitors. The interest in sub-family selective inhibitors has only been heightened by studies, including those in knockout mice, that suggest that inhibition of PDE4B is associated with the anti-inflammatory and immunomodulatory effects of PDE4 inhibitors, while inhibition of PDE4D is linked to anti-depressant effects, enhanced cognition, memory and also emesis (Jin et al. 2007; Zhang and

O'Donnell 2007). The design of highly selective inhibitors is, however, very difficult due to the fact that the catalytic sites of all the PDE4 sub-families are very similar. Ironically, the most progress has been made towards the development of PDE4D-specific inhibitors (Giembycz 2001) when it is inhibition of this sub-family that is suggested to be responsible for the emetic effect of PDE4 inhibitors (Robichaud et al. 2002). The development of isoform-specific inhibitors may help to dissect out the desirable psychopharmacological effects of PDE4D inhibitors from the undesirable emetic effects. However, PDE4 isoforms within a sub-family have identical catalytic sites, so conventional drug design targeted to the catalytic site cannot be used to distinguish between them. Overcoming the problems of isoform-specific inhibition will therefore require novel approaches and technologies.

Isoform-specific inhibition has been achieved, for the first time, in cultured cells by the use of small interfering RNA (siRNA) directed towards PDE4D3 and PDE4D5 (Lynch et al. 2005). In this manner, PDE4D5 was identified as the functionally important PDE4 species that interacts with β-arrestin to control the PKA/AKAP79-mediated switching of the β_2AR to activation of the ERK pathway. The results of this study elegantly demonstrate that particular PDE4 isoforms can have very specific, non-redundant, functional roles, such that loss of the isoform gives rise to a phenotype. While this illuminates the potential therapeutic benefits of isoform-specific inhibition, the siRNA approach does not easily translate to the clinic. Future research into PDE4 isoform-specific inhibitors should perhaps then focus on the specific interactions and regulatory mechanisms of particular PDE4 isoforms. This offers two potential routes of new drug design, one being to design novel inhibitors that can recognize the catalytic site of a specific isoform as it adopts a particular conformation mediated by its protein–protein interactions and regulatory phosphorylations. The second route is to develop compounds that do not target the catalytic site, but that instead ablate the function of a PDE4 isoform by disrupting its protein–protein interactions. This route is made more possible by the increasing knowledge of PDE4 protein–protein interactions and in particular the detailed information that can be gained from peptide array technology (see Sect. 3.1). Proof of principle for such an approach has recently been provided through the development of cell-permeable peptides able to disrupt PDE4/DISC1 interactions, PDE4D5/RACK1 and PDE4D5/barrestin in living cells (Murdoch et al. 2007; Smith et al. 2007). Meanwhile, the use of catalytically inactive PDE4 species in a so-called dominant negative approach provides proof of principal that disruption of specific interactions can give rise to functional effects (see Sect. 3.3).

3.1 Peptide Array Analyses to Identify Protein–Protein Interaction Sites

The introduction of peptide array technology has greatly increased the speed at which sites of protein–protein interaction can be mapped (Frank 2002; Bolger et al. 2006; Baillie et al. 2007). In this technique, the sequence of one of the proteins of interest is used to generate a library of overlapping 25-mer peptides, each shifted

by five amino acids, across the entire sequence of the protein. The peptides are then immobilized onto nitrocellulose and probed with a tagged, recombinant form of the interacting protein. Positive spots identify peptides that may contribute to the protein–protein binding site, with the intensity of the spots being a measure of strength of interaction. Interacting peptides can then be used as a template to design progeny peptides, each with a single amino acid substitution, usually to alanine. Substitutions that lead to loss of binding indicate those amino acids that are critical to the interaction. This information can then be used to generate mutations in the full-length proteins to confirm that the identified residues are also important in the context of the actual protein–protein interaction. Peptide array is a powerful technique that removes the guesswork from the time-consuming process of making mutants and so greatly facilitates the mapping process, which can be of particular importance when there is no structural information on which to base the generation of mutants. However, when structural information is present, then peptide array data coupled to structural information can rapidly facilitate further investigations using mutagenesis to define sites and to facilitate the generation of peptidomimetics.

This technique was recently utilized to define the binding sites for the signaling scaffold proteins, β-arrestin and RACK1, on PDE4D5 (Bolger et al. 2006) (Fig. 4). Conventional mapping methods had already shown that there was a RACK1 interaction domain (RAID1) in the unique N-terminal region of RACK1 (Yarwood et al. 1999; Bolger et al. 2002), whose structure has recently been delineated by 1H-NMR (Smith et al. 2007). Reassuringly, the peptide array technique identified the same N-terminal region, but also identified a second RACK1-binding site in the catalytic region of PDE4D5 that was later confirmed by two-hybrid analysis. Two known binding sites were also confirmed for β-arrestin, and use of scanning alanine arrays extended previous studies by pinpointing the critical residues. Simultaneous overlay of the PDE4D5 peptide array with both proteins, in conjunction with dual labeling and detection using the Odyssey system, revealed that RACK1 and β-arrestin do indeed compete for their overlapping binding sites on PDE4D5 (Bolger et al. 2006). Thus, the use of peptide arrays has allowed the elucidation of a mechanism whereby the availability of RACK1 has important implications for the regulation of the β_2AR by β-arrestin and PDE4D5 (see Sect. 2.5.6).

A peptide array approach was also used to define the binding sites for PDE4D5 on β-arrestin2 (Baillie et al. 2007). A comparison of β-arrestin2 peptide arrays overlain with either PDE4D3 or PDE4D5 revealed a region in the N-domain of β-arrestin2 that interacts with the common region of PDE4D and two sites in the C-domain that mediate specific interaction with PDE4D5. Subsequent alanine scanning peptide array analysis identified the key interacting residues at each site. Transfection of wild-type β-arrestin2 into β-arrestin1/2 knockout mouse embryonic fibroblasts was shown to result in a marked decrease in agonist-mediated PKA phosphorylation of the β_2AR. However, transfection of β-arrestin2 carrying mutations in any of the critical residues identified by peptide array failed to reduce β_2AR phosphorylation, despite their recruitment to the β_2AR upon agonist stimulation. This peptide array-based approach has therefore revealed that PDE4D5 has to interact with both the N- and C- domains of β-arrestin2 in order to effect regulation of the β_2AR.

3.2 Disruptor Therapeutics

Interacting peptides identified by peptide array analyses also have the potential to be used to develop therapeutic agents to disrupt protein–protein interactions. It has become evident that targeting and compartmentalization are fundamental to the functioning of PDE4s, such that disrupting protein–protein interactions with competing peptides can be predicted to abolish specific functioning of an isoform. Peptides themselves do not make good pharmacological agents due to problems of delivery, poor bioavailability and metabolic instability. However, interacting peptides can be used as a basis to design small molecule peptidomimetics that retain the ability to bind to the biological target, but that avoid the problems associated with natural peptides (Adessi and Soto 2002). These small molecule therapeutics will not be without their problems; for example, peptide-based drugs might be expected to be less soluble than existing PDE4 inhibitors. Additionally, the disruption of pre-existing protein–protein interactions may be problematic if their affinity is high, although in chronic disease states, continuous treatment with a small molecule disruptor could block the site of interaction on nascent proteins before they have the chance to form any high-affinity interactions. Despite these anticipated problems, this new class of PDE4 inhibitors would have the advantage of being highly specific, which would hopefully eliminate the side effects associated with current inhibitors and so be of great benefit in the treatment inflammatory disease, auto-immune disease and depression.

3.3 Dominant Negative Approach to Inhibition by Displacement

The dominant negative approach, pioneered by the Houslay laboratory, has provided proof of principle that disruption of PDE4 activity by displacement from its appropriate scaffold(s) can give rise to functional effects (Perry et al. 2002; Baillie et al. 2003; Lynch et al. 2005; McCahill et al. 2005; Bolger et al. 2006). This technique involves the over-expression of certain PDE4 isoforms that have been engineered to be catalytically inactive. Over-expression results in the displacement of the corresponding, active, endogenous PDE4 from its functionally relevant anchor site, thereby providing a dominant negative action. The displaced endogenous PDE4s are rendered non-functional, not by catalytic inhibition, but because they have been removed from their appropriate site of action. Catalytically inactive PDE4 species were generated by making point mutations in the catalytic unit of the enzyme. A number of residues were carefully selected as targets for mutagenesis based on the three-dimensional structure of the catalytic unit of PDE4B2 as determined by Xu et al. (2000). The catalytic unit is proposed to consist of 17 α-helices that form three separate sub-domains. The presumed cAMP binding site is within a deep pocket, at the bottom of which are two metal ions, essential for catalysis, which bind to amino acid residues that are conserved in all PDE4 family members. Seven amino acid

residues that were predicted to play critical roles in catalysis (Xu et al. 2000), and that were also absolutely conserved were selected for individual mutagenesis to alanine. The original test mutations were performed in PDE4D3 rather than PDE4B2 so the cognate sites were Tyr 325, His 326, His 330, His 366, His 370, Asp 484 and Glu 505. Lysates from cells transfected with these PDE4D3 point mutants were found to have PDE activity between 96 and 98% lower than lysates from cells transfected with wild type PDE4D3, indicating that all the mutated species are effectively catalytically inactive (M. Magiera, unpublished data).

Subsequently, mutations to alanine at the site equivalent to Asp 484 in PDE4D3 have been made in a number of other PDE4 isoforms to generate catalytically inactive species that have been over-expressed to gain insight into the functional role of tethered PDE4 isoforms within cells (Perry et al. 2002; Baillie et al. 2003; McCahill et al. 2005). The catalytically inactive PDE4D5 isoform (Asp 556 Ala) in particular has been used in a number of elegant studies to demonstrate the role this isoforms plays in β-adrenergic signaling (Perry et al. 2002; Baillie et al. 2003; Lynch et al. 2005; Bolger et al. 2006). In HEK293B2 cells, stimulation of the β_2AR results in β-arrestin-mediated recruitment of PDE4D5 to the β_2AR where it lowers local cAMP levels, consequently lowering PKA activity, which prevents PKA phosphorylation of the β_2AR and thus attenuates switching from Gs-mediated stimulation of AC to Gi-mediated activation of ERK (Baillie et al. 2003). RNA silencing of PDE4D5 mimicked the inhibitory effect of rolipram in that it enhanced agonist-stimulated PKA phosphorylation of the β_2AR and activation of ERK, thus indicating that PDE4D5 is the functionally important PDE4 isoform involved in modulating β_2AR signaling (Lynch et al. 2005). Although this approach identified PDE4D5 as the functionally relevant isoform, it revealed nothing of the importance of anchored PDE4D5 populations versus the entire cellular pool of PDE4D5. A dominant negative approach was therefore employed to investigate this (Fig. 5). Over-expression of catalytically inactive PDE4D5 exerted a dominant negative effect in enhancing agonist-stimulated ERK activation, presumably by displacing active endogenous PDE4D5 from its interaction with β-arrestin, preventing agonist-mediated delivery of PDE4D5 to the β_2AR, resulting in an increase in local cAMP. However, using a catalytically inactive PDE4D5 construct containing an additional point mutation in the unique N-terminal region known to prevent its interaction with β-arrestin abolished the dominant negative effect of catalytically inactive PDE4D5. This showed unequivocally that PDE4D5 must bind to β-arrestin in order to modulate agonist-stimulated ERK activation via β_2AR switching. The use of a dominant negative strategy thereby confirmed and extended the data obtained from RNA silencing in showing that PDE4D5 was indeed the functionally important isoform regulating the β_2AR, but also that compartmentalization by tethering to β-arrestin was critical for it to exert that function. The dominant negative approach is therefore a powerful technique for elucidating the functions of targeted PDE4 isoforms and can be used in conjunction with information gleaned from peptide array analyses to generate catalytically inactive constructs that also have a mutation at a critical site of protein–protein interaction and so fail to be

appropriately targeted. Furthermore, the fact that displacement of a PDE4 isoform from its cellular anchor site has been shown to be sufficient to abolish its function provides credence for the concept of generating novel inhibitors that work by disrupting protein–protein interactions and can therefore be designed to be isoform or even function specific.

4 Conclusions

The PDE4 family of cAMP-degrading enzymes has been the focus of extensive research due to the wide range of pathological conditions and disease states that could potentially benefit from treatment with PDE4 inhibitors. Significant advances have recently been made in the understanding of PDE4 biology and the role it plays in specific signaling systems, however, this has only served to highlight the complexity of the involvement of PDE4 isoforms in signaling mechanisms and so has emphasized that much more remains to be uncovered. A number of proteins that bind to PDE4 have been identified and their interactions partially characterized, but undoubtedly a myriad of interacting partners still await discovery before we can even begin to understand their functions. It is clear, however, that PDE4 enzymes are fundamental to the compartmentalization of cAMP signaling and are involved in both the spatial and temporal regulation of signal dissemination. It is also becoming increasingly clear that they are positioned to integrate crosstalk with other major signaling pathways such as the ERK MAP kinase pathway, which controls many critical cellular functions. The PDE4 family comprises some 20 distinct isoforms with it being entirely possible that the list of known PDE4 isoforms is incomplete. The isoforms identified to date have been very highly conserved between species, despite evolutionary pressure, suggesting that they will all transpire to have important functions with minimal redundancy. It is possible that the same isoform will perform different functions in different cell types dependent on the cell-specific expression of binding partners. In fact, the observed cell-specific action of certain inhibitors is likely a consequence of the availability of different interacting species that all serve to influence PDE4 localization, conformation and regulation. It will therefore be of increasing importance to the development of therapeutic inhibitors that PDE4 isoforms and their binding partners are studied in the appropriate cells or tissues that will act as targets for therapeutics, for example, cells of the CNS or inflammatory cells. Increased knowledge of the protein–protein interactions of PDE4 and the processes they regulate will be facilitated by techniques such as peptide array, dominant negative displacement and RNA silencing. This enhanced understanding will aid in the design of increasingly specific PDE4 inhibitors, which target unique isoforms by virtue of their protein–protein interactions. Hopefully, isoform-specific PDE4 inhibitors will eliminate the prohibitive side effects seen with current universal PDE4 inhibitors and thereby allow PDE4 inhibitors to fulfill their promise as therapeutic agents.

Acknowledgements This work was supported by grants from the Medical Research Council (UK) (G8604010; 0600765), the Leducq Foundation (Paris) and by the European Union (037189).

References

Adessi C, Soto C (2002) Converting a peptide into a drug: strategies to improve stability and bioavailability. Curr Med Chem 9:963–978

Ariga M, Neitzert B, Nakae S, Mottin G, Bertrand C, Pruniaux MP, Jin SL, Conti M (2004) Nonredundant function of phosphodiesterases 4D and 4B in neutrophil recruitment to the site of inflammation. J Immunol 173:7531–7538

Alvarez R, Sette C, Yang D, Eglen RM, Wilhelm R, Shelton ER, Conti M (1995) Activation and selective inhibition of camp specific phosphodiesterases, PDE4D3. Mol Pharmacol 48:616–622

Baillie GS, Houslay MD (2005) Arrestin times for compartmentalised cAMP signalling and phosphodiesterase-4 enzymes. Curr Opin Cell Biol 17:129–134

Baillie GS, Mackenzie SJ, Houslay MD (2001) PMA triggers the PKA-mediated phosphorylation and activation of the PDE4D5 cAMP phosphodiesterase in human aortic smooth muscle cells through a route involving Extracellular Signal Regulated Kinase (ERK). Mol Pharmacol 60:1100–1111.

Baillie GS, Huston E, Scotland G, Hodgkin M, Gall I, Peden AH, MacKenzie C, Houslay ES, Currie R, Pettitt TR, Walmsley AR, Wakelam MJ, Warwicker J, Houslay MD (2002) TAPAS-1, a novel microdomain within the unique N-terminal region of the PDE4A1 cAMP-specific phosphodiesterase that allows rapid, Ca2+-triggered membrane association with selectivity for interaction with phosphatidic acid. J Biol Chem 277:28298–28309

Baillie GS, Sood A, McPhee I, Gall I, Perry SJ, Lefkowitz RJ, Houslay MD (2003) beta-Arrestin-mediated PDE4 cAMP phosphodiesterase recruitment regulates beta-adrenoceptor switching from Gs to Gi. Proc Natl Acad Sci USA 100:940–945

Baillie GS, Scott JD, Houslay MD (2005) Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract. FEBS Lett 579:3264–3270

Baillie GS, Adams DR, Bahri N, Houslay TM, Vadrevu S, Meng D, Li X, Dunlop A, Milligan G, Bolger GB, Klussmann E, Houslay MD (2007) Mapping binding sites for the PDE4D5 cAMP-specific phosphodiesterase to the N- and C-domains of betaarrestin using spot-immobilised peptide arrays. Biochem J 404:71–80

Barnette MS (1999) Phosphodiesterase 4 (PDE4) inhibitors in asthma and chronic obstructive pulmonary disease (COPD). Prog Drug Res 53:193–229

Barnette MS, Underwood DC (2000) New phosphodiesterase inhibitors as therapeutics for the treatment of chronic lung disease. Curr Opin Pulm Med 6:164–169

Beard MB, O'Connell JC, Bolger GB, Houslay MD (1999) The unique N-terminal domain of the cAMP phosphodiesterase PDE4D4 allows for interaction with specific SH3 domains. FEBS Lett 460:173–177

Beard MB, Olsen AE, Jones RE, Erdogan S, Houslay MD, Bolger GB (2000) UCR1 and UCR2 domains unique to the cAMP-specific phosphodiesterase family form a discrete module via electrostatic interactions. J Biol Chem 275:10349–10358

Beard MB, Huston E, Campbell L, Gall I, McPhee I, Yarwood S, Scotland G, Houslay MD (2002) In addition to the SH3 binding region, multiple regions within the N-terminal noncatalytic portion of the cAMP-specific phosphodiesterase, PDE4A5, contribute to its intracellular targeting. Cell Signal 14:453–465

Beavo JA, Brunton LL (2002) Cyclic nucleotide research – still expanding after half a century. Nat Rev Mol Cell Biol 3:710–718

Bock KW, Kohle C (2006) Ah receptor: dioxin-mediated toxic responses as hints to deregulated physiologic functions. Biochem Pharmacol 72:393–404

Bolger GB (1994) Molecular biology of the cyclic AMP-specific cyclic nucleotide phosphodiesterases: a diverse family of regulatory enzymes. Cell Signal 6:851–859
Bolger G, Michaeli T, Martins T, St John T, Steiner B, Rodgers L, Riggs M, Wigler M, Ferguson K (1993) A family of human phosphodiesterases homologous to the dunce learning and memory gene product of Drosophila melanogaster are potential targets for antidepressant drugs. Mol Cell Biol 13:6558–6571
Bolger GB, Erdogan S, Jones RE, Loughney K, Scotland G, Hoffmann R, Wilkinson I, Farrell C, Houslay MD (1997) Characterization of five different proteins produced by alternatively spliced mRNAs from the human cAMP-specific phosphodiesterase PDE4D gene. Biochem J 328(Pt 2):539–548
Bolger GB, McCahill A, Yarwood SJ, Steele MR, Warwicker J, Houslay MD (2002) Delineation of RAID1, the RACK1 interaction domain located within the unique N-terminal region of the cAMP-specific phosphodiesterase, PDE4D5. BMC Biochem 3:24
Bolger GB, McCahill A, Huston E, Cheung YF, McSorley T, Baillie GS, Houslay MD (2003a) The unique amino-terminal region of the PDE4D5 cAMP phosphodiesterase isoform confers preferential interaction with beta-arrestins. J Biol Chem 278:49230–49238
Bolger GB, Peden AH, Steele MR, MacKenzie C, McEwan DG, Wallace DA, Huston E, Baillie GS, Houslay MD (2003b) Attenuation of the activity of the cAMP-specific phosphodiesterase PDE4A5 by interaction with the immunophilin XAP2. J Biol Chem 278:33351–33363
Bolger GB, Baillie GS, Li X, Lynch MJ, Herzyk P, Mohamed A, Mitchell LH, McCahill A, Hundsrucker C, Klussmann E, Adams DR, Houslay MD (2006) Scanning peptide array analyses identify overlapping binding sites for the signalling scaffold proteins, beta-arrestin and RACK1, in cAMP-specific phosphodiesterase PDE4D5. Biochem J 398:23–36
Bolger G, Conti M, Houslay MD (2007) Cellular functions of PDE4 enzymes. In: Beavo JA, Francis SH, Houslay MD (eds) Cyclic nucleotide phosphodiesterases in health and disease. CRC Press, Boca Raton, London, New York, pp 99–130
Bos JL (2003) Epac: a new cAMP target and new avenues in cAMP research. Nat Rev Mol Cell Biol 4:733–738
Bos JL (2006) Epac proteins: multi-purpose cAMP targets. Trends Biochem Sci 31:680–686.
Boswell-Smith V, Spina D, Page CP (2006) Phosphodiesterase inhibitors. Br J Pharmacol 147 (Suppl 1):S252–S257
Brunton LL (2003) PDE4: arrested at the border. Sci STKE 2003:PE44
Brunton LL, Hayes JS, Mayer SE (1981) Functional compartmentation of cyclic AMP and protein kinase in heart. Adv Cyclic Nucleotide Res 14:391–397
Burnouf C, Pruniaux MP (2002) Recent advances in PDE4 inhibitors as immunoregulators and anti-inflammatory drugs. Curr Pharm Des 8:1255–1296
Card GL, England BP, Suzuki Y, Fong D, Powell B, Lee B, Luu C, Tabrizizad M, Gillette S, Ibrahim PN, Artis DR, Bollag G, Milburn MV, Kim SH, Schlessinger J, Zhang KY (2004) Structural basis for the activity of drugs that inhibit phosphodiesterases. Structure 12:2233–2247
Carlisle Michel JJ, Dodge KL, Wong W, Mayer NC, Langeberg LK, Scott JD (2004) PKA-phosphorylation of PDE4D3 facilitates recruitment of the mAKAP signalling complex. Biochem J 381:587–592
Carson CC, Lue TF (2005) Phosphodiesterase type 5 inhibitors for erectile dysfunction. BJU Int 96:257–280
Carter CJ (2006) Schizophrenia susceptibility genes converge on interlinked pathways related to glutamatergic transmission and long-term potentiation, oxidative stress and oligodendrocyte viability. Schizophr Res 86:1–14
Carver LA, Bradfield CA (1997) Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophilin homolog in vivo. J Biol Chem 272:11452–11456
Chang BY, Chiang M, Cartwright CA (2001) The interaction of Src and RACK1 is enhanced by activation of protein kinase C and tyrosine phosphorylation of RACK1. J Biol Chem 276:20346–20356
Colledge M, Scott JD (1999) AKAPs: from structure to function. Trends Cell Biol 9:216–221

Conti M (2002) Specificity of the cyclic adenosine 3′,5′-monophosphate signal in granulosa cell function. Biol Reprod 67:1653–1661

Conti M, Jin SL (1999) The molecular biology of cyclic nucleotide phosphodiesterases. Prog Nucleic Acid Res Mol Biol 63:1–38

Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu Rev Biochem 76:481–511.

Conti M, Richter W, Mehats C, Livera G, Park JY, Jin C (2003) Cyclic AMP-specific PDE4 phosphodiesterases as critical components of cyclic AMP signaling. J Biol Chem 278:5493–5496

Cooper DM (2003) Regulation and organization of adenylyl cyclases and cAMP. Biochem J 375:517–529

Cooper DM (2005) Compartmentalization of adenylate cyclase and cAMP signalling. Biochem Soc Trans 33:1319–1322

Cooper DM, Crossthwaite AJ (2006) Higher-order organization and regulation of adenylyl cyclases. Trends Pharmacol Sci 27:426–431

Cowan-Jacob SW (2006) Structural biology of protein tyrosine kinases. Cell Mol Life Sci 63:2608–2625

Daaka Y, Luttrell LM, Lefkowitz RJ (1997) Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. Nature 390:88–91

Davis RL, Cherry J, Dauwalder B, Han PL, Skoulakis E (1995) The cyclic AMP system and Drosophila learning. Mol Cell Biochem 149–150:271–278

de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396:474–477

DiPilato LM, Cheng X, Zhang J (2004) Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. Proc Natl Acad Sci USA 101:16513–16518

Diviani D, Baisamy L, Appert-Collin A (2006) AKAP-Lbc: a molecular scaffold for the integration of cyclic AMP and Rho transduction pathways. Eur J Cell Biol 85:603–610

Dodge KL, Khouangsathiene S, Kapiloff MS, Mouton R, Hill EV, Houslay MD, Langeberg LK, Scott JD (2001) mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module. EMBO J 20:1921–1930

Dodge-Kafka KL, Soughayer J, Pare GC, Carlisle Michel JJ, Langeberg LK, Kapiloff MS, Scott JD (2005) The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. Nature 437:574–578

Dolwick KM, Schmidt JV, Carver LA, Swanson HI, Bradfield CA (1993) Cloning and expression of a human Ah receptor cDNA. Mol Pharmacol 44:911–917

D'Sa C, Tolbert LM, Conti M, Duman RS (2002) Regulation of cAMP-specific phosphodiesterases type 4B and 4D (PDE4) splice variants by cAMP signaling in primary cortical neurons. J Neurochem 81:745–757

Dumaz N, Marais R (2005) Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways. Based on the anniversary prize of the Gesellschaft fur Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the Special FEBS Meeting in Brussels. FEBS J 272:3491–3504

Fan Chung K (2006) Phosphodiesterase inhibitors in airways disease. Eur J Pharmacol 533:110–117

Fernandes-Alnemri T, Litwack G, Alnemri ES (1994) CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. J Biol Chem 269:30761–30764

Frank R (2002) High-density synthetic peptide microarrays: emerging tools for functional genomics and proteomics. Comb Chem High Throughput Screen 5:429–440

Gao Y, Nikulina E, Mellado W, Filbin MT (2003) Neurotrophins elevate cAMP to reach a threshold required to overcome inhibition by MAG through extracellular signal-regulated kinase-dependent inhibition of phosphodiesterase. J Neurosci 23:11770–11777

Ghavami A, Hirst WD, Novak TJ (2006) Selective phosphodiesterase (PDE)-4 inhibitors: a novel approach to treating memory deficit? Drugs R D 7:63–71
Giembycz MA (2000) Phosphodiesterase 4 inhibitors and the treatment of asthma: where are we now and where do we go from here? Drugs 59:193–212
Giembycz MA (2001) Cilomilast: a second generation phosphodiesterase 4 inhibitor for asthma and chronic obstructive pulmonary disease. Expert Opin Investig Drugs 10:1361–1379
Gretarsdottir S, Thorleifsson G, Reynisdottir ST, Manolescu A, Jonsdottir S, Jonsdottir T, Gudmundsdottir T, Bjarnadottir SM, Einarsson OB, Gudjonsdottir HM, Hawkins M, Gudmundsson G, Gudmundsdottir H, Andrason H, Gudmundsdottir AS, Sigurdardottir M, Chou TT, Nahmias J, Goss S, Sveinbjornsdottir S, Valdimarsson EM, Jakobsson F, Agnarsson U, Gudnason V, Thorgeirsson G, Fingerle J, Gurney M, Gudbjartsson D, Frigge ML, Kong A, Stefansson K, Gulcher JR (2003) The gene encoding phosphodiesterase 4D confers risk of ischemic stroke. Nat Genet 35:131–138
Hall RA (2004) Beta-adrenergic receptors and their interacting proteins. Semin Cell Dev Biol 15:281–288
Hayes JS, Brunton LL (1982) Functional compartments in cyclic nucleotide action. J Cyclic Nucleotide Res 8:1–16
Hennah W, Thomson P, Peltonen L, Porteous D (2006) Genes and schizophrenia: beyond schizophrenia: the role of DISC1 in major mental illness. Schizophr Bull 32:409–416
Hermanto U, Zong CS, Li W, Wang LH (2002) RACK1, an insulin-like growth factor I (IGF-I) receptor-interacting protein, modulates IGF-I-dependent integrin signaling and promotes cell spreading and contact with extracellular matrix. Mol Cell Biol 22:2345–2365
Higashida H, Hoshi N, Zhang JS, Yokoyama S, Hashii M, Jin D, Noda M, Robbins J (2005) Protein kinase C bound with A-kinase anchoring protein is involved in muscarinic receptor-activated modulation of M-type KCNQ potassium channels. Neurosci Res 51:231–234
Hill SJ (2006) G-protein-coupled receptors: past, present and future. Br J Pharmacol 147 (Suppl 1): S27–S37
Hill EV, Sheppard CL, Cheung YF, Gall I, Krause E, Houslay MD (2006) Oxidative stress employs phosphatidyl inositol 3-kinase and ERK signalling pathways to activate cAMP phosphodiesterase-4D3 (PDE4D3) through multi-site phosphorylation at Ser239 and Ser579. Cell Signal 18:2056–2069
Hoffmann R, Wilkinson IR, McCallum JF, Engels P, Houslay MD (1998) cAMP-specific phosphodiesterase HSPDE4D3 mutants which mimic activation and changes in rolipram inhibition triggered by protein kinase A phosphorylation of Ser-54: generation of a molecular model. Biochem J 333(Pt 1):139–149
Hoffmann R, Baillie GS, MacKenzie SJ, Yarwood SJ, Houslay MD (1999) The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser579. EMBO J 18:893–903
Houslay MD (1998) Adaptation in cyclic AMP signalling processes: a central role for cyclic AMP phosphodiesterases. Semin Cell Dev Biol 9:161–167
Houslay MD (2001) PDE4 cAMP-specific phosphodiesterases. Prog Nucleic Acid Res Mol Biol 69:249–315
Houslay MD (2005) The long and short of vascular smooth muscle phosphodiesterase-4 as a putative therapeutic target. Mol Pharmacol 68:563–567
Houslay MD (2006) A RSK(y) relationship with promiscuous PKA. Sci STKE 2006:pe32
Houslay MD, Adams DR (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. Biochem J 370:1–18
Houslay MD, Kolch W (2000) Cell-type specific integration of cross-talk between extracellular signal-regulated kinase and cAMP signaling. Mol Pharmacol 58:659–668
Houslay MD, Milligan G (1997) Tailoring cAMP-signalling responses through isoform multiplicity. Trends Biochem Sci 22:217–224
Houslay MD, Sullivan M, Bolger GB (1998) The multienzyme PDE4 cyclic adenosine monophosphate-specific phosphodiesterase family: intracellular targeting, regulation, and selective

inhibition by compounds exerting anti-inflammatory and antidepressant actions. Adv Pharmacol 44:225–342
Houslay MD, Schafer P, Zhang KY (2005) Keynote review: phosphodiesterase-4 as a therapeutic target. Drug Discov Today 10:1503–1519
Houslay MD, Baillie GS, Maurice, DH (2007) cAMP specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalised cAMP signalling. Circ Res 100:950–966
Huang Z, Ducharme Y, Macdonald D, Robichaud A (2001) The next generation of PDE4 inhibitors. Curr Opin Chem Biol 5:432–438
Huston E, Beard M, McCallum F, Pyne NJ, Vandenabeele P, Scotland G, Houslay MD (2000) The cAMP-specific phosphodiesterase PDE4A5 is cleaved downstream of its SH3 interaction domain by caspase-3. Consequences for altered intracellular distribution. J Biol Chem 275:28063–28074
Huston E, Pooley L, Julien P, Scotland G, McPhee I, Sullivan M, Bolger G, Houslay MD (1996) The human cyclic AMP-specific phosphodiesterase PDE-46 (HSPDE4A4B) expressed in transfected COS7 cells occurs as both particulate and cytosolic species that exhibit distinct kinetics of inhibition by the antidepressant rolipram. J Biol Chem 271:31334–31344
Huston E, Gall I, Houslay TM, Houslay MD (2006a) Helix-1 of the cAMP-specific phosphodiesterase PDE4A1 regulates its phospholipase-D-dependent redistribution in response to release of Ca2+. J Cell Sci 119:3799–3810
Huston E, Houslay TM, Baillie GS, Houslay MD (2006b) cAMP phosphodiesterase-4A1 (PDE4A1) has provided the paradigm for the intracellular targeting of phosphodiesterases, a process that underpins compartmentalized cAMP signalling. Biochem Soc Trans 34:504–509
Jin SL, Swinnen JV, Conti M (1992) Characterization of the structure of a low Km, rolipram-sensitive cAMP phosphodiesterase. Mapping of the catalytic domain. J Biol Chem 267:18929–18939
Jin S, Lan L, Zoudilova M, Conti M (2005) Specific role of phosphodiesterase 4B in lipopolysaccharide-induced signaling in mouse macrophages. J Immunol 175:1523–1531
Jin C, Richter W, Conti M (2007) Insights into the physiological functions of PDE4 from knockout mice. In: Beavo JA, Francis SH, Houslay MD (eds) Cyclic nucleotide phosphodiesterases in health and disease. CRC Press, Boca Raton, London, New York, pp 323–346
Johnson JA, Gray MO, Chen CH, Mochly-Rosen D (1996) A protein kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. J Biol Chem 271:24962–24966
Johnston LA, Erdogan S, Cheung YF, Sullivan M, Barber R, Lynch MJ, Baillie GS, Van Heeke G, Adams DR, Huston E, Houslay MD (2004) Expression, intracellular distribution and basis for lack of catalytic activity of the PDE4A7 isoform encoded by the human PDE4A cAMP-specific phosphodiesterase gene. Biochem J 380:371–384
Kapiloff MS, Schillace RV, Westphal AM, Scott JD (1999) mAKAP: an A-kinase anchoring protein targeted to the nuclear membrane of differentiated myocytes. J Cell Sci 112(Pt 16): 2725–2736
Karpen JW, Rich TC (2001) The fourth dimension in cellular signaling. Science 293:2204–2205
Ke H, Wang H (2007) Crystal structures of phosphodiesterases and implications on substrate specificity and inhibitor selectivity. Curr Top Med Chem 7:391–403
Kiely PA, Leahy M, O’Gorman D, O’Connor R (2005) RACK1-mediated integration of adhesion and insulin-like growth factor I (IGF-I) signaling and cell migration are defective in cells expressing an IGF-I receptor mutated at tyrosines 1250 and 1251. J Biol Chem 280:7624–7633
Kim DH, Lerner A (1998) Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. Blood 92:2484–2494
King LS, Kozono D, Agre P (2004) From structure to disease: the evolving tale of aquaporin biology. Nat Rev Mol Cell Biol 5:687–698
Klauck TM, Faux MC, Labudda K, Langeberg LK, Jaken S, Scott JD (1996) Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science 271:1589–1592

Klussmann E, Maric K, Wiesner B, Beyermann M, Rosenthal W (1999) Protein kinase A anchoring proteins are required for vasopressin-mediated translocation of aquaporin-2 into cell membranes of renal principal cells. J Biol Chem 274:4934–4938
Kooistra MR, Corada M, Dejana E, Bos JL (2005) Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. FEBS Lett 579:4966–4972
Kuzhandaivelu N, Cong YS, Inouye C, Yang WM, Seto E (1996) XAP2, a novel hepatitis B virus X-associated protein that inhibits X transactivation. Nucleic Acids Res 24:4741–4750
Lees MJ, Peet DJ, Whitelaw ML (2003) Defining the role for XAP2 in stabilization of the dioxin receptor. J Biol Chem 278:35878–35888
Le Jeune IR, Shepherd M, Van Heeke G, Houslay MD, Hall IP (2002) Cyclic AMP-dependent transcriptional up-regulation of phosphodiesterase 4D5 in human airway smooth muscle cells. Identification and characterization of a novel PDE4D5 promoter. J Biol Chem 277:35980–35989
Lerner A, Epstein PM (2006) Cyclic nucleotide phosphodiesterases as targets for treatment of haematological malignancies. Biochem J 393:21–41
Li Y, Asuri S, Rebhun JF, Castro AF, Paranavitana NC, Quilliam LA (2006) The RAP1 guanine nucleotide exchange factor Epac2 couples cyclic AMP and Ras signals at the plasma membrane. J Biol Chem 281:2506–2514
Liliental J, Chang DD (1998) Rack1, a receptor for activated protein kinase C, interacts with integrin beta subunit. J Biol Chem 273:2379–2383
Lim J, Pahlke G, Conti M (1999) Activation of the cAMP-specific phosphodiesterase PDE4D3 by phosphorylation. Identification and function of an inhibitory domain. J Biol Chem 274:19677–19685
Lipworth BJ (2005) Phosphodiesterase-4 inhibitors for asthma and chronic obstructive pulmonary disease. Lancet 365:167–175
Liu H, Palmer D, Jimmo SL, Tilley DG, Dunkerley HA, Pang SC, Maurice DH (2000) Expression of phosphodiesterase 4D (PDE4D) is regulated by both the cyclic AMP-dependent protein kinase and mitogen-activated protein kinase signaling pathways. A potential mechanism allowing for the coordinated regulation of PDE4D activity and expression in cells. J Biol Chem 275:26615–26624
Lomo J, Blomhoff HK, Beiske K, Stokke T, Smeland EB (1995) TGF-beta 1 and cyclic AMP promote apoptosis in resting human B lymphocytes. J Immunol 154: 1634–1643
Lugnier C (2006) Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. Pharmacol Ther 109:366–398
Luttrell LM, Lefkowitz RJ (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 115:455–465
Lynch MJ, Baillie GS, Mohamed A, Li X, Maisonneuve C, Klussmann E, van Heeke G, Houslay MD (2005) RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with beta arrestin to control the protein kinase A/AKAP79-mediated switching of the beta2-adrenergic receptor to activation of ERK in HEK293B2 cells. J Biol Chem 280:33178–33189
Ma Q, Whitlock JP Jr (1997) A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Biol Chem 272:8878–8884
Ma D, Wu P, Egan RW, Billah MM, Wang P (1999) Phosphodiesterase 4B gene transcription is activated by lipopolysaccharide and inhibited by interleukin-10 in human monocytes. Mol Pharmacol 55:50–57
MacKenzie SJ, Baillie GS, McPhee I, Bolger GB, Houslay MD (2000) ERK2 mitogen-activated protein kinase binding, phosphorylation, and regulation of the PDE4D cAMP-specific phosphodiesterases. The involvement of COOH-terminal docking sites and NH2-terminal UCR regions. J Biol Chem 275:16609–16617
MacKenzie SJ, Baillie GS, McPhee I, MacKenzie C, Seamons R, McSorley T, Millen J, Beard MB, van Heeke G, Houslay MD (2002) Long PDE4 cAMP specific phosphodiesterases are

activated by protein kinase A-mediated phosphorylation of a single serine residue in Upstream Conserved Region 1 (UCR1). Br J Pharmacol 136:421–433

Malbon CC, Tao J, Wang HY (2004) AKAPs (A-kinase anchoring proteins) and molecules that compose their G-protein-coupled receptor signalling complexes. Biochem J 379:1–9

Marchmont RJ, Houslay MD (1980) Insulin trigger, cyclic AMP-dependent activation and phosphorylation of a plasma membrane cyclic AMP phosphodiesterase. Nature 286:904–906

Martinez JM, Afshari CA, Bushel PR, Masuda A, Takahashi T, Walker NJ (2002) Differential toxicogenomic responses to 2,3,7,8-tetrachlorodibenzo-p-dioxin in malignant and nonmalignant human airway epithelial cells. Toxicol Sci 69:409–423

Martey CA, Baglole CJ, Gasiewicz TA, Sime PJ, Phipps RP (2005) The aryl hydrocarbon receptor is a regulator of cigarette smoke induction of the cyclooxygenase and prostaglandin pathways in human lung fibroblasts. Am J Physiol Lung Cell Mol Physiol 289:L391–L399

Mayr B, Montminy M (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2:599–609

McCahill A, Warwicker J, Bolger GB, Houslay MD, Yarwood SJ (2002) The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. Mol Pharmacol 62:1261–1273

McCahill A, McSorley T, Huston E, Hill EV, Lynch MJ, Gall I, Keryer G, Lygren B, Tasken K, van Heeke G, Houslay MD (2005) In resting COS1 cells a dominant negative approach shows that specific, anchored PDE4 cAMP phosphodiesterase isoforms gate the activation, by basal cyclic AMP production, of AKAP-tethered protein kinase A type II located in the centrosomal region. Cell Signal 17:1158–1173

McConkey DJ, Orrenius S, Jondal M (1990) Agents that elevate cAMP stimulate DNA fragmentation in thymocytes. J Immunol 145:1227–1230

McPhee I, Yarwood SJ, Scotland G, Huston E, Beard MB, Ross AH, Houslay ES, Houslay MD (1999) Association with the SRC family tyrosyl kinase LYN triggers a conformational change in the catalytic region of human cAMP-specific phosphodiesterase HSPDE4A4B. Consequences for rolipram inhibition. J Biol Chem 274:11796–11810

Meschia JF, Brott TG, Brown RD Jr, Crook R, Worrall BB, Kissela B, Brown WM, Rich SS, Case LD, Evans EW, Hague S, Singleton A, Hardy J (2005) Phosphodiesterase 4D and 5-lipoxygenase activating protein in ischemic stroke. Ann Neurol 58:351–361

Meyer BK, Petrulis JR, Perdew GH (2000) Aryl hydrocarbon (Ah) receptor levels are selectively modulated by hsp90-associated immunophilin homolog XAP2. Cell Stress Chaperones 5:243–254

Millar JK, Christie S, Porteous DJ (2003) Yeast two-hybrid screens implicate DISC1 in brain development and function. Biochem Biophys Res Commun 311:1019–1025

Millar JK, Pickard BS, Mackie S, James R, Christie S, Buchanan SR, Malloy MP, Chubb JE, Huston E, Baillie GS, Thomson PA, Hill EV, Brandon NJ, Rain JC, Camargo LM, Whiting PJ, Houslay MD, Blackwood DH, Muir WJ, Porteous DJ (2005) DISC1 and PDE4B are interacting genetic factors in schizophrenia that regulate cAMP signaling. Science 310:1187–1191

Monaco L, Vicini E, Conti M (1994) Structure of two rat genes coding for closely related rolipram-sensitive cAMP phosphodiesterases. Multiple mRNA variants originate from alternative splicing and multiple start sites. J Biol Chem 269:347–357

Morales JL, Perdew GH (2007) Carboxyl terminus of hsc70-interacting protein (CHIP) can remodel mature aryl hydrocarbon teceptor (AhR) complexes and mediate ubiquitination of both the AhR and the 90 kDa heat-shock protein (hsp90) in vitro. Biochemistry 46:610–621

Murdoch H, Mackie S, Collins DM, Hill EV, Bolger GG, Klussmann E, Porteous DJ, Millar JK, Houslay MD (2007) Isoform selective susceptibility of DISC1/phosphodiesterase-4 (PDE4) complexes to dissociation by elevated intracellular cAMP levels. J Neurosci 27:9513–9524.

Nagata S (1997) Apoptosis by death factor. Cell 88:355–365

Nemoz G, Sette C, Conti M (1997) Selective activation of rolipram-sensitive, cAMP-specific phosphodiesterase isoforms by phosphatidic acid. Mol Pharmacol 51:242–249

Nikolaev VO, Bunemann M, Hein L, Hannawacker A, Lohse MJ (2004) Novel single chain cAMP sensors for receptor-induced signal propagation. J Biol Chem 279:37215–37218
O'Connell JC, McCallum JF, McPhee I, Wakefield J, Houslay ES, Wishart W, Bolger G, Frame M, Houslay MD (1996) The SH3 domain of Src tyrosyl protein kinase interacts with the N-terminal splice region of the PDE4A cAMP-specific phosphodiesterase RPDE-6 (RNPDE4A5). Biochem J 318(Pt 1):255–261
O'Donnell JM, Zhang HT (2004) Antidepressant effects of inhibitors of cAMP phosphodiesterase (PDE4). Trends Pharmacol Sci 25:158–163
Oesch-Bartlomowicz B, Oesch F (1990) Phosphorylation of cytochrome P450 isoenzymes in intact hepatocytes and its importance for their function in metabolic processes. Arch Toxicol 64: 257–261
Olsen AE, Bolger GB (2000) Physical mapping and promoter structure of the murine cAMP-specific phosphodiesterase pde4a gene. Mamm Genome 11:41–45
Parkes JD, Thompson C, Brennan L, Gajraj N, Howcroft B, Ruiz J (1984) Rolipram in Parkinson's disease. Adv Neurol 40:563–565
Pawson T, Gish GD (1992) SH2 and SH3 domains: from structure to function. Cell 71:359–362
Pearse DD, Pereira FC, Marcillo AE, Bates ML, Berrocal YA, Filbin MT, Bunge MB (2004) cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat Med 10:610–616
Perry SJ, Baillie GS, Kohout TA, McPhee I, Magiera MM, Ang KL, Miller WE, McLean AJ, Conti M, Houslay MD, Lefkowitz RJ (2002) Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. Science 298:834–836
Petrulis JR, Perdew GH (2002) The role of chaperone proteins in the aryl hydrocarbon receptor core complex. Chem Biol Interact 141:25–40
Pierce KL, Premont RT, Lefkowitz RJ (2002) Seven-transmembrane receptors. Nat Rev Mol Cell Biol 3:639–650
Pooley L, Shakur Y, Rena G, Houslay MD (1997) Intracellular localization of the PDE4A cAMP-specific phosphodiesterase splice variant RD1 (RNPDE4A1A) in stably transfected human thyroid carcinoma FTC cell lines. Biochem J 321(Pt 1):177–185
Porteous DJ, Millar JK (2006) Disrupted in schizophrenia 1: building brains and memories. Trends Mol Med 12:255–261
Ravipati G, McClung JA, Aronow WS, Peterson SJ, Frishman WH (2007) Type 5 phosphodiesterase inhibitors in the treatment of erectile dysfunction and cardiovascular disease. Cardiol Rev 15:76–86
Rena G, Begg F, Ross A, MacKenzie C, McPhee I, Campbell L, Huston E, Sullivan M, Houslay MD (2001) Molecular cloning, genomic positioning, promoter identification, and characterization of the novel cyclic amp-specific phosphodiesterase PDE4A10. Mol Pharmacol 59:996–1011
Richter W, Conti M (2002) Dimerization of the type 4 cAMP-specific phosphodiesterases is mediated by the upstream conserved regions (UCRs). J Biol Chem 277:40212–40221
Richter W, Conti M (2004) The oligomerization state determines regulatory properties and inhibitor sensitivity of type 4 cAMP-specific phosphodiesterases. J Biol Chem 279:30338–30348
Robichaud A, Tattersall FD, Choudhury I, Rodger IW (1999) Emesis induced by inhibitors of type IV cyclic nucleotide phosphodiesterase (PDE IV) in the ferret. Neuropharmacology 38:289–297
Robichaud A, Stamatiou PB, Jin SL, Lachance N, MacDonald D, Laliberte F, Liu S, Huang Z, Conti M, Chan CC (2002) Deletion of phosphodiesterase 4D in mice shortens alpha(2)-adrenoceptor-mediated anesthesia, a behavioral correlate of emesis. J Clin Invest 110:1045–1052
Ron D, Chen CH, Caldwell J, Jamieson L, Orr E, Mochly-Rosen D (1994) Cloning of an intracellular receptor for protein kinase C: a homolog of the beta subunit of G proteins. Proc Natl Acad Sci USA 91:839–843
Scotland G, Houslay MD (1995) Chimeric constructs show that the unique N-terminal domain of the cyclic AMP phosphodiesterase RD1 (RNPDE4A1A; rPDE-IVA1) can confer membrane association upon the normally cytosolic protein chloramphenicol acetyltransferase. Biochem J 308(Pt 2):673–681

Sette C, Conti M (1996) Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. Involvement of serine 54 in the enzyme activation. J Biol Chem 271:16526–16534

Seybold J, Newton R, Wright L, Finney PA, Suttorp N, Barnes PJ, Adcock IM, Giembycz MA (1998) Induction of phosphodiesterases 3B, 4A4, 4D1, 4D2, and 4D3 in Jurkat T-cells and in human peripheral blood T-lymphocytes by 8-bromo-cAMP and Gs-coupled receptor agonists. Potential role in beta2-adrenoreceptor desensitization. J Biol Chem 273: 20575–20588

Shakur Y, Pryde JG, Houslay MD (1993) Engineered deletion of the unique N-terminal domain of the cyclic AMP-specific phosphodiesterase RD1 prevents plasma membrane association and the attainment of enhanced thermostability without altering its sensitivity to inhibition by rolipram. Biochem J 292(Pt 3):677–686

Shakur Y, Wilson M, Pooley L, Lobban M, Griffiths SL, Campbell AM, Beattie J, Daly C, Houslay MD (1995) Identification and characterization of the type-IVA cyclic AMP-specific phosphodiesterase RD1 as a membrane-bound protein expressed in cerebellum. Biochem J 306(Pt 3):801–809

Sharrocks AD, Yang SH, Galanis A (2000) Docking domains and substrate-specificity determination for MAP kinases. Trends Biochem Sci 25:448–453

Shepherd MC, Baillie GS, Stirling DI, Houslay MD (2004) Remodelling of the PDE4 cAMP phosphodiesterase isoform profile upon monocyte-macrophage differentiation of human U937 cells. Br J Pharmacol 142:339–351

Sikorski RS, Boguski MS, Goebl M, Hieter P (1990) A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. Cell 60:307–317

Smith KJ, Scotland G, Beattie J, Trayer IP, Houslay MD (1996) Determination of the structure of the N-terminal splice region of the cyclic AMP-specific phosphodiesterase RD1 (RNPDE4A1) by 1H NMR and identification of the membrane association domain using chimeric constructs. J Biol Chem 271:16703–16711

Smith FD, Langeberg LK, Scott JD (2006) The where's and when's of kinase anchoring. Trends Biochem Sci 31:316–323

Smith KJ, Baillie GS, Hyde EI, Li X, Houslay TM, McCahill A, Dunlop AJ, Bolger GB, Klussmann E, Adams DR, Houslay MD (2007) 1H-NMR structural and functional characterisation of a cAMP-specific phosphodiesterase-4D5 (PDE4D5) N-terminal region peptide that disrupts PDE4D5 interaction with the signalling scaffold proteins, βarrestin and RACK1. Cell Signal 19:2612–2624.

Sondek J, Siderovski DP (2001) Ggamma-like (GGL) domains: new frontiers in G-protein signaling and beta-propeller scaffolding. Biochem Pharmacol 61:1329–1337

Souness JE, Rao S (1997) Proposal for pharmacologically distinct conformers of PDE4 cyclic AMP phosphodiesterases. Cell Signal 9:227–236

Souness JE, Aldous D, Sargent C (2000) Immunosuppressive and anti-inflammatory effects of cyclic AMP phosphodiesterase (PDE) type 4 inhibitors. Immunopharmacology 47:127–162

Steele MR, McCahill A, Thompson DS, MacKenzie C, Isaacs NW, Houslay MD, Bolger GB (2001) Identification of a surface on the beta-propeller protein RACK1 that interacts with the cAMP-specific phosphodiesterase PDE4D5. Cell Signal 13:507–513

Stefan E, Wiesner B, Baillie GS, Mollajew R, Henn V, Lorenz D, Furkert J, Santamaria K, Nedvetsky P, Hundsrucker C, Beyermann M, Krause E, Pohl P, Gall I, MacIntyre AN, Bachmann S, Houslay MD, Rosenthal W, Klussmann E (2007) Compartmentalization of cAMP-dependent signaling by phosphodiesterase-4D is involved in the regulation of vasopressin-mediated water reabsorption in renal principal cells. J Am Soc Nephrol 18:199–212

Stehlik J, Movsesian MA (2006) Inhibitors of cyclic nucleotide phosphodiesterase 3 and 5 as therapeutic agents in heart failure. Expert Opin Investig Drugs 15:733–742

Sturton G, Fitzgerald M (2002) Phosphodiesterase 4 inhibitors for the treatment of COPD. Chest 121:192S–196S

Sullivan M, Olsen AS, Houslay MD (1999) Genomic organisation of the human cyclic AMP-specific phosphodiesterase PDE4C gene and its chromosomal localisation to 19p13.1, between RAB3A and JUND. Cell Signal 11:735–742
Swinnen JV, Tsikalas KE, Conti M (1991) Properties and hormonal regulation of two structurally related cAMP phosphodiesterases from the rat Sertoli cell. J Biol Chem 266:18370–18377
Tasken K, Aandahl EM (2004) Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol Rev 84:137–167
Tasken KA, Collas P, Kemmner WA, Witczak O, Conti M, Tasken K (2001) Phosphodiesterase 4D and protein kinase a type II constitute a signaling unit in the centrosomal area. J Biol Chem 276:21999–22002
Taylor SS, Kim C, Vigil D, Haste NM, Yang J, Wu J, Anand GS (2005) Dynamics of signaling by PKA. Biochim Biophys Acta 1754:25–37
Terasawa H, Kohda D, Hatanaka H, Tsuchiya S, Ogura K, Nagata K, Ishii S, Mandiyan V, Ullrich A, Schlessinger J et al (1994) Structure of the N-terminal SH3 domain of GRB2 complexed with a peptide from the guanine nucleotide releasing factor Sos. Nat Struct Biol 1:891–897
Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J et al (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature 356:768–774
Thornton C, Tang KC, Phamluong K, Luong K, Vagts A, Nikanjam D, Yaka R, Ron D (2004) Spatial and temporal regulation of RACK1 function and N-methyl-D-aspartate receptor activity through WD40 motif-mediated dimerization. J Biol Chem 279:31357–31364
Uzawa S, Samejima I, Hirano T, Tanaka K, Yanagida M (1990) The fission yeast cut1+ gene regulates spindle pole body duplication and has homology to the budding yeast ESP1 gene. Cell 62: 913–925
Vandecasteele G, Rochais F, Abi-Gerges A, Fischmeister R (2006) Functional localization of cAMP signalling in cardiac myocytes. Biochem Soc Trans 34:484–488
Verde I, Pahlke G, Salanova M, Zhang G, Wang S, Coletti D, Onuffer J, Jin SL, Conti M (2001) Myomegalin is a novel protein of the golgi/centrosome that interacts with a cyclic nucleotide phosphodiesterase. J Biol Chem 276:11189–11198
Vicini E, Conti M (1997) Characterization of an intronic promoter of a cyclic adenosine 3′,5′-monophosphate (cAMP)-specific phosphodiesterase gene that confers hormone and cAMP inducibility. Mol Endocrinol 11:839–850
Wallace DA, Johnston LA, Huston E, MacMaster D, Houslay TM, Cheung YF, Campbell L, Millen JE, Smith RA, Gall I, Knowles RG, Sullivan M, Houslay MD (2005) Identification and characterization of PDE4A11, a novel, widely expressed long isoform encoded by the human PDE4A cAMP phosphodiesterase gene. Mol Pharmacol 67:1920–1934
Wang P, Wu P, Ohleth KM, Egan RW, Billah MM (1999) Phosphodiesterase 4B2 is the predominant phosphodiesterase species and undergoes differential regulation of gene expression in human monocytes and neutrophils. Mol Pharmacol 56:170–174
Wang D, Deng C, Bugaj-Gaweda B, Kwan M, Gunwaldsen C, Leonard C, Xin X, Hu Y, Unterbeck A, De Vivo M (2003) Cloning and characterization of novel PDE4D isoforms PDE4D6 and PDE4D7. Cell Signal 15:883–891
Wang H, Peng M, Chen Y, Robinson H, Houslay MD, Cai J, Ke H (2007) Structures of the four sub-families of phosphodiesterase-4 provide insight into the selectivity of their inhibitors. Biochem J 408:193–201
Wong W, Scott JD (2004) AKAP signalling complexes: focal points in space and time. Nat Rev Mol Cell Biol 5:959–970
Xiang Y, Naro F, Zoudilova M, Jin S, Conti M, Kobilka B (2005) Phosphodiesterase 4D is required for beta(2) adrenoceptor subtype-specific signaling in cardiac myocytes. Proc Natl Acad Sci USA 102:909–914
Xu RX, Hassell AM, Vanderwall D, Lambert MH, Holmes WD, Luther MA, Rocque WJ, Milburn MV, Zhao Y, Ke H, Nolte RT (2000) Atomic structure of PDE4: insights into phosphodiesterase mechanism and specificity. Science 288:1822–1825

Xu RX, Rocque WJ, Lambert MH, Vanderwall DE, Luther MA, Nolte RT (2004) Crystal structures of the catalytic domain of phosphodiesterase 4B complexed with AMP, 8-Br-AMP, and rolipram. J Mol Biol 337:355–365

Yaka R, Thornton C, Vagts AJ, Phamluong K, Bonci A, Ron D (2002) NMDA receptor function is regulated by the inhibitory scaffolding protein, RACK1. Proc Natl Acad Sci USA 99:5710–5715

Yarwood SJ, Steele MR, Scotland G, Houslay MD, Bolger GB (1999) The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform. J Biol Chem 274:14909–14917

Yedovitzky M, Mochly-Rosen D, Johnson JA, Gray MO, Ron D, Abramovitch E, Cerasi E, Nesher R (1997) Translocation inhibitors define specificity of protein kinase C isoenzymes in pancreatic beta-cells. J Biol Chem 272:1417–1420

Zaccolo M, Pozzan T (2002) Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. Science 295:1711–1715

Zeller E, Stief HJ, Pflug B, Sastre-y-Hernandez M (1984) Results of a phase II study of the antidepressant effect of rolipram. Pharmacopsychiatry 17:188–190

Zhang HT, O'Donnell JM (2007) Phosphodiesterase-4 as a pharmacological target mediating antidepressant and cognitive effects on behavior. In: Beavo JA, Francis SH, Houslay MD (eds) Cyclic nucleotide phosphodiesterases in health and disease. CRC Press, Boca Raton, London, New York, pp 539–558

Zhang J, Ma Y, Taylor SS, Tsien RY (2001) Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. Proc Natl Acad Sci USA 98:14997–15002

Zhang HT, Zhao Y, Huang Y, Dorairaj NR, Chandler LJ, O'Donnell JM (2004) Inhibition of the phosphodiesterase 4 (PDE4) enzyme reverses memory deficits produced by infusion of the MEK inhibitor U0126 into the CA1 subregion of the rat hippocampus. Neuropsychopharmacology 29:1432–1439

Zhu J, Mix E, Winblad B (2001) The antidepressant and antiinflammatory effects of rolipram in the central nervous system. CNS Drug Rev 7:387–398

G-Protein-Coupled Receptor-Signaling Components in Membrane Raft and Caveolae Microdomains

H.H. Patel, F. Murray, and P.A. Insel(✉)

Contents

1 Cellular Compartments as Signaling Microdomains: A General Overview 167
2 Specialized Plasma Membrane Compartments:
Membrane/Lipid Rafts vs. Caveolae 169
3 Determinants of Membrane Raft/Caveolae Localization 170
4 Membrane Rafts/Caveolae in Signal Transduction 171
5 GPCR/G-Proteins 172
5.1 Specific Signal Transduction Pathways 174
6 Questions to Consider for Future Investigations 178
References 179

Abstract The efficiency of signal transduction in cells derives in part from subcellular, in particular plasma membrane, microdomains that organize signaling molecules and signaling complexes. Two related plasma membrane domains that compartmentalize G-protein coupled receptor (GPCR) signaling complexes are lipid (membrane) rafts, domains that are enriched in certain lipids, including cholesterol and sphingolipids, and caveolae, a subset of lipid rafts that are enriched in the protein caveolin. This review focuses on the properties of lipid rafts and caveolae, the mechanisms by which they localize signaling molecules and the identity of GPCR signaling components that are organized in these domains.

1 Cellular Compartments as Signaling Microdomains: A General Overview

A large number of pharmacologically important signaling molecules localize in the plasma membrane, and as such, differential expression of such molecules in various cell types and tissues is an over riding principle that dominates drug development

P.A. Insel
University of California, San Diego Department of Pharmacology, 9500 Gilman Drive, BSB 3076, La Jolla, CA 92093-0636, USA
pinsel@ucsd.edu

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,

© Springer-Verlag Berlin Heidelberg 2008

and usage. The plasma membrane is one of several cellular organelles (others include the nucleus, Golgi apparatus and mitochondria) that can be readily recognized using light and/or electron microscopy. Subcellular fractionation methods have provided the principal starting point for the application of biochemical and biophysical methods to assess the properties and functions of such organelles. Substantial evidence, however, supports the view that preparations derived from the use of fractionation methods are quite heterogeneous, with differences in protein and lipid composition, as well as in organelle-specific functional activities. Thus, there are generally no "gold standard" methods to isolate "pure" organelles, in particular, the plasma membrane and its sub-domains. Such plasma membrane regions show unique patterns of expression of molecular components and functional activities, as well as, in some cases, unique anatomic features; examples include the apical and basolateral membranes of epithelial cells, luminal and abluminal membranes of endothelial cells, and dendritic, axonal and cell body membranes of neurons.

Plasma membrane receptors are critical sites of action for a large number of currently used drugs and drugs in development. Such receptors include G-protein-coupled receptors (GPCR), the largest membrane receptor super-family in eukaryotic genomes, and post-GPCR components, e.g., heterotrimeric G-proteins and G-protein-regulated effector molecules, which are the three necessary and sufficient components that mediate signal transduction by GPCR. In native cell systems, the level of expression of the three key components is: GPCR, typically <10,000^{-1} cell for individual GPCR that link to G_s and G_i; G-proteins (~1,000,000^{-1} cell) and effectors (~30,000^{-1} cell in the case of adenylyl cyclase), thus yielding a stoichiometric ratio of ~1:100:3 for GPCR that act via G_s and G_i to regulate the activity of adenylyl cyclase (Ostrom et al. 2000). Limited data are available for the stoichiometric ratios of other classes of heterotrimeric G-proteins and the GPCRs and effectors with which they interact.

Knowledge of these stoichiometric relationships among the critical GPCR signaling components is not sufficient information to explain the speed, fidelity and extent of response (measured as second messenger generation) to GPCR agonists, including the selectivity of activation of distal effectors. Such observations have provided a rationale in support of the concept of compartmentation of GPCR and components distal to the receptors (Lucero and Robbins 2004; Ostrom and Insel 2004; Pike 2004). The identification of plasma membrane domains that organize and compartmentalize GPCR and post-GPCR signaling components has helped provide both anatomic and functional evidence for membrane microdomains involved in signal transduction by GPCR. Such microdomains include clathrin-coated pits, membrane/lipid rafts, teteraspanins and caveolae ("little caves"), a subset of membrane/lipid rafts. In this review, we focus on rafts and caveolae. Other articles provide overviews on clathrin-coated pits (Lefkowitz and Shenoy 2005; Luttrell and Lefkowitz 2002; von Zastrow 2003) and tetraspanins (Berditchevski and Odintsova 2007).

2 Specialized Plasma Membrane Compartments: Membrane/Lipid Rafts vs. Caveolae

Membrane/lipid rafts are defined by their enrichment in cholesterol and other lipids, including glycosphingolipids, in the outer leaflet of the lipid bilayer of the plasma membrane. This specialized lipid composition creates membrane regions that have greater order and less fluidity than more disordered portions with less densely packed phospholipids (Pike 2003). Membrane/lipid rafts are found in virtually every eukaryotic cell, but cannot be readily visualized by either light or electron microscopy. Accordingly, some believe their existence is more operational than anatomic (Carver and Schnitzer 2003; Munro 2003). A recent, consensus definition replaced the name "lipid rafts" with "membrane rafts" and defined them as "small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes," noting that caveolae are a subset of membrane rafts (Pike 2006). Precise information about the biology of membrane rafts (the term that we shall use throughout the remainder of this review) remains limited, in part because of the limitations of techniques available for their study (Helms and Zurzolo 2004; Jacobson et al. 2007).

A challenge in defining the biological role of membrane rafts derives from the lack of universally accepted methods for their isolation and, as a result, controversies with respect to their expression and properties (Carver and Schnitzer 2003; Jacobson et al. 2007; Lucero and Robbins 2004; Munro 2003; Pike 2003, 2004). Nevertheless, substantial data support the existence of membrane rafts and have contributed to important revisions in ideas regarding the organization of plasma membrane components. Older models of membrane organization hypothesized a lipid "sea" with protein "islands" (e.g., the fluid mosaic model, Singer and Nicolson 1972) and that required the collision of protein components ("collision coupling," Bergman and Hechter 1978) to facilitate biochemical reactions, including signal transduction events. Such notions have been modified to accommodate findings that imply an organization of the plasma membrane with co-localization of signaling components (Marguet et al. 2006). Co-localization in lipid microdomains helps explain the efficiency of certain biochemical events (e.g., signal transduction by GPCR) that occur in the plasma membrane environment and that might otherwise be constrained by both the low concentration and relative inaccessibility of reactants.

Unlike the virtually ubiquitous expression of membrane rafts in eukaryotic cells, many cell types (with certain exceptions, e.g., erythrocytes, lymphocytes and neurons) also express caveolae. Caveolae were first identified by electron microscopy as ~100-nm invaginations of the plasma membrane (Palade 1953; Yamada 1955). Caveolae have a lipid composition similar to that of membrane rafts, but in addition, caveolae possess other proteins, including an organelle-specific, structural protein, caveolin (Kurzchalia et al. 1992; Rothberg et al. 1992) and more recently identified, cavin (Hill et al. 2008; Liu and Pilch 2008). As a molecular tag for caveolae, caveolin facilitates biochemical, cell and molecular biological and pharmacological analyses of caveolar microdomains, as well as contributing to the functions of caveolae

(Cohen et al. 2004; Liu et al. 2002; Morris et al. 2004; Ostrom and Insel 2004; van Deurs et al. 2003). Other proteins may also be uniquely expressed in caveolae. One example is flotillins/reggies, but since they are also found in cells that lack caveolae and in non-caveolar membranes, flotillins/reggies cannot be considered "caveolae-specific" proteins (Lucero and Robbins 2004). The three known caveolins, caveolin-1, -2 and -3, have a similar overall structure, but differ in primary sequence and tissue expression (van Deurs et al. 2003). An important feature of caveolin-3 is its unique expression in myocytes, in particular skeletal and cardiac myocytes. A large number of studies that have been published regarding caveolins involve the use of biochemical and cell biological approaches, or in recent years, analyses of mice in which each caveolin has been knocked out; results of such studies indicate that the three caveolins are non-identical in their ability to regulate enzymatic and other functional activities (Cohen et al. 2004; Insel and Patel 2007; van Deurs et al. 2003).

3 Determinants of Membrane Raft/Caveolae Localization

In spite of the localization of certain GPCR and post-receptor signaling components in rafts or caveolae (as we shall discuss in more detail subsequently), the precise determinants of this localization are not known. Certain GPCR and post-receptor signaling components show cell-selective patterns of localization in those microdomains, but no generally accepted explanation for such patterns is as yet available. Possible explanations for these patterns include:

Protein–protein interaction: Interactions of particular proteins might be favored because of charge, size and/or steric factors, but why such factors should differ in a cell-type-selective manner is not clear. For GPCR, the ability to form oligomers with different composition may contribute to such localization (Nichols 2003). It has been proposed that the localization of receptor tyrosine kinases (RTK) in membrane microdomains is attributable to protein–protein binding by sequences in the extracellular domain of the receptors; for the EGFR a 60-amino-acid region mediates targeting to rafts/caveolae (Pike 2005; Yamabhai and Anderson 2002).

Lipid–protein interaction: Lipid composition is important for rafts and caveolae with differences among different cell types or in the nature of the lipids in different portions of the plasma membrane (Park et al. 2004; Rothberg et al. 1992). Although such differences might contribute to changes in localization of proteins during states of altered lipid composition, or perhaps to cell-specific patterns of localization, direct evidence for this idea has not been provided. Lipid modification of proteins, perhaps most importantly palmitoylation and myristoylation, contribute to the localization of G-protein-signaling components in raft/caveolae domains (Ratajczak et al. 2003; Razzaq et al. 2004; Resh 2006; Rodgers et al. 2005).

Caveolin–associated proteins: Caveolin-associated proteins might also contribute to differences in the localization of signaling molecules. The caveolin scaffolding domain (CSD), a hydrophobic region in the cytoplasmic amino terminal tail that

interacts with protein "partners" through hydrophobic interactions, has been proposed as a – or perhaps the – critical region by which signaling proteins interact with caveolins (Becher and McIlhinney 2005; Chini and Parenti 2004), but it is difficult to fathom how such an ~20-amino-acid domain accommodates such a large number and array of proteins (see below). Are there other regions on caveolins that bind signaling proteins or is there a supramolecular assembly, whereby multiple signaling proteins create a "caveolin signaling particle," akin to protein complexes involved in other cellular events (transcription, translation, secretion, etc.)? Use of proteomic methods to analyze caveolin-bound proteins in cells treated under various experimental conditions should prove useful in defining the existence (or not) of "caveolin signaling particles." Proteomic methods have shown that a large number of proteins localize to caveolae (Banfi et al. 2006; Durr et al. 2004; Foster et al. 2003; McMahon et al. 2006; Sprenger and Horrevoets 2007), either via their interaction with the CSD or by localization in the caveolar lipid microenvironment. Proteomic approaches should prove useful in revealing the full range of protein partners in caveolae, as well as changes in the amount and nature of these partners that occur in physiological states, with drug treatment or in disease.

4 Membrane Rafts/Caveolae in Signal Transduction

Membrane rafts and caveolae regulate a wide variety of cellular activities, including nutrient transport, endocytosis, exocytosis, transcytosis, viral entry and budding, as well as receptor and ion channel expression, activation and desensitization (Cohen et al. 2004; Liu et al. 2002; Morris et al. 2004; Parton and Simons 2007; van Deurs et al. 2003). The term "caveosome" has been proposed to identify a caveolin-containing endosome that mediates endocytosis in a manner akin to, but independent of, the clathrin-coated pit/clathrin-coated vesicle endocytic pathway (Carver and Schnitzer 2003; Nichols 2003). Certain proteins, e.g., glycosylphosphatidylinositol (GPI)-linked proteins, preferentially localize in membrane rafts/caveolae. Important receptor-regulated events, e.g., shear-stress and calcium release, are among the cellular responses that occur in caveolae. Moreover, changes in expression or activity of rafts/caveolae have been implicated in aging and disease-associated alterations in cell function, including in signal transduction (Gratton et al. 2004; Park et al. 2004; Patel et al. 2007a,b; Ratajczak et al. 2003; Schutzer et al. 2005; Simons and Ehehalt 2002; van Deurs et al. 2003).

The raft/caveolar localization of signaling components, including GPCR pathway components, provides an arrangement whereby agonists can promote rapid, high fidelity activation of signaling pathways, especially pathways that require interaction of multiple proteins. The "caveolin signaling hypothesis" proposes that interaction of signaling proteins with the CSD (Fig. 1) regulates signal transduction events by sequestering components away from their signal transduction partners, such that in the "basal" state signaling is inhibited, but co-localization in caveolae facilitates the interaction of components upon activation of the signaling pathway (Cohen et al. 2004; Liu et al. 2002; Okamoto et al. 1998; van Deurs et al. 2003).

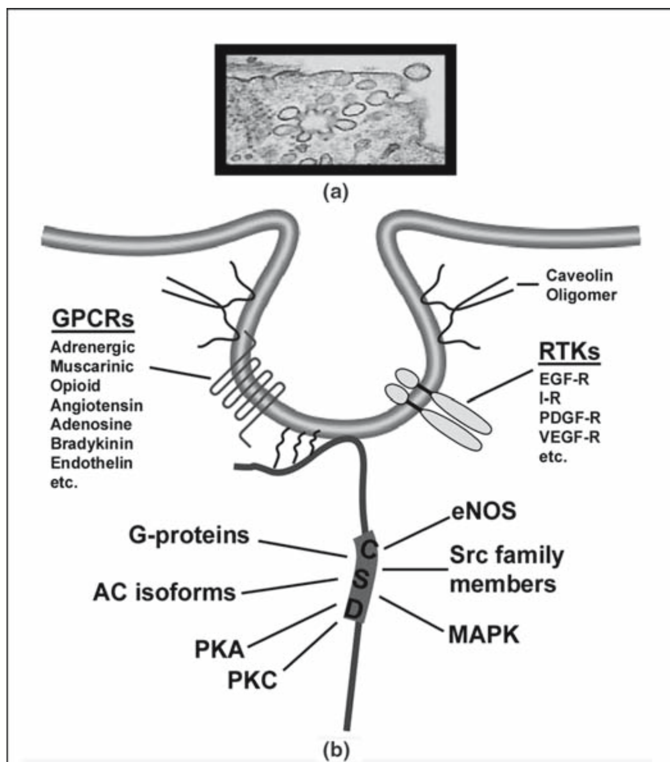

Fig. 1 **(a)** Electron micrograph showing caveolae on pulmonary artery smooth muscle cells. **(b)** Schematic depicting caveolae, caveolae resident proteins and proteins interacting with the caveolin scaffolding domain (*CSD*). G-protein coupled receptor (*GPCR*); adenylyl cyclase (*AC*); protein kinase A (*PKA*); protein kinase C (*PKC*); receptor tyrosine kinase (*RTK*); epidermal growth factor receptor (*EGF-R*); insulin receptor (*I-R*); platelet-derived growth factor receptor (*PDGF-R*); vascular endothelial growth factor receptor (*VEGF-R*); endothelial nitric oxide synthase (*eNOS*); mitogen activated protein kinase (*MAPK*)

The co-expression of plasma membrane-localized GPCR, heterotrimeric (and certain low molecular weight) G-proteins and G-protein-regulated effectors in caveolae likely contributes to efficient activation and amplification of GPCR signaling events. In the following sections, we discuss classes of signal transduction proteins that localize in rafts/caveolae.

5 GPCR/G-Proteins

A large number of GPCRs localize in membrane rafts/caveolae either before (e.g., ß-adrenergic receptor sub-types) or after treatment with agonists (e.g., certain muscarinic cholinergic and bradykinin receptors, Table 1). Agonists of certain GPCRs can promote the entry of receptors into raft/caveolar domains, while for other

Table 1 Examples of G-protein-coupled receptors that localize in lipid raft/caveolae prior to ("pre-agonist") and/or after ("post-agonist") treatment with agonists

	Pre-agonist	Post-agonist
Endothelin (ETA, ETB)	+	+
Cholecystokinin (CCK)		+
Muscarinic cholinergic	+	+
Bradykinin (B_1 and B_2)	+	+
Lysophosphatidic acid (LPA-1)	+	
Angiotensin II (AT-1)		+
β_1- and β_2- adrenergic	+	
P_2Y (P_2Y_1)	+	
Adenosine A1	+	+
Sphingosine 1-phosphate (EDG-1)	+	+
Smoothened/patched	+	
Serotonin ($5HT_{2A}$)	+	
Calcium-sensing receptor	+	
α_1-adrenergic (α_{1B})	+	
Chemokine CCR_2		+
Metabotropic glutamate (mGlu1)	+	
Gonadotrophin-releasing hormone (GnRH)		+
Oxytocin	+	
Growth-hormone releasing-hormone		+
Dopamine (D_1; D(1A))	+	+
Mu-opioid receptor	+	
Neurokinin 1	+	

GPCRs, agonists promote the exit from these domains, perhaps as a prelude to receptor internalization/endocytosis and desensitization (Cohen et al. 2004; van Deurs et al. 2003). While certain GPCR internalize in response to agonist activation following phosphorylation by G-protein-receptor kinase, interaction with ß-arrestins and internalization via clathrin-coated pits, other GPCR are phosphorylated by protein kinase A (PKA) and then internalized from caveolae (Morris et al. 2004). The precise contribution of rafts/caveolae to GPCR desensitization and internalization, in terms of the full-range of GPCR and cell types, has not yet been defined (Chini and Parenti 2004). The angiotensin-1 receptor is an example of a GPCR for which receptor-caveolin interaction appears to be important for sorting and delivery to the plasma membrane (Wyse et al. 2003). Other such examples have not been reported, although palmitoylation of a GPCR, for example, of the 5-HT1A receptor, can target it to rafts and facilitate receptor-mediated signal transduction (Renner et al. 2007).

As implied by the caveolin-signaling hypothesis, these microdomains bring "downstream" effectors and receptors together so as to facilitate receptor-, tissue- and cell-specific signal transduction (Cohen et al. 2004; Insel and Patel 2007; Ostrom and Insel 2004). The interaction of effectors with caveolins (via the CSD) or other caveolae-associated proteins are thought to be key to their targeting to caveolae. In addition, in response to stimulation, reversible palmitoylation may aid in caveolar localization of proteins by helping to regulate the movement of molecules into and out of caveolae (Lee et al. 2001; Parat and Fox 2001; Song et al. 1997).

Heterotrimeric G-proteins localize to caveolae and directly interact with caveolin (Insel et al. 2005; Ostrom and Insel 2004) in a manner that helps maintain Gα-proteins in an inactive, GDP-bound state (Couet et al. 1997; Li et al. 1995, 1996; Murthy and Makhlouf 2000). Agonist stimulation can promote exchange of GTP for GDP and Gα redistribution to the cytoplasm, an effect blocked by the CSD (Murthy and Makhlouf 2000), suggesting that binding to the CSD regulates G-protein function. Localization of Gα-proteins in caveolae may facilitate coupling to particular signaling pathways (Bhatnagar et al. 2004; Head et al. 2005; Iiri et al. 1996; Li et al. 1995; Oh and Schnitzer 2001). Certain heterotrimeric G-protein subunits, such as Gαs, show a diffuse distribution on sucrose density gradients that are used to isolate caveolin-rich fractions; the Gα proteins can be detected in both caveolin-enriched (buoyant) and non-buoyant fractions (Cho et al. 2003; Cohen et al. 2004; Ostrom and Insel 2006). Heterotrimeric G-protein subunits can differentially localize to caveolae vs. membrane rafts (Head et al. 2005; Li et al. 1995; Oh and Schnitzer 2001). Palmitoylation enhances targeting of Gα subunits to caveolae (Song et al. 1997); Gβγ can increase the palmitoylation of Gα, suggesting that this covalent modification may enhance interaction of caveolae and Gα (Iiri et al. 1996). Localization of Gα subunits in caveolae and interaction with the CSD is thus likely important for heterotrimeric G-protein signaling (Oh and Schnitzer 2001).

Certain small GTP-binding proteins (e.g., H-ras) also reside in caveolae, perhaps via palmitoylation of the C-terminal hypervariable region (CAAX motif) (Prior et al. 2001; Roy et al. 2005) and/or via interaction with the CSD (Li et al. 1995; Song et al. 1996). Activating mutations in H-Ras (e.g., G12V) prevent its interaction with caveolin and maintain an active conformation of the protein; such mutations are found in human cancers, suggesting that agents able to mimic this inhibitory action of caveolin have therapeutic potential in Ras-expressing cancers (Cavallo-Medved et al. 2005; Engelman et al. 1998).

5.1 Specific Signal Transduction Pathways

5.1.1 NO Signaling

Binding of eNOS to caveolin and the inhibition of eNOS activity by this interaction is the most well-studied example of a caveolin-signaling partner interaction (Feron et al. 1998; Feron and Balligand 2006; Venema et al. 1997). Regions on caveolins-1 and -3 that correspond to the CSD can suppress eNOS activity (Garcia-Cardena et al. 1997). Loss of caveolin expression increases eNOS activity (Razani et al. 2001), which implies that caveolin regulates this activity under basal conditions and that disruption of the binding of eNOS to caveolin would increase NO production. The interaction of eNOS and caveolin thus appears to have two consequences: direct interaction with caveolin basally to maintain an inactive enzyme and enrichment of eNOS in caveolae to provide for a rapid, high-fidelity response upon stimulation (Sbaa et al. 2005).

5.1.2 cAMP Pathway

Adenylyl cyclase (AC), cyclic nucleotide phosphodiesterase and PKA, all of which are important signaling components in the cAMP pathway, are among a number of downstream effectors of GPCR signaling that can target to rafts/caveoale and modulate cellular function. Such organization can compartmentalize cAMP signals by creating cellular microdomains that have greater (or lesser) levels of cAMP (see below). As with eNOS, the CSD region of caveolin, in particular of caveolin-1 and -3, can inhibit mediators involved in cAMP signaling; moreover, increases in cAMP can down-regulate the expression of caveolin (Yamamoto et al. 1999).

Willoughby and Cooper (2007) have recently reviewed the regulation of AC in specific microdomains. Caveolin (in particular the CSD region of caveolins-1 and -3) can inhibit activity of AC isoforms; this inhibition is AC isoform-specific, for example, with greater inhibition of AC types 5 and 3 (Toya et al. 1998; Willoughby and Cooper 2007). The cytosolic domains of the Ca^{2+}-sensitive ACs (AC5 and AC8) are particularly important for targeting the cyclases to rafts (Crossthwaite et al. 2005). Certain isoforms of AC and regulators of AC (e.g., GPCR, G-proteins, eNOS, etc.) are enriched in caveolae, helping to modulate cAMP generation in this membrane domain, especially because various GPCRs couple with different efficiencies to AC in caveolae (Ostrom et al. 2001, 2004). Superactivation of AC, which can occur with opiate treatment of cells, may depend on the long-term stimulation of opioid receptors that localize to rafts/caveolae along with AC (Zhao et al. 2006). Disruption of membrane rafts/caveolae using methyl-β-cyclodextrin (MβCD, a cholesterol depleting agent, Fig. 2), colchicine (which disrupts cellular microtubules) or cytochalasin D (which disrupts microfilaments) relocates AC to non-buoyant membranes in parallel with an

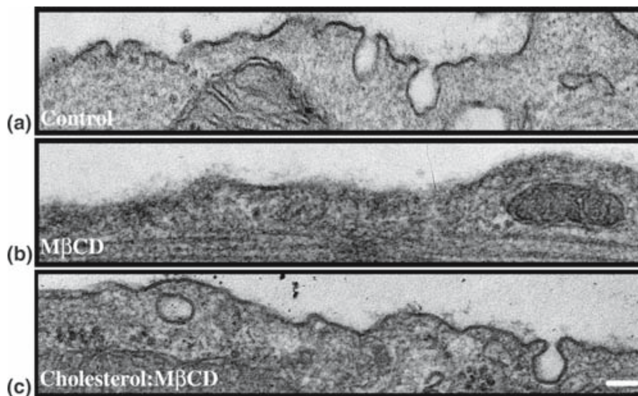

Fig. 2 Electron micrographs of adult rat cardiac myocytes showing effects of the cholesterol-depleting agent methyl-beta-cyclodextrin (*MβCD*). **(a)** control; **(b)** MβCD; **(c)** cholesterol-loaded MβCD. MβCD is able to deplete membrane cholesterol and thereby disrupt caveolae. Cholesterol-loaded MβCD serves as a control for effects of MβCD that are independent of cholesterol removal

enhancement in β-adrenergic agonist-stimulated cAMP generation; such results imply that interaction of cytoskeletal components with membrane rafts/caveolae regulates activation of AC by GPCR (Head et al. 2006). Because stimulation of cAMP synthesis can reduce the expression of caveolin mRNA and protein (Yamamoto et al. 1999), a feedback loop may exist between cAMP levels and caveolin expression. The findings with the cytoskeletal inhibitors, some of which are used therapeutically (e.g., colchicine for gout and vinblastine for cancer chemotherapy), suggest that regulation of membrane rafts/caveolae contributes to the therapeutic utility of such agents.

The enzymes that hydrolyze cAMP and cGMP, cyclic nucleotide phosphodiesterases (PDEs), help compartmentalize cyclic nucleotide signals in cells by acting as barriers that establish cyclic nucleotide gradients, thereby influencing post-cAMP responses (Baillie et al. 2005; Houslay et al. 2007). PDEs can link to scaffolding complexes in subcellular compartments, in part due to PDE isoform-unique N-terminal regions (Lynch et al. 2006) (e.g., PDE4), and can associate with membrane rafts before or after agonist stimulation (Abrahamsen et al. 2004). At least one PDE isoform, PDE3B, localizes to caveolae and co-immunoprecipitates with caveolin-1 (Nilsson et al. 2006); it is unknown whether other PDEs also localize to caveolae.

The targeting of PDEs to membrane microdomains in the basal state seems counterintuitive since such localization would be predicted to enhance cyclic nucleotide hydrolysis, thereby inhibiting signal transduction. However, because PDE3B expression is reduced in adipocytes from caveolin-1-knockout mice or when caveolin-1 expression is lowered by MβCD, interaction with caveolin-1 may stabilize or activate PDE3B. Such findings contrast with evidence that caveolin-1 associates with PDE5 (a cGMP-specific PDE) in pulmonary artery smooth muscle cells in which overexpression of caveolin-1 decreases, while siRNA-promoted decrease in caveolin-1 increases PDE5 expression (Murray et al. 2006). This inverse relationship may occur by indirect mechanisms, perhaps by adapter proteins, as typified by the interaction of PDE4A4 with the SH3-domain of Src protein tyrosine kinases (McPhee et al. 1999).

Both the CSD and C-terminus of caveolin-1 can interact with PKA and inhibit its activity (Razani and Lisanti 2001; Razani et al. 1999). Caveolae-localized PKA may contribute to its regulation of proteins, such as ATP-dependent K^+ channels in smooth muscle cells (Sampson et al. 2004) and eNOS in endothelial cells (Heijnen et al. 2004). The interaction of caveolin-1, PKA and eNOS via the CSD may organize these entities into a multi-component complex in caveolin oligomers that produces inhibited enzyme activity (Levin et al. 2007). De-oligomerization of caveolins and release of the "caged" enzymes would then lead to enzyme activation.

The results showing that both cAMP generation and hydrolysis can occur in near-membrane environments implies that cells possess different "pools" of cAMP and perhaps of proteins that contribute to cAMP action (e.g., PKA, Epac, cyclic nucleotide-gated channels, A-kinase-anchoring proteins; Fischmeister 2006; Lynch et al. 2006; Malbon et al. 2004; Razani and Lisanti 2001; Younes et al. 2008). Cells may thus have local pools of cAMP levels and response that depend on membrane rafts and caveolae.

5.1.3 PKC and PI3K/PKB Pathways

Caveolar localization and interaction of caveolin with other protein kinases, such as PKC and PI3K/PKB, may regulate the phosphorylation of other caveolar resident proteins and as a consequence modulate cellular processes.

The PKC family translocates to cellular compartments in response to stimuli (Peart and Headrick 2007); ceramide can recruit and activate caveolar PKC (Fox et al. 2007). In turn, isoforms of PKC can target to caveolae to enhance the regulation of caveolar-localized proteins (Rybin et al. 1999). Caveolae can also influence the inactivation of PKCα signaling via facilitation of endosomal delivery (Prevostel et al. 2000).

Phosphatidylinositol-3-kinase/protein kinase B (PI3K/PKB, Akt) interacts with caveolin and can regulate a number of cellular events, including cell survival. For example, caveolin maintains Akt in an activated state in prostate cancer (Zhuang et al. 2002). Phosphorylation of Akt is thought to occur via interaction of the CSD with, and inhibition of, protein phosphatase 1 and 2A (Li et al. 2003). Caveolin expression can enhance cell death via activation of Akt, for example, sensitizing HepG2 cells to killing by TNF-α (Ono et al. 2004) or arsenite (Shack et al. 2003). Other data indicate that there is a correlation between increased expression of caveolin-1 and Akt activity in colorectal cancer (Kim et al. 2006). In skeletal muscle cells, cell survival can be regulated by a balance between caveolin-3 expression and activation of the PI3K/Akt pathway (Smythe and Rando 2006). Interaction of Akt may be recruited by PI3K, which binds to caveolin (Krajewska and Maslowska 2004). Caveolin and Akt can also contribute to insulin signaling in muscle cells (Ha and Pak 2005), an effect that may be altered by insulin resistance (Cohen et al. 2003b; Ha and Pak 2005; Oshikawa et al. 2004). Caveolin, in particular its phosphorylated form, is involved in EGF receptor transactivation, which is dependent on Src and Akt phosphorylation and for which caveolin helps integrate the signaling cascade (Zhang et al. 2007). Such integration is also important for cyclical stretch of smooth muscle, in which PI3K/Akt activation depends on caveolin expression (Sedding et al. 2005).

5.1.4 Receptor Tyrosine Kinase (RTK) Pathway

In addition to GPCR a number of receptor tyrosine kinases (RTK) localize to caveolae (e.g., receptors for EGF, NGF, IGF, PGDF and insulin; Couet et al. 1997b; Huang et al. 1999; Pike 2005). Considerable attention has been devoted to studies of GPCR-RTK "cross talk": activation of GPCR that in turn activates RTK through mechanisms that are not fully defined, but appear to include activation of metalloproteinases that cleave membrane-bound ligands, such as heparin-binding EGF-like growth factor (HB-EGF), which then interacts with EGF receptors. Conceivably such events may occur in rafts/caveolae, but limited data support this conjecture.

Of note, though, various MAPK are downstream effectors of RTK and certain GPCR and regulation of MAPK can occur in rafts/caveolae; activation of MAPK contributes to numerous cellular processes, including shear, mechanical and

osmotic stress (Boyd et al. 2003; Volonte et al. 2001), lung fibrosis (Wang et al. 2006), cellular proliferation (Gosens et al. 2006) and angiogenesis (Sonveaux et al. 2004). Caveolae localize the p42/44 MAP kinases, and caveolin-1 can inhibit such kinases (Engelman et al. 1998; Galbiati et al. 1998). Consistent with this action, caveolin-1 and caveolin-3 KO mice have increased activation of p42/44 ERK (Cohen et al. 2003a; Woodman et al. 2002). One can observe differential activation of p42/44 ERK and p38 MAPK in caveolar fractions, implying differences in the regulation of these kinases by caveolins (Ballard-Croft et al. 2006). In addition, up-regulation of caveolin-1, secondary to the activation of p38 MAPK, contributes to the anti-proliferative properties of carbon monoxide (Kim et al. 2005).

GPCR and TKR also interact with non-receptor tyrosine kinases, in particular Src family members c-Src, Fyn and Lyn, which are enriched in caveolae. Caveolar localization of such kinases occurs via N-terminal myristoylation and formation of complexes with caveolin; Cys^{156}-palmitorylation of caveolin-1 regulates its interaction with c-Src (Lee et al. 2001; Song et al. 1997). The CSD of caveolin-1 suppresses the activity of c-Src and Fyn (Li et al. 1996). Tyrosine phosphorylation of caveolin-1 (Tyr^{14}) and caveolin-2 (Tyr^{19}) facilitates the recruitment of SH2 domain-containing proteins, such as Grb7 or matrix metalloproteinases (Lee et al. 2000; Li et al. 1996b). Phosphocaveolin-1 localizes to focal adhesions, which are important sites of tyrosine kinase signaling that mediate cytoskeleton rearrangement (Del Pozo and Schwartz 2007; Lee et al. 2000; Swaney et al. 2006). Phosphorylation of caveolin by Src can alter cellular shape, lead to muscle degeneration and inflammatory gene expression and has been implicated in cancer and ischemic injury (Patel et al. 2007a; Smythe et al. 2003; Volonte et al. 2001). Caveolin-1 contributes to the association of integrins with Src kinases, which can activate ERK and promote cell cycle progression (Del Pozo and Schwartz 2007; Echarri and Del Pozo 2006).

6 Questions to Consider for Future Investigations

This review provides examples of the roles of membrane rafts and caveolae in the organization and regulation of signal transduction, especially by GPCR. In spite of considerable data that document such roles, a number of unanswered questions remain. These include:

- What is the full range of signaling proteins and signaling cascades that are organized in caveolae and membrane rafts, and do physiological states, drugs or disease alter the identity of these raft/caveolae "partners"?
- What are the mechanisms/interactions that determine co-localization and functional regulation of signaling proteins in those microdomains?
- Is the CSD the only means by which signaling proteins associate with caveolae, and if so, how does this association occur for so many signaling and other proteins?
- Is there a signaling role for cellular pools of caveolins that exist outside of caveolae (as reviewed in Head and Insel 2007)?

– Does the compartmentation of GPCR and post-receptor components in caveolae/rafts contribute to disease manifestations and therapeutic responses in disease?

Investigations that attempt to answer such questions are predicted to reveal new insights into the physiological, pathophysiological and pharmacological role of rafts and caveolae. The answers will likely also reveal insights regarding compartmentation of signal transduction, in particular by GPCR. In addition, the findings may yield novel targets for drug action, such as sites that contribute to the protein–protein and protein–lipid interactions that appear to be critical for membrane raft and caveolar interaction with protein partners.

References

Abrahamsen H, Baillie G, Ngai J et al (2004) TCR- and CD28-mediated recruitment of phosphodiesterase 4 to lipid rafts potentiates TCR signaling. J Immunol 173:4847–4858

Baillie GS, Scott JD, Houslay MD (2005) Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract. FEBS Lett 579:3264–3270

Ballard-Croft C, Locklar AC, Kristo G et al (2006) Regional myocardial ischemia-induced activation of MAPKs is associated with subcellular redistribution of caveolin and cholesterol. Am J Physiol Heart Circ Physiol 291:H658–H667

Banfi C, Brioschi M, Wait R et al (2006) Proteomic analysis of membrane microdomains derived from both failing and non-failing human hearts. Proteomics 6:1976–1988

Becher A, McIlhinney RA (2005) Consequences of lipid raft association on G-protein-coupled receptor function. Biochem Soc Symp 72:151–164

Berditchevski F, Odintsova E (2007) Tetraspanins as regulators of protein trafficking. Traffic 8:89–96

Bergman RN, Hechter O (1978) Neurohypophyseal hormone-responsive renal adenylate cyclase. IV. A random-hit matrix model for coupline in a hormone-sensitive adenylate cyclase system. J Biol Chem 253:3238–3250

Bhatnagar A, Sheffler DJ, Kroeze WK et al (2004) Caveolin-1 interacts with 5-HT2A serotonin receptors and profoundly modulates the signaling of selected Galphaq-coupled protein receptors. J Biol Chem 279:34614–34623

Boyd NL, Park H, Yi H et al (2003) Chronic shear induces caveolae formation and alters ERK and Akt responses in endothelial cells. Am J Physiol Heart Circ Physiol 285:H1113–H1122

Carver LA, Schnitzer JE (2003) Caveolae: mining little caves for new cancer targets. Nat Rev Cancer 3:571–581

Cavallo-Medved D, Mai J, Dosescu J et al (2005) Caveolin-1 mediates the expression and localization of cathepsin B, pro-urokinase plasminogen activator and their cell-surface receptors in human colorectal carcinoma cells. J Cell Sci 118:1493–1503

Chini B, Parenti M (2004) G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there? J Mol Endocrinol 32:325–338

Cho KA, Ryu SJ, Park JS et al (2003) Senescent phenotype can be reversed by reduction of caveolin status. J Biol Chem 278:27789–27795

Cohen AW, Park DS, Woodman SE et al (2003a) Caveolin-1 null mice develop cardiac hypertrophy with hyperactivation of p42/44 MAP kinase in cardiac fibroblasts. Am J Physiol Cell Physiol 284:C457–C474

Cohen AW, Razani B, Wang XB et al (2003b) Caveolin-1-deficient mice show insulin resistance and defective insulin receptor protein expression in adipose tissue. Am J Physiol Cell Physiol 285:C222–C235

Cohen AW, Hnasko R, Schubert W et al (2004) Role of caveolae and caveolins in health and disease. Physiol Rev 84:1341–1379

Couet J, Li S, Okamoto T et al (1997a) Identification of peptide and protein ligands for the caveolin-scaffolding domain. Implications for the interaction of caveolin with caveolae-associated proteins. J Biol Chem 272:6525–6533
Couet J, Sargiacomo M, Lisanti MP (1997b) Interaction of a receptor tyrosine kinase, EGF-R, with caveolins. Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities. J Biol Chem 272:30429–30438
Crossthwaite AJ, Seebacher T, Masada N et al (2005) The cytosolic domains of Ca2+-sensitive adenylyl cyclases dictate their targeting to plasma membrane lipid rafts. J Biol Chem 280:6380–6391
Del Pozo MA, Schwartz MA (2007) Rac, membrane heterogeneity, caveolin and regulation of growth by integrins. Trends Cell Biol 17:246–250
Durr E, Yu J, Krasinska KM et al (2004) Direct proteomic mapping of the lung microvascular endothelial cell surface in vivo and in cell culture. Nat Biotechnol 22:985–992
Echarri A, Del Pozo MA (2006) Caveolae internalization regulates integrin-dependent signaling pathways. Cell Cycle 5:2179–2182
Engelman JA, Chu C, Lin A et al (1998) Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade in vivo. A role for the caveolin-scaffolding domain. FEBS Lett 428:205–211
Feron O, Balligand JL (2006) Caveolins and the regulation of endothelial nitric oxide synthase in the heart. Cardiovasc Res 69:788–797
Feron O, Dessy C, Opel DJ et al (1998) Modulation of the endothelial nitric-oxide synthase-caveolin interaction in cardiac myocytes. Implications for the autonomic regulation of heart rate. J Biol Chem 273:30249–30254
Fischmeister R (2006) Is cAMP good or bad?: Depends on where it's made. Circ Res 98:582–584
Foster LJ, De Hoog CL, Mann M (2003) Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. Proc Natl Acad Sci USA 100:5813–5818
Fox TE, Houck KL, O'Neill SM et al (2007) Ceramide recruits and activates protein kinase C zeta (PKC zeta) within structured membrane microdomains. J Biol Chem 282:12450–12457
Galbiati F, Volonte D, Engelman JA et al (1998) Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade. EMBO J 17:6633–6648
Garcia-Cardena G, Martasek P, Masters BS et al (1997) Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. J Biol Chem 272:25437–25440
Gosens R, Stelmack GL, Dueck G et al (2006) Role of caveolin-1 in p42/p44 MAP kinase activation and proliferation of human airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 291:L523–L534
Gratton JP, Bernatchez P, Sessa WC (2004) Caveolae and caveolins in the cardiovascular system. Circ Res 94:1408–1417
Ha H, Pak Y (2005) Modulation of the caveolin-3 and Akt status in caveolae by insulin resistance in H9c2 cardiomyoblasts. Exp Mol Med 37:169–178
Head BP, Insel PA (2007) Do caveolins regulate cells by actions outside of caveolae? Trends Cell Biol 17:51–57
Head BP, Patel HH, Roth DM et al (2005) G-protein-coupled receptor signaling components localize in both sarcolemmal and intracellular caveolin-3-associated microdomains in adult cardiac myocytes. J Biol Chem 280:31036–31044
Head BP, Patel HH, Roth DM et al (2006) Microtubules and actin microfilaments regulate lipid raft/caveolae localization of adenylyl cyclase signaling components. J Biol Chem 281:26391–26399
Heijnen HF, Waaijenborg S, Crapo JD et al (2004) Colocalization of eNOS and the catalytic subunit of PKA in endothelial cell junctions: a clue for regulated NO production. J Histochem Cytochem 52:1277–1285
Helms JB, Zurzolo C (2004) Lipids as targeting signals: lipid rafts and intracellular trafficking. Traffic 5:247–254

Hill MM, Bastiani M, Luetterforst R et al (2008) PTRF-Cavin, a conserved cytoplasmic protein required for caveola formation and function. Cell 132:113–124
Houslay MD, Baillie GS, Maurice DH (2007) cAMP-Specific phosphodiesterase-4 enzymes in the cardiovascular system: a molecular toolbox for generating compartmentalized cAMP signaling. Circ Res 100:950–966
Huang CS, Zhou J, Feng AK et al (1999) Nerve growth factor signaling in caveolae-like domains at the plasma membrane. J Biol Chem 274:36707–36714
Iiri T, Backlund PS Jr, Jones TL et al (1996) Reciprocal regulation of Gs alpha by palmitate and the beta gamma subunit. Proc Natl Acad Sci USA 93:14592–14597
Insel PA, Patel HH (2007) Do studies in caveolin-knockouts teach us about physiology and pharmacology or instead, the ways mice compensate for ‘lost proteins’? Br J Pharmacol 150:251–254
Insel PA, Head BP, Patel HH et al (2005) Compartmentation of G-protein-coupled receptors and their signalling components in lipid rafts and caveolae. Biochem Soc Trans 33:1131–1134
Jacobson K, Mouritsen OG, Anderson RG (2007) Lipid rafts: at a crossroad between cell biology and physics. Nat Cell Biol 9:7–14
Kim HP, Wang X, Nakao A et al (2005) Caveolin-1 expression by means of p38beta mitogen-activated protein kinase mediates the antiproliferative effect of carbon monoxide. Proc Natl Acad Sci USA 102:11319–11324
Kim HA, Kim KH, Lee RA (2006) Expression of caveolin-1 is correlated with Akt-1 in colorectal cancer tissues. Exp Mol Pathol 80:165–170
Krajewska WM, Maslowska I (2004) Caveolins: structure and function in signal transduction. Cell Mol Biol Lett 9:195–220
Kurzchalia TV, Dupree P, Parton RG et al (1992) VIP21, a 21-kD membrane protein is an integral component of trans-Golgi-network-derived transport vesicles. J Cell Biol 118:1003–1014
Lee H, Volonte D, Galbiati F et al (2000) Constitutive and growth factor-regulated phosphorylation of caveolin-1 occurs at the same site (Tyr-14) in vivo: identification of a c-Src/Cav-1/Grb7 signaling cassette. Mol Endocrinol 14:1750–1775
Lee H, Woodman SE, Engelman JA et al (2001) Palmitoylation of caveolin-1 at a single site (Cys-156) controls its coupling to the c-Src tyrosine kinase: targeting of dually acylated molecules (GPI-linked, transmembrane, or cytoplasmic) to caveolae effectively uncouples c-Src and caveolin-1 (TYR-14). J Biol Chem 276:35150–35158
Lefkowitz RJ, Shenoy SK (2005) Transduction of receptor signals by beta-arrestins. Science 308:512–517
Levin AM, Murase K, Jackson PJ et al (2007) Double barrel shotgun scanning of the caveolin-1 scaffolding domain. ACS Chem Biol 2:493–500
Li S, Okamoto T, Chun M et al (1995) Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. J Biol Chem 270:15693–15701
Li S, Couet J, Lisanti MP (1996a) Src tyrosine kinases, Galpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. J Biol Chem 271:29182–29190
Li S, Seitz R, Lisanti MP (1996b) Phosphorylation of caveolin by src tyrosine kinases. The alpha-isoform of caveolin is selectively phosphorylated by v-Src in vivo. J Biol Chem 271:3863–3868
Li L, Ren CH, Tahir SA et al (2003) Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. Mol Cell Biol 23:9389–9404
Liu L, Pilch PF (2008) A critical role of cavin (polymerase I and transcript release factor) in caveolae formation and organization. J Biol Chem 283:4314–4322.
Liu P, Rudick M, Anderson RG (2002) Multiple functions of caveolin-1. J Biol Chem 277:41295–41298
Lucero HA, Robbins PW (2004) Lipid rafts-protein association and the regulation of protein activity. Arch Biochem Biophys 426:208–224
Luttrell LM, Lefkowitz RJ (2002) The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 115:455–465

Lynch MJ, Hill EV, Houslay MD (2006) Intracellular targeting of phosphodiesterase-4 underpins compartmentalized cAMP signaling. Curr Top Dev Biol 75:225–259
Malbon CC, Tao J, Shumay E et al (2004) AKAP (A-kinase anchoring protein) domains: beads of structure-function on the necklace of G-protein signalling. Biochem Soc Trans 32:861–864
Marguet D, Lenne PF, Rigneault H et al (2006) Dynamics in the plasma membrane: how to combine fluidity and order. EMBO J 25:3446–3457
McMahon KA, Zhu M, Kwon SW et al (2006) Detergent-free caveolae proteome suggests an interaction with ER and mitochondria. Proteomics 6:143–152
McPhee I, Yarwood SJ, Scotland G et al (1999) Association with the SRC family tyrosyl kinase LYN triggers a conformational change in the catalytic region of human cAMP-specific phosphodiesterase HSPDE4A4B. Consequences for rolipram inhibition. J Biol Chem 274:11796–11810
Morris R, Cox H, Mombelli E et al (2004) Rafts, little caves and large potholes: how lipid structure interacts with membrane proteins to create functionally diverse membrane environments. Subcell Biochem 37:35–118
Munro S (2003) Lipid rafts: elusive or illusive? Cell 115:377–388
Murray F, Patel H, Suda R et al (2006) Caveolar localization and caveolin-1 regulation of PDE5 in human pulmonary artery smooth muscle cells. FASEB J. 20:A543
Murthy KS, Makhlouf GM (2000) Heterologous desensitization mediated by G protein-specific binding to caveolin. J Biol Chem 275:30211–30219
Nichols B (2003) Caveosomes and endocytosis of lipid rafts. J Cell Sci 116:4707–4714
Nilsson R, Ahmad F, Sward K et al (2006) Plasma membrane cyclic nucleotide phosphodiesterase 3B (PDE3B) is associated with caveolae in primary adipocytes. Cell Signal 18:1713–1721
Oh P, Schnitzer JE (2001) Segregation of heterotrimeric G proteins in cell surface microdomains. G(q) binds caveolin to concentrate in caveolae, whereas G(i) and G(s) target lipid rafts by default. Mol Biol Cell 12:685–698
Okamoto T, Schlegel A, Scherer PE et al (1998) Caveolins, a family of scaffolding proteins for organizing "preassembled signaling complexes" at the plasma membrane. J Biol Chem 273:5419–5422
Ono K, Iwanaga Y, Hirayama M et al (2004) Contribution of caveolin-1 alpha and Akt to TNF-alpha-induced cell death. Am J Physiol Lung Cell Mol Physiol 287:L201–L209
Oshikawa J, Otsu K, Toya Y et al (2004) Insulin resistance in skeletal muscles of caveolin-3-null mice. Proc Natl Acad Sci USA 101:12670–12675
Ostrom RS, Insel PA (2004) The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. Br J Pharmacol 143:235–245
Ostrom RS, Insel PA (2006) Methods for the study of signaling molecules in membrane lipid rafts and caveolae. Methods Mol Biol 332:181–191
Ostrom RS, Post SR, Insel PA (2000) Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving G(s). J Pharmacol Exp Ther 294:407–412
Ostrom RS, Gregorian C, Drenan RM et al (2001) Receptor number and caveolar co-localization determine receptor coupling efficiency to adenylyl cyclase. J Biol Chem 276:42063–42069
Ostrom RS, Bundey RA, Insel PA (2004) Nitric oxide inhibition of adenylyl cyclase type 6 activity is dependent upon lipid rafts and caveolin signaling complexes. J Biol Chem 279:19846–19853
Palade G (1953) Fine structure of blood capilaries. J Appl Physiol 24:1424–1436
Parat MO, Fox PL (2001) Palmitoylation of caveolin-1 in endothelial cells is post-translational but irreversible. J Biol Chem 276:15776–15782
Park SC, Cho KA, Jang IS et al (2004) Functional efficiency of the senescent cells: replace or restore? Ann N Y Acad Sci 1019:309–316
Parton RG, Simons K (2007) The multiple faces of caveolae. Nat Rev Mol Cell Biol 8:185–194
Patel HH, Tsutsumi YM, Head BP et al (2007a) Mechanisms of cardiac protection from ischemia/reperfusion injury: a role for caveolae and caveolin-1. FASEB J 21:1565–1574
Patel HH, Zhang S, Murray F et al (2007b) Increased smooth muscle cell expression of caveolin-1 and caveolae contribute to the pathophysiology of idiopathic pulmonary arterial hypertension. FASEB J 21:2970–2979

Peart JN, Headrick JP (2007) Adenosinergic cardioprotection: multiple receptors, multiple pathways. Pharmacol Ther 114:208–221
Pike LJ (2003) Lipid rafts: bringing order to chaos. J Lipid Res 44:655–667
Pike LJ (2004) Lipid rafts: heterogeneity on the high seas. Biochem J 378:281–292
Pike LJ (2005) Growth factor receptors, lipid rafts and caveolae: an evolving story. Biochim Biophys Acta 1746:260–273
Pike LJ (2006) Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. J Lipid Res 47:1597–1598
Prevostel C, Alice V, Joubert D et al (2000) Protein kinase C(alpha) actively downregulates through caveolae-dependent traffic to an endosomal compartment. J Cell Sci 113(Pt 14):2575–2584
Prior IA, Harding A, Yan J et al (2001) GTP-dependent segregation of H-ras from lipid rafts is required for biological activity. Nat Cell Biol 3:368–375
Ratajczak P, Damy T, Heymes C et al (2003) Caveolin-1 and -3 dissociations from caveolae to cytosol in the heart during aging and after myocardial infarction in rat. Cardiovasc Res 57:358–369
Razani B, Lisanti MP (2001) Two distinct caveolin-1 domains mediate the functional interaction of caveolin-1 with protein kinase A. Am J Physiol Cell Physiol 281:C1241–C1250
Razani B, Rubin CS, Lisanti MP (1999) Regulation of cAMP-mediated signal transduction via interaction of caveolins with the catalytic subunit of protein kinase A. J Biol Chem 274:26353–26360
Razani B, Engelman JA, Wang XB et al (2001) Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. J Biol Chem 276:38121–38138
Razzaq TM, Ozegbe P, Jury EC et al (2004) Regulation of T-cell receptor signalling by membrane microdomains. Immunology 113:413–426
Renner U, Glebov K, Lang T et al (2007) Localization of the mouse 5-hydroxytryptamine(1A) receptor in lipid microdomains depends on its palmitoylation and is involved in receptor-mediated signaling. Mol Pharmacol 72:502–513
Resh MD (2006) Palmitoylation of ligands, receptors, and intracellular signaling molecules. Sci STKE 2006:re14
Rodgers W, Farris D, Mishra S (2005) Merging complexes: properties of membrane raft assembly during lymphocyte signaling. Trends Immunol 26:97–103
Rothberg KG, Heuser JE, Donzell WC et al (1992) Caveolin, a protein component of caveolae membrane coats. Cell 68:673–682
Roy S, Plowman S, Rotblat B et al (2005) Individual palmitoyl residues serve distinct roles in H-ras trafficking, microlocalization, and signaling. Mol Cell Biol 25:6722–6733
Rybin VO, Xu X, Steinberg SF (1999) Activated protein kinase C isoforms target to cardiomyocyte caveolae: stimulation of local protein phosphorylation. Circ Res 84:980–988
Sampson LJ, Hayabuchi Y, Standen NB et al (2004) Caveolae localize protein kinase A signaling to arterial ATP-sensitive potassium channels. Circ Res 95:1012–1018
Sbaa E, Frerart F, Feron O (2005) The double regulation of endothelial nitric oxide synthase by caveolae and caveolin: a paradox solved through the study of angiogenesis. Trends Cardiovasc Med 15:157–162
Schutzer WE, Reed JF, Mader SL (2005) Decline in caveolin-1 expression and scaffolding of G protein receptor kinase-2 with age in Fischer 344 aortic vascular smooth muscle. Am J Physiol Heart Circ Physiol 288:H2457–H2464
Sedding DG, Hermsen J, Seay U et al (2005) Caveolin-1 facilitates mechanosensitive protein kinase B (Akt) signaling in vitro and in vivo. Circ Res 96:635–642
Shack S, Wang XT, Kokkonen GC et al (2003) Caveolin-induced activation of the phosphatidylinositol 3-kinase/Akt pathway increases arsenite cytotoxicity. Mol Cell Biol 23:2407–2414
Simons K, Ehehalt R (2002) Cholesterol, lipid rafts, and disease. J Clin Invest 110:597–603
Singer SJ, Nicolson GL (1972) The fluid mosaic model of the structure of cell membranes. Science 175:720–731
Smythe GM, Rando TA (2006) Altered caveolin-3 expression disrupts PI(3) kinase signaling leading to death of cultured muscle cells. Exp Cell Res 312:2816–2825

Smythe GM, Eby JC, Disatnik MH et al (2003) A caveolin-3 mutant that causes limb girdle muscular dystrophy type 1C disrupts Src localization and activity and induces apoptosis in skeletal myotubes. J Cell Sci 116:4739–4749

Song KS, Li S, Okamoto T et al (1996) Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. J Biol Chem 271:9690–9697

Song KS, Sargiacomo M, Galbiati F et al (1997) Targeting of a G alpha subunit (Gi1 alpha) and c-Src tyrosine kinase to caveolae membranes: clarifying the role of N-myristoylation. Cell Mol Biol 43:293–303

Sonveaux P, Martinive P, DeWever J et al (2004) Caveolin-1 expression is critical for vascular endothelial growth factor-induced ischemic hindlimb collateralization and nitric oxide-mediated angiogenesis. Circ Res 95:154–161

Sprenger RR, Horrevoets AJ (2007) Proteomic study of caveolae and rafts isolated from human endothelial cells. Methods Mol Biol 357:199–213

Swaney JS, Patel HH, Yokoyama U et al (2006) Focal adhesions in (myo)fibroblasts scaffold adenylyl cyclase with phosphorylated caveolin. J Biol Chem 281:17173–17179

Toya Y, Schwencke C, Couet J et al (1998) Inhibition of adenylyl cyclase by caveolin peptides. Endocrinology 139:2025–2031

van Deurs B, Roepstorff K, Hommelgaard AM et al (2003) Caveolae: anchored, multifunctional platforms in the lipid ocean. Trends Cell Biol 13:92–100

Venema VJ, Ju H, Zou R et al (1997) Interaction of neuronal nitric-oxide synthase with caveolin-3 in skeletal muscle. Identification of a novel caveolin scaffolding/inhibitory domain. J Biol Chem 272:28187–28190

Volonte D, Galbiati F, Pestell RG et al (2001) Cellular stress induces the tyrosine phosphorylation of caveolin-1 (Tyr(14)) via activation of p38 mitogen-activated protein kinase and c-Src kinase. Evidence for caveolae, the actin cytoskeleton, and focal adhesions as mechanical sensors of osmotic stress. J Biol Chem 276:8094–8103

von Zastrow M (2003) Mechanisms regulating membrane trafficking of G protein-coupled receptors in the endocytic pathway. Life Sci 74:217–224

Wang XM, Zhang Y, Kim HP et al (2006) Caveolin-1: a critical regulator of lung fibrosis in idiopathic pulmonary fibrosis. J Exp Med 203:2895–2906

Willoughby D, Cooper DM (2007) Organization and Ca2+ regulation of adenylyl cyclases in cAMP microdomains. Physiol Rev 87:965–1010

Woodman SE, Park DS, Cohen AW et al (2002) Caveolin-3 knock-out mice develop a progressive cardiomyopathy and show hyperactivation of the p42/44 MAPK cascade. J Biol Chem 277:38988–38997

Wyse BD, Prior IA, Qian H et al (2003) Caveolin interacts with the angiotensin II type 1 receptor during exocytic transport but not at the plasma membrane. J Biol Chem 278:23738–23746

Yamabhai M, Anderson RG (2002) Second cysteine-rich region of epidermal growth factor receptor contains targeting information for caveolae/rafts. J Biol Chem 277:24843–24846

Yamada E (1955) The fine structure of the gall bladder epithelium of the mouse. J Biophys Biochem Cytol 1:445–458

Yamamoto M, Okumura S, Oka N et al (1999) Downregulation of caveolin expression by cAMP signal. Life Sci 64:1349–1357

Younes A, Lyashkov AE, Graham D et al (2008) Ca 2+-stimulated basal adenylyl cyclase activity localization in membrane lipid microdomains of cardiac sinoatrial nodal pacemaker cells. J Biol Chem [ePub ahead of print 10.1074/jbc.M707540200]

Zhang B, Peng F, Wu D et al (2007) Caveolin-1 phosphorylation is required for stretch-induced EGFR and Akt activation in mesangial cells. Cell Signal 19:1690–1700

Zhao H, Loh HH, Law PY (2006) Adenylyl cyclase superactivation induced by long-term treatment with opioid agonist is dependent on receptor localized within lipid rafts and is independent of receptor internalization. Mol Pharmacol 69:1421–1432

Zhuang L, Lin J, Lu ML et al (2002) Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. Cancer Res 62:2227–2231

Protein Scaffolds, Lipid Domains and Substrate Recognition in Protein Kinase C Function: Implications for Rational Drug Design

J.W. Walker

Contents

1 Introduction . . . 186
2 Targeting to Cellular Lipids and Proteins . . . 186
3 Lipids and Lipid Domains . . . 187
4 PKC Substrates . . . 189
5 RACKs . . . 190
6 PICK1 . . . 191
7 Actin . . . 192
8 Cypher/ZASP/Oracle . . . 193
9 AKAPs . . . 193
10 STICKs . . . 194
11 Atypical PKCs: A World Apart . . . 194
12 PKC Translocation Mechanisms . . . 196
13 Rational Drug Design . . . 197
References . . . 198

Abstract Protein kinase C (PKC) represents a family of lipid-regulated protein kinases with ubiquitous expression throughout the animal kingdom. High fidelity in PKC phosphorylation of intended target substrates is crucial for normal cell and tissue function. Therefore, it is likely that multiple interdependent factors contribute to determining substrate specificity in vivo, including divalent cation binding, substrate recognition motifs, local lipid heterogeneity and protein scaffolds. This review provides an overview of targeting mechanisms for the three subclasses of PKC isoforms, conventional, novel and atypical, with an emphasis on how they bind to substrates, lipids/lipid microdomains and multifunctional protein scaffolds. The diversity of interactions between PKC isoforms and their immediate environment is extensive, suggesting that systems biology approaches including proteomics and network modeling may be important strategies for rational drug design in the future.

J.W. Walker
Department of Physiology, Director of Human Proteomics Program, University of Wisconsin, 1300 University Avenue, Madison, WI 53706, USA
jwalker@physiology.wisc.edu

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.* Handbook of Experimental Pharmacology 186,

© Springer-Verlag Berlin Heidelberg 2008

1 Introduction

Protein kinase C (PKC) was discovered in the late 1970s as a lipid-regulated protein kinase activity (Nishizuka 1995) and was subsequently found to be ubiquitously expressed in eukaryotic tissues. PKC quickly captured the attention of the biomedical research community in a big way with the realization that it was a major target of the tumor-promoting phorbol esters (Castagna et al. 1982) and was therefore likely to be a central player in cellular transformation and cancer. In the intervening years, phorbol esters became key research tools for exploring PKC function in a variety of systems. Intense interest in PKC led to the identification and characterization of a family of related PKC isoforms with variations on the theme of lipid-regulated protein kinases (Bataini and Mochly-Rosen 2007). By comparison to other well-known protein kinases such as cyclic AMP-dependent protein kinase (PKA), the substrate specificity of PKCs appeared to be considerably broader. This in turn led to the discovery of a number of scaffolds or anchoring proteins that were hypothesized to provide specificity in PKC signaling by targeting the active kinase to preferred substrates (Mochly-Rosen 1995; Jaken and Parker 2000). Predictably, this may have led to a downplaying of the importance of membrane-resident lipids in targeting PKCs to critical locations in the cell. It is now well recognized that membrane lipids are heterogeneously distributed throughout various cellular organelles and even within microdomains in a given organelle (i.e., lipid rafts) (Dibble et al. 1996; Sando and Chertihin 1996; Corbin et al. 2007; Escriba et al. 2007). It is therefore likely that lipids and proteins cooperate to determine both subcellular localization and function for many of the PKC isozymes. Modern systems biology techniques have also revealed that PKC substrate specificity may not be as broad as early studies suggested (Fuji et al. 2004). An integrated understanding of substrate-recognition motifs combined with targeting mechanisms including roles for lipid microdomains and protein scaffolds will almost certainly provide the most biological insight into the PKC signaling system and its roles in health and disease.

2 Targeting to Cellular Lipids and Proteins

The idea that membrane-associated lipids such as the acidic phospholipids (phosphoinositides, phosphatidylserine) or the neutral lipid messenger diacylglycerol (DAG) can influence PKC targeting to subcellular sites has received strong experimental support throughout the history of PKC research (Corbin et al. 2007; Escriba et al. 2007). On the other hand, evidence for organizing PKC and other signaling proteins on protein scaffolds has been substantial and is concisely summarized in recent reviews (Mochly-Rosen 1995; Jaken and Parker 2000; Vondriska et al. 2004). Since scaffolds are the central theme of this volume, a goal of this

chapter is to consider models that balance the importance of membrane lipid structure/function with the benefits of employing protein scaffolds. Multipoint attachment is a common strategy in biomolecular interactions because it provides for enhanced specificity, versatility and reversibility combined with high-affinity binding. Another common strategy in biological regulation (especially well characterized in the nervous system) is coincidence detection, which serves to ensure high-fidelity signal transmission in a noisy environment (Campbell and King 2004). As a central regulatory kinase involved in many cellular processes, PKC almost certainly employs both multisite attachment and a form of coincidence detection in its bag of tricks.

Thirty years after the discovery of PKC, what is most intriguing about PKC scaffolding proteins is the realization that these proteins are much more than PKC scaffolds. Indeed, the fact that PKC binds quite specifically to a single protein is often *not* among the most impressive roles for the protein in question. These proteins are often still referred to as PKC/PKA scaffolds or anchoring proteins in recognition of the context in which they were initially discovered (e.g., RACK1-receptor for activated C kinase, Mochly-Rosen 1995; PICK1-protein interacting with C kinase, Dev and Henley 2006; AKAPs-A kinase anchoring proteins, Lester and Scott 1997, etc.). Perhaps the more interesting concept that has emerged is that PKCs are often components of vast signaling complexes made up of a network of multi-domain proteins that facilitate crosstalk among various signaling pathways and various cellular processes. The idea of signaling pathways (by analogy with metabolic pathways) is rapidly being replaced by the idea of signaling networks in which highly modular components interact with a high degree of complexity in time and space (Locasale et al. 2007; Woolf et al. 2005; Bose et al. 2006). By using newer high-throughput technologies such as functional proteomics (Edmondson et al. 2004; Brajenovic et al. 2004; Ptacik and Snyder 2006; Schelzke and White 2006; Chung and Walker 2007), we will almost certainly know the detailed composition of these signaling complexes as well as their inter-connectivity in the near future. Making sense out of the input/output behavior of such complex signaling networks represents a major challenge for the future and will rely heavily on the emerging field of systems biology with its emphasis on computer modeling of self-associating network systems (Brajenovic et al. 2004; Locasale et al. 2007; Woolf et al. 2005; Bose et al. 2006). At this stage, however, careful classification of PKC scaffolds, or more broadly, PKC-interacting lipids and proteins represents an important step toward the goal of a complete understanding of signal transduction mechanisms involving PKC.

3 Lipids and Lipid Domains

It is well established that all PKC isoforms except the two atypical isoforms (λ and ζ) require phosphatidylserine and DAG for optimal enzyme activity (Nishizuka 1995; Bataini and Mochly-Rosen 2007). The finding that PKC has strict stereospecific

requirements for both of these membrane-resident lipids suggests that the lipids interact stereospecifically within binding pockets on the enzyme. Phosphatidyl-L-serine is greatly preferred over its enantiomer phosphatidyl-D-serine (Newton and Keranen 1994), but a high degree of cooperativity in the response to this phospholipid has made it difficult to define a single site of interaction with PKC. 1,2-Diacylglycerols bind to one or both of the C1 domains (C1a or C1b), whereas the enantiomeric 2,3-diacylglycerols do not bind or activate (Sreekumar et al. 1997), nor do the isomeric 1,3-diacylglycerols. Among the C1a/b-containing PKC isoforms, substantial variation exists in terms of the affinity for DAG as well as C1a/C1b subdomain interactions. For example, the affinity of C1 for DAG in the conventional PKC isoforms is relatively low, but together with Ca^{2+} bridging between C2 domains and membrane phosphatidylserine, these interactions are adequate to ensure selective lipid targeting as well as relatively long duration anchoring to membranes (Corbin et al. 2007; Oancea and Meyer 1998). The novel PKC isoforms that lack a Ca^{2+}-binding C2 domain display a much higher affinity for DAG, a difference that can be traced to a single amino acid change in the binding pocket (Dries et al. 2007). This high-affinity interaction with DAG effectively increases the residence time for activated PKC once it binds to a target membrane. This difference in the C1 lipid binding domain may play a key role in anchoring certain PKC isoforms to DAG-rich subcellular compartments such as the Golgi (Giorione et al. 2006).

The importance of Ca^{2+}, DAG and negatively charged phospholipids for subcellular targeting of PKC isoforms should not be underestimated. Localized and transient elevations of DAG are produced by a variety of phospholipase C isoforms with considerable diversity in their regulatory properties (Drin and Scarlata 2007). Similarly, a variety of mechanisms are known for producing localized and transient Ca^{2+} elevation. Phosphatidylserine may not change rapidly in response to environmental cues, but the DAG precursor phosphatidylinositol-4,5-bisphosphate (PIP_2) does undergo rapid and transient changes that can dynamically influence PKC targeting. The potential for complex isoform-specific control of PKC activity at a membrane surface is substantial without the need to invoke scaffolding proteins. Moreover, other lipids such as *cis*-unsaturated fatty acids, lysophospholipids, ceramide and phosphatidic acid are known to influence PKC activity with some preference for certain isoforms over others (Huang et al. 1997; Pi and Walker 2000).

Studies of defined lipid mixtures using physico-chemical methods have revealed that the physical properties of membranes have a large impact on PKC function (Jimenez-Monreal et al. 1999). Membrane curvature, head-group spacing, head-group hydration, acyl chain fluidity and the presence of non-bilayer structures such as hexagonal phases have all been correlated with PKC activity (Dibble et al. 1996; Sando and Chertihin 1996; Corbin et al. 2007; Escriba et al. 2007; Jimenez-Monreal et al. 1999). A common feature of these physical characteristics of lipids targeted by PKC (small head groups such as DAG, high membrane curvature as in small vesicles, and bulky unsaturated acyl chains) is crowding of acyl chains within the hydrophobic core of the bilayer. An intriguing hypothesis is that such lateral crowding forces can cause local “splaying” of membrane lipids such that individual

acyl chains are squeezed out of the bilayer (Escriba et al. 2007). This unusual hypothetical structure featuring acyl chains protruding away from the membrane surface could then be stabilized by acyl chain-binding proteins such as PKC.

This kind of correlation between physical properties of membranes and membrane protein activity is not unique to PKC, but is common among enzymes that function at the 2D boundary between the cell membrane and aqueous cytosol (Hurley 2006), such as phospholipases (Drin and Scarlata 2007) and proteins involved in membrane fission/fusion (Adreeva et al. 2006). Lipid heterogeneity within cell membranes is well documented with the inner leaflet known to contain regions with a high level of phosphatidylserine, PIP_2 or DAG (Escriba et al. 2007). With their unique physical properties such as high charge density, divalent cation binding and a tendency to form non-bilayer structures, such lipid mixtures could play a major role in PKC targeting and subsequent substrate phosphorylation, even if such lipids come together only transiently.

Lipid rafts are cell membrane subdomains containing a unique combination of lipids such as cholesterol and sphingolipids, often stabilized by specific proteins (Michel and Backovic 2007). Caveolae represent one type of lipid raft characterized microscopically as cell surface invaginations and biochemically as detergent insoluble membrane microdomains rich in the small oligomeric scaffolding protein caveolin (Couet et al. 1997a,b; see also Patel et al., this volume). Caveolae may play a role in transcytosis across epithelial cell barriers, endocytotic recycling of surface molecules or organization of signaling pathways (Michel and Backovic 2007). PKC isoforms have been shown to interact reversibly with caveolae in cardiac myocytes and to stimulate phosphorylation of other signaling molecules in the vicinity (Rybin et al. 1999), but it remains unclear how the lipid requirements for PKC activity are met in this environment. The more traditional view is that signaling proteins [e.g., kinases, G-protein coupled receptors (GPCRs)] are inactive when associated with caveolae (Couet, et al. 1997b). Small peptide sequence motifs that mediate interactions between caveolin and PKC isoforms have been described (Couet et al. 1997a). Other lipid rafts rich in PIP_2 and phosphatidyinositol-3,4,5-trisphosphate (PIP_3) have been suggested to play a key role in establishing cell polarity in conjunction with aPKCζ and PI3-kinase/PTEN (the kinase and phosphatase that interconvert these polyphosphoinositides) (Comer and Parent 2007), although membrane proteins almost certainly play a role (see below).

4 PKC Substrates

Recent work on substrate preferences for PKC isoforms is also revising perceptions of the PKC family as an overly promiscuous group. Oriented peptide libraries have revealed significant preferences for PKC isoforms from different subclasses (conventional, novel or atypical), but also within the same subclass (Nishikawa et al. 1997). PKC-ε and PKC-δ have consistently been shown to have distinct substrate

preferences, and a recent detailed analysis of preferred as well as disfavored residues have led to the proposal of rather specific recognition motifs for PKC-δ and PKCζ (Fuji et al. 2004). The motif for the two atypical PKC (aPKC) isoforms is different enough from all others that a selective pseudosubstrate peptide can be used to selectively inhibit these isoforms (Couet et al. 1997a). Lest we become too comfortable with the idea that substrate preferences are strictly encoded in the primary sequence of PKC isoforms, there is also emerging evidence that the substrate preferences for PKC depend on many factors (Jaken and Parker 2000). For instance, the substrate specificity of PKC-δ can change as a result of tyrosine phosphorylation on its catalytic domain (Konishi et al. 2001). Tyrosine phosphorylation of PKC-δ also greatly reduces its requirements for activation by lipids leading to a "quasi-constitutively active" PKC species no longer dependent upon being near a membrane (Kikkawa et al. 2002).

Phosphorylation of intended target substrates with high fidelity is crucial for normal cell function. Therefore, multiple inter-dependent factors contribute to phosphorylation specificity in vivo, including specific lipid–protein interactions and active site recognition motifs. Some of our perception that PKCs as a family exhibit low substrate selectivity may have arisen as a result of widespread use of (1) phorbol esters, (2) short peptide substrate motifs, (3) kinase overexpression strategies and (4) kinase catalytic domains proteolytically removed from regulatory domains. Phorbol esters potently and permanently activate many PKC isoforms as well as other signaling molecules (Griner and Kazanietz 2007), possibly contributing to non-specific phosphorylation. Full length enzymes often contain conformational features that contribute to substrate specificity (Pears et al. 1991), as do the immediate lipid and protein environment. These "secondary" factors are lost when constitutively active catalytic domains are used experimentally. Overexpression of kinases is very likely to promote excessive (and possibly non-physiological) crosstalk between signaling systems. As usual, caution and multiple independent strategies are needed for a balanced interpretation of experiments of this nature.

5 RACKs

The pioneering work of Mochly-Rosen and colleagues (1995) introduced the concept of a class of intracellular proteins whose purpose was to localize PKC near its substrates. The first protein of this class to be discovered and thoroughly characterized was RACK1 (receptor for activated C kinase 1) (Mochly-Rosen et al. 1995). It featured isoform-specific and high-affinity binding of PKC only in its activated form, with the interesting twist that RACK1 was not a substrate for the kinase activity (Mochly-Rosen et al. 1995). In vitro, purified RACK1 was shown to bind PKC-βII with a K_d of 1 nM, and only in the presence of the PKC activators Ca^{2+}, DAG and phosphatidylserine. The binding affinity for other PKC isoforms was much lower. Many important advances building on this concept followed from the Mochly-Rosen group, including characterization of the PKC-RACK1 binding

interface and identification and use of peptides to disrupt PKC-βII anchoring in cells to address biological function (Ron et al. 1995).

Cloning and sequence analysis revealed that RACK1 was a member of a WD40 gene superfamily that included the β-subunit of heterotrimeric G-proteins (Ron et al. 1994). These WD40 repeat proteins form a β-propeller structure with a variable number of propeller blades that assemble to form a circular disc. In the case of RACK1, its seven blades form a circular disc from which loops with variable size and sequence protrude above and below the plane of the disc. It is interesting to speculate that RACK1 exhibits an analogous structural organization to GPCRs, which contain seven transmembrane α-helices bundled into a barrel-like structure with loops of variable size and sequence protruding away from the membrane surface on both sides (Escriba et al. 2007).

In short order, a second protein was identified that displayed the requisite properties of a PKC-ε selective RACK and was duly christened RACK2 (Csukai et al. 1997). DAG/phosphatidylserine activated PKC-ε bound RACK2 with ~10 nM K_d, was not a substrate and led directly to the development of peptide reagents that could either block or promote PKC-ε anchoring (Csukai et al. 1997). Many investigators have now used these peptides successfully to explore PKC-ε function in complex biological preparations, including cells (Robia et al. 2005), tissues (Johnson et al. 1996) and transgenic mice (Mochly-Rosen et al. 2000). Further characterization of RACK2 revealed it to be identical to a β'-COP subunit of the coatamer assembly involved in membrane trafficking through the Golgi apparatus (Csukai et al. 1997). RACK proteins for the other remaining ten or so PKC isoforms have not yet been identified. This may be in part because the Mochly-Rosen group has focused its attention on therapeutic applications of its powerful peptide reagents (Kikkawa et al. 2002), and in part because understanding of kinase anchoring proteins was evolving beyond the simple idea of a specific RACK for each PKC isoform.

A good example of PKC and its scaffold being part of a vast complex or supramolecular assembly was illustrated by the recent finding that RACK1 binds tightly to the ribosome (Sengupta et al. 2004). RACK1 could therefore be considered a ribosomal protein. Further evidence suggests that ribosome-bound RACK1 recruits conventional PKC isoforms for the purpose of regulating protein synthesis (Sengupta et al. 2004). RACK1 no longer seems like a static anchor, but perhaps more like an adaptor that allows certain PKC isoforms to attach to and regulate a variety of processes, including the protein synthesis machinery. In the case of the ribosome, RACK1 is but a tiny cog in the wheel of a supramolecular assembly.

6 PICK1

Among the earliest PKC-interacting proteins to be identified in a yeast two-hybrid screen was PICK1 (protein interacting with C kinase 1) (Staudinger et al. 1995). PICK1 bound PKC-α with high affinity and yet did not become phosphorylated

upon PKC-α activation, suggesting that its role was to position PKC-α near key substrates within the cell. Sequence and 3D structural analysis of PICK1 revealed that in addition to binding PKC, it contained a PDZ domain and a BAR domain, providing further clues to its overall function (Tarricone et al. 2001; Peter et al. 2004; Xu and Xia 2007). PDZ domains have emerged in recent years as canonical protein–protein interaction domains that typically recognize a four-residue motif at the C-terminus of its binding partners (Dev 2004). The PDZ domain of PICK1 appears to recognize its own preferred C-terminal motif, which does not fit neatly into the original type-1 and type-2 PDZ recognition motif classification (Madsen et al. 2005). In contrast, BAR domains oligomerize into a coiled-coil stabilized boomerang-shaped structure that is thought to recognize and/or dictate the curvature of membranes (Tarricone et al. 2001). As such, BAR domains are thought to play critical roles in endocytosis and tubulation of membranes. The BAR domain of PICK1 was initially not recognized to be a BAR domain based only upon sequence analysis. When the crystal structure of a BAR-like domain from arfaptin (a homolog of PICK1) was compared to amphiphysin, another well-characterized tubulating protein, it was clear that both formed boomerang-shaped oligomers with lysine/arginine residues located at critical positions for interacting with membrane surfaces (Tarricone et al. 2001; Peter et al. 2004; Xu and Xia 2007) Thus, with low sequence homology, but high structural homology, PICK1 was deemed to contain a membrane-curvature-sensing BAR domain. PICK1 is the only protein known to contain both a PDZ domain and a BAR domain, suggesting a unique role in organizing the protein machinery involved in endocytosis, membrane traffic (Wang et al. 2003) and synaptic plasticity (Gardiner et al. 2005). PKC-α is recruited to these complexes by binding its C-terminus to the PDZ domain of PICK1 (Staudinger et al. 1997; Dev et al. 2004), with additional contributions from lipid-binding domains on PKC-α that are also sensitive to membrane curvature and/or the presence of non-bilayer structures (Jimenez-Monreal et al. 1999).

7 Actin

Filamentous actin (F-actin) is the primary constituent of stress fibers that dictates the shape, structure and contractility/motility of many cell and tissue types. Similarly, in many cases PKC regulates F-actin assembly (Liu et al. 2007), stress fiber formation (Prekeris et al. 1996), neurite outgrowth (Zeidman et al. 2002) and cell motility, suggesting that PKC may be intimately associated with F-actin. Indeed, a number of investigators have characterized direct interactions between PKC isoforms (notably PKC-ε and PKC-βII) and purified F-actin in vitro. Perekis et al. (1996) reported that PKC-ε bound to F-actin via a specific interaction with its C2 domain that could be specifically disrupted with a small peptide. These authors provided evidence that PKC-ε/F-actin interactions were critical in regulating the neuronal cytoskeleton and thus synaptic function. Huang et al. (1997) confirmed a

specific high-affinity interaction between PKC-ε and F-actin, but interestingly, the PKC bound even when inactive and then became catalytically active upon binding (even without addition of lipid activators). This PKC-ε/F-actin interaction did not account for anchoring of PKC-ε to cardiac myofibrils despite high levels of F-actin in myofibrils. Blobe et al. (1996) characterized high-affinity binding of PKC-βII to F-actin in vitro. It is interesting to note that F-actin is a highly acidic (negatively charged) protein and that PKC isoforms are fond of similarly negatively charged phospholipids, such as phosphoinositides. Therefore, reports of direct binding of PKC to F-actin in vitro should not be a surprise. It remains an open question, however, whether direct PKC/actin binding interactions play a central role in regulating cytoskeletal dynamics in vivo.

8 Cypher/ZASP/Oracle

Cypher proteins (also known as ZASP or oracle proteins) are found only in striated muscles (cardiac and skeletal muscles) where they associate with the cytoskeleton and interact with PKC through one or more LIM domains (Zhou et al. 1999). The interaction does not appear to be highly PKC isoform-specific, raising questions as to the selectivity and perhaps physiological relevance of their PKC anchoring capabilities. Cypher/ZASP/oracle proteins also contain a single PDZ domain presumably for promoting and organizing protein assemblies. In striated muscle, cypher/ZASP/oracle localizes predominantly to the Z-disc, a central anchoring point for sarcomeric F-actin and a compartment rich in cytoskeletal and signaling proteins (Chung and Walker 2007), including calcineurin (Frey and Olson 2002). Ablation of cypher in the mouse is embryonically lethal (Zhou et al. 2001), suggesting a critical role in sarcomere assembly or other aspects of muscle development. Moreover, mutations in cypher that impair PKC binding have been linked with dilated cardiomyopathy in humans (Arimura et al. 2004).

9 AKAPs

AKAPs (A kinase anchoring proteins) are described in detail in other chapters of this volume, so they will be discussed only briefly here in the context of PKC. AKAPs represent a very large family of proteins that function as scaffolds for ubiquitous PKA and related signaling molecules (Lester and Scott 1997). AKAPs bind regulatory domains of the PKA tetramer such that the inactive tetramer is localized near its target substrates. The active catalytic subunits are then released upon elevation of free cyclic AMP in the vicinity. It is now clear that AKAPs recruit a variety of signaling molecules, including phosphodiesterases, phosphatases and other kinases, including PKC, into an organized complex (Klauck et al. 1996; Hagashida

et al. 2005). In contrast to RACKs, however, AKAPs appear to take up a more "permanent" residence in specific subcellular compartments rather than undergoing a co-translocation with PKA and other signaling molecules. In this regard, AKAPs may represent examples of the more traditional concept of anchoring proteins that provide a stable non-moving platform for assembly of multi-component signaling complexes. AKAPs also appear to be substrates for PKA and PKC (Klauck et al. 1996), further distinguishing them from RACKs.

10 STICKs

Another interesting and important class of PKC binding partners have been termed STICKs (substrates interacting with C kinase) (Jaken and Parker 2000). This group of proteins exhibits high-affinity interactions with regulatory domains on PKC, but they are also good substrates for PKC. Intriguingly, some STICKs also bind phosphatidylserine, and others bind F-actin, suggesting opportunities for multisite interactions to enhance PKC targeting. Among the widely studied members of this group are MARCKS (myristolyated alanine rich C-kinase substrate) (Fujise et al. 1994), vinculin/talin (Hyatt et al. 1994), GAP43 (Dekker and Parker 1997), gravin (Nauert et al. 1997), AKAP79 (Klauck et al. 1996) and annexins (Hyatt et al. 1994). These proteins interact with PKC isoforms in at least two ways, through PKC's regulatory domains for high-affinity binding and through PKC's catalytic domain to become phosphorylated.

11 Atypical PKCs: A World Apart

Much of what has been discussed to this point applies only to the DAG-responsive PKC isoforms, which include the conventional (α, βI, βII, γ) and novel (δ, ε, θ, η) subgroups. Two atypical PKC isoforms (λ and ζ) live in a world of their own, including a unique set of scaffolds and a distinct relationship with lipids (Suzuki et al. 2003; Moscat and Diaz-Meco 2000; Hirai and Chida 2003). The atypical PKCs are classified within the PKC family on the basis of modest sequence homology in the kinase catalytic domain with conventional and novel PKC isoforms (Suzuki et al. 2003). Atypical PKCs show very low sequence homology with other PKCs in their regulatory domains except for a single cysteine-rich C1 motif that appears to bind PIP_3 rather than DAG. A PB1 domain unique to atypical PKCs represents a focal point in dictating isotype-specific protein–protein interactions (Hirai and Chida 2003).

Atypical PKC (aPKC) has been shown to play a role in signaling downstream of insulin receptors (Liu et al. 2007), which nicely highlights the involvement of lipids in regulating this subclass of PKCs. A common downstream effector recruited to active tyrosine phosphorylated insulin receptors is PI3-kinase. This enzyme

converts the membrane-resident phospholipid PIP_2 to PIP_3. In turn, PIP_3 can then bind and activate a number of kinases, including aPKCζ, PDK1 and the abundant and widely studied growth regulating kinase Akt/PKB (Moscat and Diaz-Meco 2000; Hirai and Chida 2003). Of these, PDK1 has the highest affinity for PIP_3. However, aPKCζ becomes activated as a result of coincident PIP_3 binding to its C1 domain and phosphorylation of its activation loop residue T410 by PDK1 (Liu et al. 2007). This activation of aPKCζ is critical for insulin-dependent recruitment of the GLUT4 glucose transporter to the cell surface, which promotes glucose uptake in insulin target tissues (Liu et al. 2007).

The multitude of proteins involved in insulin receptor signaling is vast, and the nature of protein assemblies involving aPKCs may be easier to illustrate for other growth-signaling pathways. For example, the TNF-α (tumor necrosis factor-α) receptor signals through a complex made up of the proteins TRAF2, RIP, ZIP/p62 and aPKCζ (Moscat and Diaz-Meco 2000). Through this complex, TNF-α stimulates aPKCζ-mediated phosphorylation of the NF-kB regulatory complex, thereby initiating gene expression patterns involved in inflammatory responses. Analogous signaling complexes featuring aPKCζ may explain intracellular signaling resulting from binding of IL-1 (interleukin-1) and NGF (nerve growth factor) to their respective cell surface receptors (Moscat and Diaz-Meco 2000). These functionally relevant protein–protein interactions involving aPKCζ are mediated through its PB1 subdomain at the N-terminus of the regulatory domain.

A major role for atypical PKCs in embryonic development was revealed by genetic and RNAi experiments in *C. elegans*, and subsequently recapitulated in mouse knockout models. In *C. elegans*, at least three mutants were found to be defective in anterior–posterior polarization of embryos during early development: PKC-3, PAR-3 and PAR-6. PKC-3 is the *C. elegans* homolog of aPKC. PAR-3 is a scaffolding protein with three PDZ domains that binds PKC-3 in its catalytic domain. The mammalian homolog of PAR-3, known as ASIP, binds (through a PDZ domain) proteins such as JAM that are critical in the formation of tight junctions in polarized epithelial cells (Suzuki et al. 2003). Interestingly, JAM is also a direct molecular partner of PICK1, suggesting that conventional and atypical PKC isoforms may converge during the formation of cell–cell contacts such as in epithelial cell tight junctions (Reymond et al. 2005). PAR-6 also appears to be a scaffold protein, but it binds aPKC through a specific sequence motif in the N-terminal PB1 domain, such that PAR-6 and PAR-3 can bind to aPKC simultaneously. PAR-6 contains interaction sites for the small G-proteins cdc42 and rac1, which regulate actin cytoskeletal dynamics and may link the cell polarity machinery to cellular transformation by ras, or to modulation by E-cadherin-mediated cell–cell contacts (Moscat and Diaz-Meco 2000; Hirai and Chida 2003). In mouse models, germ-line-targeted knockout of aPKCλ is embryonically lethal (Suzuki et al. 2003), suggesting essential functions in early development, whereas aPKCζ knockouts survive, but display extensive defects in cell–cell junctions and cell polarity (Suzuki et al. 2003).

This brief overview of aPKCs barely scratches the surface of a rapidly developing literature on aPKC signaling in mammalian tissues. The emphasis here has

been on illustrating the involvement of the critical membrane lipids PIP_2 and PIP_3, as well as on providing examples of scaffolding proteins that organize large signaling complexes in which aPKCs function to regulate cell shape, cell polarity, cell survival and many other cellular processes. It is also well established that protein–protein interactions involving aPKCs directly impact other critical downstream signaling pathways, including MAP kinase cascades and src (Moscat and Diaz-Meco 2000; Hirai and Chida 2003).

12 PKC Translocation Mechanisms

One salient feature of PKC is its ability to translocate from a cytosolic (soluble) compartment to a membrane-bound (particulate) compartment in response to coincident elevation of the classical second messengers Ca^{2+} and DAG (Nishizuka 1995; Bataini and Mochly-Rosen 2007). This property has been widely exploited as a surrogate of PKC activation in a variety of studies. However, defining precise and generally applicable mechanisms of PKC translocation has been elusive. Ca^{2+} and phosphatidylserine recruit conventional PKC isoforms rapidly (seconds) and reversibly enough to be consistent with a diffusion controlled process for PKC translocation over normal intracellular distances (≤ 50 μm). Novel PKC isoforms, which lack the Ca^{2+}-mediated membrane targeting capability, may translocate over minutes or longer due to rate-limiting conformational changes in the PKC regulatory domain itself (Robia et al. 2001). Similarly, multi-step translocation processes have been suggested for novel PKC isoforms based upon an antibody that recognizes a weakly membrane-bound, but inactive PKC conformation (Souroujon et al. 2004). Annexin V-microtubule interactions have also been shown to play a critical role in a slow multistep PKC-δ translocation process (Kheifets et al. 2006). Other types of translocation of PKC isoforms may depend upon trafficking of membranes through endosomal and Golgi compartments (Alvi et al. 2007). Alternatively, direct interactions of PKC isoforms with F-actin (Huang et al. 1997; Prekeris et al. 1996; Blobe et al. 1996) may reflect a form of directed one-dimensional diffusion as has been proposed for transcription factors moving along stretches of DNA (Elf et al. 2007). Thus, PKC may interact with membranes to restrict its influence to membrane proteins and by analogy with F-actin to limit its influence (under different circumstances) to the actin cytoskeleton. Such an intriguing possibility for PKC/F-actin interactions must await thorough experimental verification. Nevertheless, it now seems unlikely given the diverse cellular functions regulated by PKC that it utilizes a single general mechanism to control subcellular localization or transport. Such versatility in regulatory strategies may simply be yet another manifestation of the evolutionary success of the PKC family.

Atypical PKCs are readily distinguished from conventional and novel PKCs by *not* undergoing translocation in response to phorbol esters or DAGs (Bataini and

Mochly-Rosen 2007; Suzuki et al. 2003; Moscat and Diaz-Meco 2000; Hirai and Chida 2003). However, the existence of nuclear localization and export signals in both aPKCζ and aPKCλ is consistent with evidence that they undergo dynamic nuclear-cytoplasmic shuttling (Perander et al. 2001). So, while extensive protein–protein interaction networks appear to be a central feature of aPKC signaling, these PKC family members are also versatile and not restricted to functioning only in large stable macromolecular complexes.

13 Rational Drug Design

In many ways, the RACK-inspired peptides developed by Mochly-Rosen and colleagues represent the epitome of drug leads that have emerged from a rational approach to targeting protein–protein interfaces (Kheifets and Mochly-Rosen 2007). The use of cell-permeant carrier peptides (e.g., TAT, antennapedia) to deliver the bioactive peptides across cell membranes also addressed a potentially serious drawback of peptide-based therapeutics (i.e., poor access to intracellular targets) (Kheifets and Mochly-Rosen 2007). The RACK-inspired peptides are working their way through clinical trials for potential use against various cardiovascular diseases (Bataini and Mochly-Rosen 2007).

Medicinal chemists have long been systematically targeting C1 and C2 domains of PKCs for potential therapeutic purposes with some notable success (Marquez et al. 1999; Tamamura et al. 2004). One lesson for future drug design might include that PKC achieves functional specificity by interacting with many neighbors, including lipid microdomains and 3D assemblies containing a host of distinct proteins, some substrates (Jaken and Parker 2000; Klauck et al. 1996; Hagashida et al. 2005; Fujise et al. 1994; Hyatt et al. 1994; Dekker and Parker 1997; Nauert et al. 1997), others only anchors (Mochly-Rosen 1995; Kheifets and Mochly-Rosen 2007; Sengupta et al. 2004; Staudinger et al. 1995; Tarricone et al. 2001). Thus, specificity in drug targeting might be achieved with multivalency – incorporating several pharmacophores into the same molecule. Systems biology approaches to drug discovery will be more prominent in the future. For example, screens for drug candidates should be more powerful if they contain more than just the target kinase activity in solution, in recognition of both the importance and complexity of the immediate environment in which PKC isoforms function.

Finally, it is important to recognize that considerable success has been achieved with a PKC inhibitor that simply targets the kinase activity of PKC-β (Idris et al. 2001; Kelly et al. 2003). The compound LY333531 (ruboxistaurin) has shown considerable efficacy toward improving the symptoms of diabetes, including its devastating cardiovascular complications (Kelly et al. 2003); ruboxistaurin is well along in clinical trials. Further rational drug design by targeting PKC binding proteins is an appealing strategy, especially within the basic science

community. Therefore, developing a fundamental understanding of the cellular and molecular biology underlying major human diseases will continue to be a top research priority, as will clarifying potential roles of PKC isoforms in these disease states. Identifying and minimizing off-target detrimental side effects of various classes of drugs will also continue to be important. At the end of the day, however, if a drug improves important endpoints in human trials and is safe, then it will find use in the clinic whether we know exactly how it works or not. Moreover, it may be impossible to predict pleiotropic effects of drugs (Spector et al. 2007), but a drug's ability to influence multiple cellular systems or processes can often carry the day for widespread use in a diverse human population (Kang et al. 2007).

Recent review articles have enumerated upwards of hundreds of well-characterized PKC interacting proteins including substrates and scaffolds (Jaken and Parker 2000; Edmondson et al. 2004; Poole et al. 2004); moreover, the number of candidate molecular interactions involving PKC continues to expand at a remarkable pace. Therefore, strategies that embrace integrated systems biology approaches may offer the greatest promise. For example, the selectivity of protein loss-of-function studies (e.g., using RNAi, Sahin et al. 2007) combined with the global perspective of functional proteomics (Edmondson et al. 2004; Brajenovic et al. 2004; Ptacik and Snyder 2006; Schelzke and White 2006; Chung and Walker 2007) and the power of computational biology (Locasale et al. 2007; Woolf et al. 2005; Bose et al. 2006) should permit more effective modeling of complex signaling networks containing PKC interacting lipids, proteins and substrates. The goal of such studies would be to develop new fundamental insights into the input/output behavior of PKC networks. Without such systems biology tools, it may be virtually impossible to predict and then systematically test which PKC interfaces to target therapeutically in different disease settings.

Acknowledgements The author would like to thank the Wisconsin Partnership for a Healthy Future and the National Heart Lung and Blood Institute for generous research funding.

References

Adreeva AV, Kutuzov MA, Voyno-Yasenetskaya TA (2006) A ubiquitous membrane fusion protein αSNAP: a potential therapeutic target for cancer, diabetes and neurological disorders? Expert Opin Ther Targets 10:723–733

Alvi F, Idkowiak-Baldys J, Baldys A, Raymond JR, Hannun YA (2007) Regulation of membrane trafficking and endocytosis by protein kinase C: emerging role of the pericentron, a novel protein kinase C-dependent subset of recycling endosomes. Cell Mol Life Sci 64:263–270

Arimura T, Hayashi T, Terada H, Lee SY, Zhou Q, Takahashi M, Ueda K, Nouchi T, Hohda S, Shibutani M, Hirose M, Chen J, Park JE, Yasunami M, Hayashi H, Kimura A (2004) A cipher/ZASP mutation associated with dilated cardiomyopathy alters the binding affinity to protein kinase C. J Biol Chem 279:6746–6752

Bataini F, Mochly-Rosen D (2007) Happy Birthday protein kinase C: Past, present and future of a superfamily. Pharmacol Res 55:461–466

Blobe GC, Stribling DS, Fabbro D, Stabel S, Hannun YA (1996) Protein kinase C β II specifically binds to and is activated by F-actin. J Biol Chem 271:15823–15830

Bose R, Molina H, Patterson AS, Bitok JK, Periswamy B, Bader JS, Powdey A, Cole PA (2006) Phosphoproteomic analysis of Her2/neu signaling and inhibition. Proc Natl Acad Sci USA 103:9773–9778

Brajenovic M, Joberty G, Kuster B, Bouwmeester T, Drewes G (2004) Comprehensive proteomics analysis of human PAR proteins reveals an interconnected protein network. J Biol Chem 279:12804–12811

Campbell RA, King AJ (2004) Auditory neuroscience: a time for coincidence? Curr Biol 26: R886–R888

Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol ester. J Biol Chem 257:7847–7851

Chung KY, Walker JW (2007) Interaction and inhibitory cross-talk between endothelin and erbB receptors in the adult heart. Mol Pharm 71:1494–1502

Comer FI, Parent CA (2007) Phosphoinositides specify polarity during epithelial organ development. Cell 128:239–240

Corbin JA, Evans JH, Landgraf KE, Falke JJ (2007) Mechanism of specific membrane targeting of C2 domains: localized pools of target lipids enhance calcium affinity. Biochemistry 46:4322–4336

Couet J, Li S, Okamoto T, Ikezu T, Lisanti MP (1997a) Identification of peptide and protein ligands for the caveolin-scaffolding domain. J Biol Chem 272:6525–6533

Couet J, Sargiacomo M, Lisanti MP (1997b) Interaction of a receptor tyrosine kinase, EGF-R, with caveolins. Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities. J Biol Chem 272:30429–30438

Csukai M, Chen CH, DeMatteis MA, Mochly-Rosen (1997) The coatamer protein β’-COP, a selective binding protein (RACK) for protein kinase C ε. J Biol Chem 272:29200–29206

Dekker LV, Parker PJ (1997) Regulated binding of the protein kinase C substrate GAP-43 to the VO/C2 region of protein kinase C-δ. J Biol Chem 272:12747–12753

Dev KK (2004) Making protein interactions druggable: targeting PDZ domains. Nat Rev Drug Discov 3:1047–1056

Dev KK, Henley JM (2006) The schizophrenic faces of PICK1. Trends Pharm Sci 27: 574–579

Dev K, Nakanishi S, Henley JM (2004) The PDZ domain of PICK1 differentially accepts protein kinase C and GluR2 as interacting ligands. J Biol Chem 279:41393–41397

Dibble ARG, Hinderliter AK, Sando JJ, Biltonen RL (1996) Lipid lateral heterogeneity in phosphatidylcholine/phosphatidylserin/diacylglycerol vesicles and its influence on protein kinase C activation. Biophys J 71:1877–1890

Dries DR, Gallegos LL, Newton AC (2007) A single residue in the C1 domain sensitizes novel PKC isoforms to cellular diacylglycerol formation. J Biol Chem 282:826–830

Drin G, Scarlata S (2007) Stimulation of phospholipase C-β by membrane interactions, interdomain movement and G-protein binding. How many ways can you activate an enzyme? Cell Signal 19:1383–1392

Edmondson RD, Vondriska TM, Biederman KJ, Zhang J, Jones RC, Zheng Y, Allen DJ, Xiu JX, Cardwell EM, Pisano MR, Ping P (2004) Protein kinase C ε signaling complexes include metabolism- and transcription/translation-related proteins. Mol Cell Proteomics 1:421–433

Elf J, Li GW, Xie XS (2007) Probing transcription factor dynamics at the single molecule level in a living cell. Science 316:1191–1194

Escriba PV, Wedegaertaener PB, Goni F, Volger O (2007) Lipid–protein interactions in GPCR-associated signaling. Biochem Biophys Acta 1768:836–852

Frey N, Olson EN (2002) Calsarscin, a novel skeletal muscle-specific member of the calsarsin family, interacts with multiple Z disc proteins. J Biol Chem 277:13998–134004

Fuji K, Zhu G, Liu Y, Hallam J, Chen L, Herrero J, Shaw S (2004) Kinase peptide specificity: improved determination and relevance to protein phosphorylation. Proc Natl Acad Sci USA 101:13744–13749

Fujise A, Mizuno K, Ueda Y, Osada S, Hirai S, Takayangi A, Shimizu, N, Owadal MK, Nakajima H, Ohno S (1994) Specificity of high affinity interaction of protein kinase C with a physiological substrate, myristoylated alanine-rich protein kinase C substrate. J Biol Chem 269:31642–31648

Gardiner SM, Takamiya K, Xia J, Suh JG, Johnson R, Yu S, Huganir RL (2005) Calcium-permeable AMPA receptor plasticity is mediated by subunit specific interactions with PICK1 and NSF. Neuron 45:903–915

Giorione JR, Lin JH, McCammon A, Newton AC (2006) Increased membrane affinity of the C1 domain of protein kinase C δ compensates for the lack of involvement of its C2 domain in membrane recruitment. J Biol Chem 281:1660–1669

Griner EM, Kazanietz MG (2007) Protein kinase C and other diacylglycerol effectors in cancer. Nature Rev Cancer 7:281–294

Hagashida H, Hoshi N, Zhang JS, Yokoyama S, Hashii M, Jin D Noda M, Robbins J (2005) Protein kinase C bound with A kinase anchoring protein is involved in muscarinic receptor-activated modulation of M-type KCNQ channels. Neurosci Res 51:231–234

Hirai T, Chida K (2003) Protein kinase C ζ: activation mechanisms and cellular functions. J. Biochem 133:1–7

Huang XP, Pi Y, Lokuta AJ, Greaser ML, Walker JW (1997) Arachidonic acid stimulates protein kinase C-ε redistribution in heart cells. J Cell Sci 110:1625–1634

Hurley JH (2006) Membrane binding domains. Biochem Biophys Acta 1761:805–811

Hyatt SL, Liao L, Chapline C, Jaken S (1994) Identification and characterization of α-protein kinase C binding proteins in normal and transformed REF52 cells. Biochemistry 33:1223–1228

Idris I, Gray S, Donnelly R (2001) PKC activation: isozyme-specific effects on metabolic and cardiovascular complications in diabetes. Diabetolgia 44:659–673

Jaken S, Parker PJ (2000) Protein kinase C binding partners. BioEssays 22:245–254

Jimenez-Monreal AM, Aranda FJ, Micol V, Sanchez-Pinera P, de Godos A, Gomez-Fernandez JC (1999) Influence of the physical state of the membrane on the enzymatic activity and energy of activation of protein kinase C α. Biochemistry 38:7747–7754

Johnson JA, Gray M, Chen CH, Mochly-Rosen D (1996) A protein kinase C translocation inhibitor as an isozyme-selective inhibitor of cardiac function. J Biol Chem 271:24962–24966

Kang M, Chung KY, Walker JW (2007) G-protein coupled receptor signaling in myocardium: not for the faint of heart. Physiology 22:174–184

Kelly DJ, Zhang Y, Hepper C, Gow RM, Jaworski K, Kemp BE Wilkinson-Berka JL, Gilbert RE (2003) Protein kinase C β inhibition attenuates the progression of experimental diabetic nephropathy in the presence of continued hypertension. Diabetes 52:512–518

Kheifets V, Mochly-Rosen D (2007) Insight into intra- and inter-molecular interactions of PKC: design of specific modulators of kinase function. Pharmacol Res 55:467–476

Kheifets V, Bright R, Inagaki K, Schechtman D, Mochly-Rosen D (2006) Protein kinase C-δ-annexin V interaction: a required step in δ-PKC translocation and function. J Biol Chem 281:23218–23224

Kikkawa U, Matsuzaki H, Yamamoto T (2002) Protein kinase C-δ: activation mechanisms and function. J Biochem 132:831–839

Klauck TM, Faux MC, Labudda K, Langeberg LK, Jaken S, Scott JD (1996) Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science 271: 1589–1592

Konishi H, Yamauchi E, Taniguchi H, Yamamoto T, Matsuzaki H, Takemura Y, Ohmae K, Kikkawa U, Nishizuka Y (2001) Phosphorylation sites of protein kinase C δ in H_2O_2 treated cells and its activation by tyrosine kinase in vitro. Proc Natl Acad Sci USA 98:6587–6592

Lester LB, Scott JD (1997) Anchoring and scaffold proteins for kinases and phosphatases. Recent Prog Horm Res 52:409–429

Liu XJ, Yang C, Gupta N, Zou J, Chang YS, Fang FD (2007) Protein kinase C ζ regulation of GLUT-4 translocation through actin remodeling in CHO cells. J Mol Med 85: 851–861

Locasale JW, Shaw AS, Chakraborty AK (2007) Scaffold proteins confer diverse regulatory properties to protein kinase cascades. Proc Natl Acad Sci USA 104:13307–13312

Madsen KL, Beuming T, Niv MY, Chang CW, Dev KK, Weinstein H, Gether U (2005) Molecular determinants for the complex binding specificity of the PDZ domain of PICK1. J Biol Chem 280:20539–20548

Marquez VE, Nacro K, Benzaria S, Lee J, Sharma R, Teng K, Milne GW, Bienfait B, Wang S, Lewin NE, Blumberg PM (1999) The transition from a pharmacophore guided approach to a receptor guided approach in the design of potent PKC ligands. Pharmacol Ther 82:251–261

Michel V, Backovic M (2007) Lipid rafts in health and disease. Biol Cell 99:129–140

Mochly-Rosen D (1995) Localization of protein kinases by anchoring proteins: a theme in signal transduction. Science 268:247–251

Mochly-Rosen D, Smith BL, Chen CH, Disatnik MH, Ron D (1995) Interaction of protein kinase C with RACK1, a receptor for activated C-kinase: a role in β protein kinase C mediated signaling transduction. Biochem Soc Trans 23:596–600

Mochly-Rosen D, Wu G, Hahn H, Osinska H, Liron T, Lorenz JN, Yatani A, Robbins J, Dorn GW (2000) Cardiotrophic effects of protein kinase C ε. Analysis by in vivo modulation of PKC ε translocation. Circ Res 86:1173–1179

Moscat J, Diaz-Meco M (2000) The atypical protein kinase Cs: functional specificity mediated by specific protein adapters. EMBO Rep 1:399–403

Nauert JB, Klauck TM, Langeberg LK, Scott JD (1997) Gravin, an autoantigen recognized by serum from myasthenia gravis patients, is a kinase scaffold protein. Curr Biol 7:52–62

Newton AC, Keranen LM (1994) Phosphatidyl-L-serine is necessary for protein kinase C's high affinity interaction with diacylglycerol-containing membranes. Biochemistry 33:6651–6658

Nishizuka Y (1995) Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 9:484–496

Nishikawa K, Toker A, Johannes FJ, Songyang Z, Cantley LL (1997) Determination of the specific substrate sequence motifs for protein kinase C. J Biol Chem 272:952–960

Oancea E, Meyer T (1998) Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. Cell 95:307–318

Pears C, Schaap D, Parker PJ (1991) The regulatory domain of protein kinase C ε restricts the catalytic domain specificity. Biochem J 276:257–260

Perander M, Bjorkoy G, Johansen T (2001) Nuclear import and export signals enable rapid nucleocytoplasmic shuttling of the atypical protein kinase C λ. J Biol Chem 276: 13015–13024

Peter BJ, Kent HM, Mills IG, Vallis Y, Butler PJG, Evans PR, McMahon HT (2004) BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. Science 303:495–499

Pi Y, Walker JW (2000) Diacylglycerol and fatty acids synergistically increase cardiomyocyte contraction via activation of PKC. Am J Physiol 279:H26–H34

Poole AW, Pula G, Hers I, Crosby D, Jones ML (2004) PKC-interacting proteins: from function to pharmacology. Trends Pharm Sci 25:528–535

Prekeris R, Mayhew MW, Cooper JB, Terrian DM (1996) Identification and localization of an actin-binding motif unique to PKC ε and participates in regulation of synaptic function. J Cell Biol 132:77–90

Ptacik J, Snyder M (2006) Charging it up:global analysis of protein phosphorylation. Trends Genet 22:545–554

Reymond N, Garrido-Urbani S, Borg J-P, Dubreuil P, Lopez M (2005) PICK-1: A scaffold protein that interacts with Nectins and JAMS at cell junctions. FEBS Lett 579:2243–2249

Robia SL Ghanta J, Robu V, Walker JW (2001) Localization and kinetics of protein kinase C-ε anchoring in cardiac myocytes. Biophys J 80:2140–2151

Robia SL, Kang M, Walker JW (2005) Novel determinant of PKC ε anchoring at cardiac Z-lines. Am J Physiol 281:H1941–H1950

Ron D, Chen CH, Caldwell J, Jamieson L, Orr E, Mochly-Rosen (1994) Cloning of an intracellular receptor for protein kinase C: a homolog of the β subunit of G-proteins. Proc Natl Acad Sci USA 91:839–843

Ron D, Luo J, Mochly-Rosen D (1995) C2 region-derived peptides inhibit translocation and function of β protein kinase C in vivo. J Biol Chem 270:24180–24187

Rybin VO, Xu X, Steinberg SF (1999) Activated protein kinase C isoforms target to cardiomyocyte caveolae: stimulation of local protein phosphorylation. Circ Res 84:980–988

Sahin O, Lobke C, Korf U, Appelhans H, Sultmann H, Poustka A, Wiemann S, Arlt D (2007) Combinatorial RNAi for quantitative protein network analysis. Proc Natl Acad Sci USA 104:6579–6584

Sando JJ, Chertihin OI (1996) Activation of protein kinase C by lysophosphatidic acid: dependence on composition of lipid vesicles. Biochem J 317:583–588

Schelzke K, White FM (2006) Phosphoproteomic approach to elucidate cell signaling networks. Curr Opin Biotech 17:406–414

Sengupta J, Nilsson J, Gursky R, Spahn CM, Nissen P, Frank J (2004) Identification of the versatile scaffold RACK1 on the eukaryotic ribosome by cryo EM. Nat Struct Biol 11:957

Souroujon MC, Yao L, Chen H, Endemann G, Khaner H, Geeraert V, Schechtman D, Gordon AS, Diamond I, Mochly-Rosen (2004) State-specific monoclonal antibodies identify an intermediate state in ε protein kinase C activation. J Biol Chem 17617–17624

Spector NL, Yarden Y, Smith B, Ljuba L, Trusk P, Pry K, Hill JE, Xia W, Seger R, Bacus SS (2007) Activation of AMP-activated protein kinase by human EGF receptor 2/EGR receptor tyrosine kinase inhibitor protects cardiac cells. Proc Natl Acad Sci USA 104: 10607–10612

Sreekumar R, Pi YQ, Huang XP, Walker JW (1997) Stereospecific protein kinase C activation by photolabile diglycerides. Bioorg Med Chem Lett 7:341–346

Staudinger J, Zhou J, Burgess R, Elledge SJ, Olson EN (1995) PICK1: a perinuclear binding protein and substrate for protein kinase C isolated by the yeast two-hybrid system. J Cell Biol 128:263–271

Staudinger J, Lu J, Olson EN (1997) Specific interaction of the PDZ domain of PICK1 with the COOH-terminus of protein kinase C α. J Biol Chem 272:32019–32024

Suzuki A, Akimoto K, Ohno S (2003) Protein kinase C λ/ι: a PKC isotype essential for the development of multicellular organisms. J Biochem 133:9–16

Tamamura H, Sigano DM, Lewin NE, Peach ML, Nicklaus MC, Blumberg PM, Marquez VE (2004) Conformationally constrained analogues of DAG. Hydrophobic ligand protein interactions versus ligand lipid interactions of DAG lactones with PKC. J Med Chem 23:4858–4864

Tarricone C, Xiao B, Justin N, Walker PA, Rittinger K, Gamblin SJ, Smerdon SJ (2001) Structural basis of arfaptin mediated cross-talk between Rac and Arf signaling pathways. Nature 411:215–219

Vondriska TM, Pass JM, Ping P (2004) Scaffold proteins and assembly of mulitprotein complexes. J Mol Cell Cardiol 37:391–397

Wang WL, Yeh SF, Chang YI, Hsiao SF, Lian WN, Lin CH, Huang CYF, Lin WJ (2003) PICK1, an anchoring protein that specifically targets protein kinase C to mitochondria selectively upon serum stimulation in NIH 3T3 cells. J Biol Chem 278:37705–37712

Woolf PJ, Prudhomme W, Daheron L, Daley GQ, Lauffenburger DA (2005) Bayesian analysis of signaling networks governing embryonic stem cell fate decisions. Bioinformatics 21:741–753

Xu J, Xia J (2007) Structure and function of PICK1. Neurosignals 15:190–201

Zeidman R, Troller U, Raghunath A, Pahlman S, Larsson C (2002) Protein kinase Cε actin binding is important for neurite outgrowth during neuronal differentiation. Mol Biol Cell 13:12–24

Zhou Q, Ruiz-Lozano P, Marton ME, Chien J (1999) Cypher: a striated muscle-restricted PDZ and LIM domain-containing protein, binds to a-actinin-2 and protein kinase C. J Biol Chem 274:19807–19813

Zhou Q, Chu PH, Huang C, Cheng CF, Martone ME, Knoll G, Shelton GD, Evans S, Chen J (2001) Ablation of cipher, a PDZ-LIM domain Z-line protein, causes a severe form of congenital myopathy. J Cell Biol 155:605–612

Compartmentalised MAPK Pathways

M.D. Brown and D.B. Sacks(✉)

Contents

1 Introduction 206
1.1 The MEK/ERK Cascade 206
1.2 JNK Pathway 209
1.3 p38 Pathway 209
2 Specificity of MAPK Signalling 209
2.1 Receptor-Specific Pathways 209
2.2 Signalling Kinetics 210
2.3 Integration of Multiple Pathways 211
2.4 Tissue-Specific Downstream Effectors 211
3 Compartmentalisation of the MAPK Pathway 212
3.1 Plasma Membrane 212
3.2 Endosomes 214
3.3 Golgi and Endoplasmic Reticulum 215
4 Molecular Scaffolds 216
4.1 Kinase Suppressor of Ras 216
4.2 IQGAP1 218
4.3 MEK Partner-1 219
4.4 β-Arrestins 219
4.5 Similar Expression to FGF 220
5 MAPK as Drug Targets 220
5.1 MAPK and Oncogenes 220
5.2 MAPK and Inflammatory Diseases 223
5.3 Targeting the MAPK Pathway in Other Diseases 224
5.4 Future Direction for Therapies Directed Against the MAPK Cascade 225
6 Perspectives 227
References 229

Abstract The mitogen-activated protein kinase (MAPK) pathway provides cells with the means to interpret external signal cues or conditions, and respond accordingly. This cascade regulates many cell functions such as differentiation, proliferation and migration. Through modulation of both the amplitude and duration

D.B. Sacks
Brigham and Women's Hospital and Harvard Medical School, Thorn 530,
75 Francis St, Boston, MA 02115, USA
dsacks@rics.bwh.harvard.edu

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

of MAPK signalling, cells can control their responses to the multiple activators of the pathway. In addition, recent work has highlighted the importance of the cellular compartment from which the signalling occurs. Cells have developed intricate systems that enable them to localise MAPK components to specific subcellular domains in response to a particular stimulus. Consequently, different factors can activate the same kinase in separate locations. Crucial to this ability are molecular scaffolds, which act as signalling modules for MAPKs, confining them to the desired compartment. The participation of the MAPK network in fundamental physiological processes, such as cell proliferation and inflammation, and the derangement of the homeostasis that occurs in disease processes, renders MAPK a highly desirable target for therapeutic intervention. As we enhance our comprehension of scaffolds and other regulatory molecules, novel targets for drug design may be discovered that will afford selective and specific MAPK modulation.

1 Introduction

The mitogen-activated protein kinase (MAPK) pathway is a highly studied intracellular signalling cascade that regulates multiple cellular functions, such as cell proliferation and differentiation, in response to diverse external cues (Pearson et al. 2001). A large number of growth factors and cytokines, including epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin, neurotrophins and inflammatory cytokines, are known to activate MAPKs. Through these factors, and their modulation of MAPK signalling, cells are able to respond to changes in their environment, and modify their behaviour accordingly. Five MAPK pathways have been identified, namely MEK/ERK, JNK, p38, ERK5 and ERK3 (Fig. 1). Through phosphorylation and activation of both cytosolic and nuclear targets, MAPKs can control non-nuclear events, including cytoskeletal reorganisation, and gene regulation. Although the different MAPK pathways all contribute to the constellation MAPK signalling, they have distinct roles in cellular function. Here we discuss the compartmentalisation of MAPKs and the involvement of molecular scaffolds. In addition, we examine how drugs are being targeted against specific MAPK components in an effort to develop selective therapies for cancer and other diseases.

1.1 The MEK/ERK Cascade

The MEK/ERK cascade, the best characterised of the MAPK pathways, is activated in response to protein tyrosine kinase receptors, such as EGF receptor (EGFR) or VEGF receptor (VEGFR) (Pearson et al. 2001). Growth factor binding induces receptor dimerisation and phosphorylation of the cognate receptor by intrinsic tyrosine

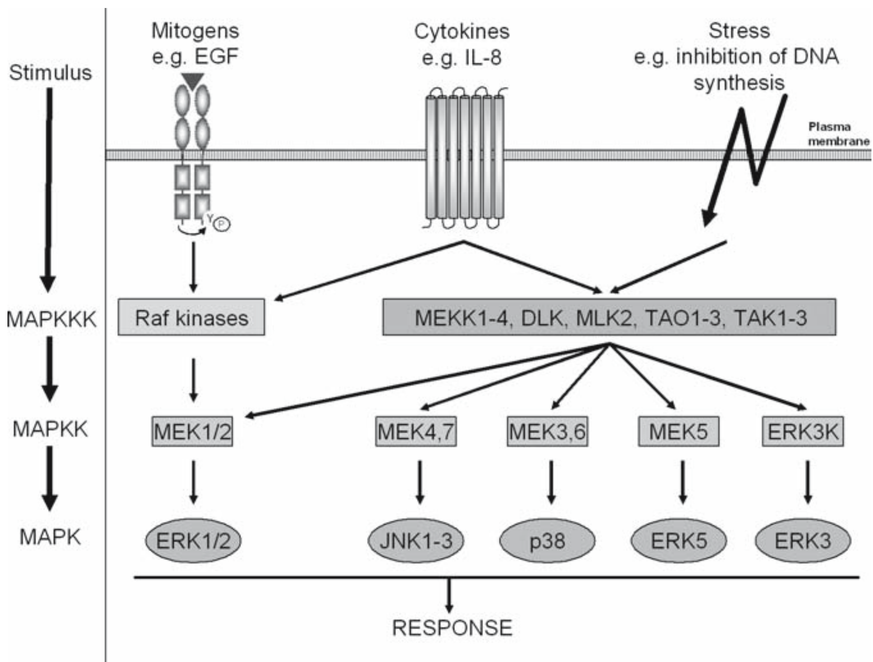

Fig. 1 The MAPK signalling cascades. The MAPK networks and pathways are stimulated in response to a variety of external cues. The five families of MAPK, MEK/ERK, JNK, p38, ERK5 and ERK3, are induced by stimuli such as growth factors, cytokines and stress. Although these pathways can be activated by different environmental conditions, crosstalk occurs, enabling this complex network to control cellular behaviour. Adapted from (Raman and Cobb 2003). *MAPKKK*, MAPK kinase kinase; *MAPKK*, MAPK kinase; *ERK*, extracellular regulated kinase; *MEK*, MAPK/ERK kinase; *JNK*, c-Jun N-terminal kinase; *MEKK*, mitogen ERK kinase kinase; *DLK*, dual leucine zipper-bearing kinase; *MLK2*, mixed lineage kinase; *TAO*, thousand-and-one amino acids; *TAK*, transforming growth factor-beta-activated kinase

kinases. Tyrosine phosphorylation of the receptor induces recruitment of proteins that contain SH2 (Src homology 2) domains, including the adaptor protein Grb2. Grb2 is constitutively bound to the Ras activator Sos and is normally localised to the cytosol. This relocation activates Sos, which in turn activates Ras. Ras is a GTPase and hydrolyses guanosine triphosphate (GTP) to guanosine diphosphate (GDP) (Fig. 2). When bound to GTP, Ras is able to bind to, and activate, downstream effectors, allowing propagation of signalling. Therefore, by regulating the GTP/GDP-bound state of Ras, cells have tight control over its activity. Two classes of proteins provide this control. GTPase-activating proteins (GAPs) upregulate the intrinsic GTPase activity of Ras (and therefore the rate of GTP hydrolysis), reducing the pool of GTP-bound Ras. Consequently, Ras GAPs act as “off” switches and reduce Ras activity. Guanine-nucleotide exchange factors (GEFs), in contrast, promote the exchange of GDP for GTP, increasing the pool of GTP-bound Ras, thereby

increasing Ras activity. Mammalian cells contain three Ras isoforms, H-Ras, K-Ras and N-Ras. At their C-terminal ends, Ras proteins contain a CAAX motif, in which a cysteine (C) is followed by two aliphatic amino acids (A) and any other amino acid (X). This motif is farnesylated by farnesyl transferase, resulting in membrane localisation of Ras GTPases (Mor and Philips 2006).

When GTP bound, Ras recruits the kinase Raf to the membrane, where is becomes active. There are three known isoforms of Raf, namely A-Raf, B-Raf and C-Raf (also termed Raf-1). The Raf proteins share common architecture and all function as serine/threonine kinases, but have distinct functions (Wellbrock et al. 2004). Raf catalyses the phosphorylation and activation of the dual specificity kinases, MAPK/ERK kinase 1 and 2 (MEK1 and MEK2), which in turn activate extracellular regulated kinases, ERK1 and ERK2 (Fig. 2). Once active, ERKs dimerise and either translocate to the nucleus, where they phosphorylate transcription factors, such as the Ets family, or remain in the cytosol, where they catalyse the phosphorylation of substrates in multiple cellular compartments (Fig. 2) (reviewed in Roux and Blenis 2004). The predominant sequelae of MEK/ERK signalling are proliferation and differentiation.

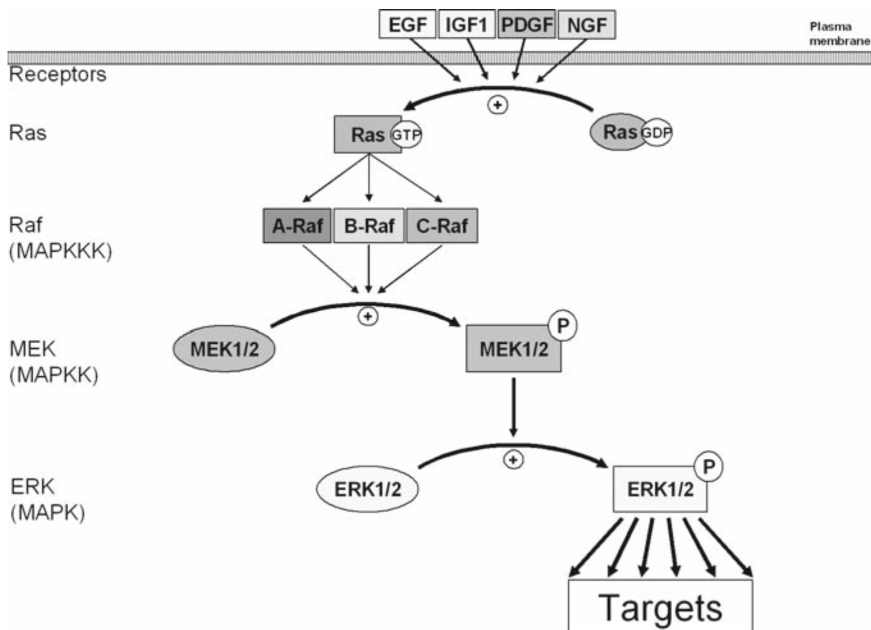

Fig. 2 The MEK/ERK pathway. Growth factors, such as EGF, PDGF and NGF, induce the exchange of GDP for GTP on Ras, thereby activating Ras. GTP-Ras activates the Raf kinases, which then phosphorylate the downstream targets, MEK1 and MEK2. MEK1/2 in turn phosphorylates ERK1 and ERK2, which activate cytosolic and nuclear substrates by catalysing their phosphorylation

1.2 JNK Pathway

The c-Jun N-terminal kinase (JNK) family consists of three ubiquitously expressed members, termed JNK1, JNK2 and JNK3 (Fig. 1) (Roux and Blenis 2004). The JNKs are strongly activated in response to cytokines, selected G-protein coupled receptors (GPCR) and cell stress, such as inhibition of DNA or protein synthesis. JNK is phosphorylated by either MEK4 or MEK7, which are themselves phosphorylated by several kinases, including MEKK1-4, MLK2/3 and DLK (Fig. 1). Following activation, JNK is translocated to the nucleus where it phosphorylates and upregulates several transcription factors, including c-Jun, ATF-2, STAT3 and HSF-1 (Roux and Blenis 2004). JNKs control apoptosis and the development of multiple cell types in the immune system.

1.3 p38 Pathway

There are four members of the p38 kinase family, namely α, β, γ and δ. Cytokines, hormones, G-protein-coupled receptors and cell stress, for example, heat or osmotic shock, activate these enzymes (Pearson et al. 2001). p38 kinases, which are targets of both MEK3 and MEK6 (Fig. 1), have numerous substrates, including MAPK interacting kinases (Mnk) 1 and Mnk 2, and eukaryotic initiation factor 4e (eIF4e). p38 regulates angiogenesis, cell proliferation, inflammation and cytokine production.

2 Specificity of MAPK Signalling

One of the fundamental questions concerning MAPK signalling is that of specificity. As mentioned above, diverse environmental, chemical and molecular factors all activate the MAPK cascade, but each induces a distinct cellular response (Fig. 3). Therefore, cells have developed systems by which a specific activator produces a specific response (reviewed in Tan and Kim 1999; Sacks 2006). Although poorly understood, elucidating these mechanisms is a prerequisite to comprehend how the MAPK pathway is integrated into physiological systems. Several schemes have been proposed by which specificity is achieved. These explanations, which are not mutually exclusive, are briefly described below.

2.1 Receptor-Specific Pathways

Signalling specificity can occur at the level of the receptor. In this model, the outcome of receptor activation is modulated by different intracellular components. For example, two types of cell may express the same receptor, but have distinct signalling

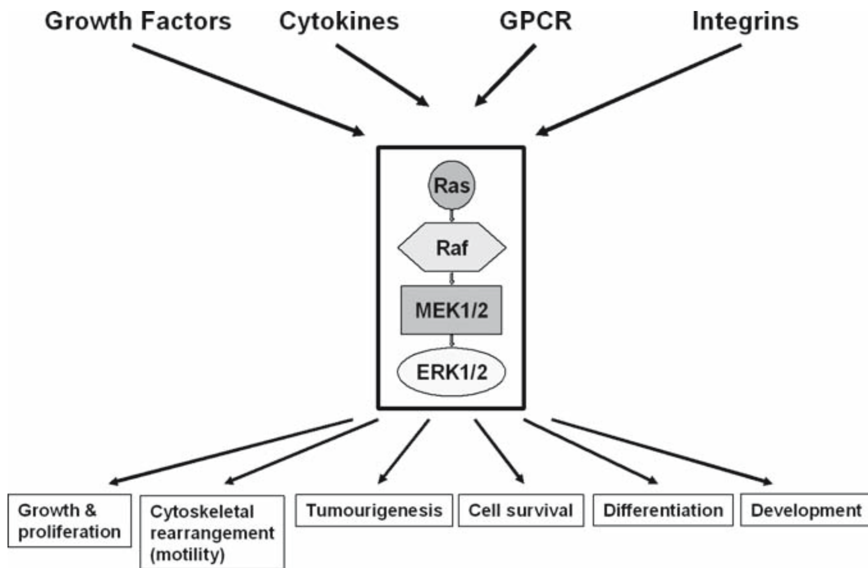

Fig. 3 MAPK specificity. Diverse extracellular inputs stimulate the Ras/Raf/MEK/ERK cascade, and each can elicit a distinct cellular outcome. Although models have been proposed to explain how a specific signalling cue produces a specific response, this aspect of MAPK function is poorly understood. *GPCR*, G-protein coupled receptor

molecules. Therefore, a single kind of receptor would activate distinct signalling cascades in separate tissues. Consequently, the physiological outcome of receptor stimulation will be different. While conceptually appealing, there is limited evidence to support this theory. Some adaptor proteins, such as the Rac1 GEF, VAV (Adams et al. 1992) that are associated with receptors, are tissue-specific. In addition, the receptor tyrosine kinase LET-23 controls development of five different tissues in *C. elegans*, but the MAPK pathway participates in only four of these. Therefore, although LET-23 activates MAPK in some tissues, it also activates alternative signalling cascades in others (Tan and Kim 1999). Consequently, a degree of specificity for MAPK signalling can occur at the level of the receptor. How important receptor specificity is for other activators of MAPKs is unknown.

2.2 *Signalling Kinetics*

Modulation of signalling kinetics, namely the duration and/or strength of activation, elicits distinct cellular responses. The classic example of this mechanism can be found in the neuronal cell line PC12 (pheochromocytoma). PC12 cells proliferate in response to EGF, while nerve growth factor (NGF) induces differentiation (Marshall 1995). Although very different, both sequelae are mediated via the MEK/ERK pathway (Tan and Kim 1999). Further investigation has shown that EGF-dependant MAPK signalling is transient, lasting minutes, while NGF-dependant signalling is

sustained, continuing for hours (Marshall 1995). Through stimulation of the TrkA receptor, NGF activates the MAPK cascade by two mechanisms, the Grb2/SOS pathway and the Rap1 pathway (York et al. 1998). Rap1, a member of the Ras family, modulates integrin-mediated cell adhesion and cadherin-mediated cell junction formation by regulating several effectors, including B-Raf (Bos 2005). In response to NGF, Grb2/SOS produces a transient signal due to a negative feedback mechanism, while Rap1-dependant signalling produces sustained activation of B-Raf. However, EGF can activate only the Grb2/SOS pathway, resulting in a transient activation of MAPK (York et al. 1998). Consequently, these two growth factors induce distinct kinetics of MAPK signalling, culminating in disparate cellular outcomes. Despite the progress in our understanding, why or how these differences in signalling kinetics bring about such divergent cell behaviour is not known.

2.3 Integration of Multiple Pathways

In a living organism, cells are unlikely to be stimulated by a single growth factor or cytokine, but rather will respond to a plethora of signals at one time. Consequently, cells have to integrate diverse inputs to produce a coordinated outcome. Different pathways may converge on a single transcription factor or promoter to regulate gene expression (Tan and Kim 1999). An example of this occurs in *Drosophila* where both EGF and DPP (decapentaplegic) signalling induce the expression of a gene termed *labial*, but each one alone is insufficient to promote expression (Szuts et al. 1998). In addition to converging on single targets, separate pathways can intersect on complexes of transcription factors. The complex of serum-response factor (SRF) and ELK-1 allows integration of Rho-mediated (Marais et al. 1993) and ERK-mediated (Hill et al. 1995) signalling pathways. Different signalling pathways could also intersect on proteins. This has been observed for the oestrogen receptor, which requires both oestrogen binding and phosphorylation by MAPK for maximal activation (Kato et al. 1995).

As well as synergistic relationships, crosstalk among signalling pathways may culminate in inhibition. In this situation, the inhibiting pathway would abrogate the activating signal. A response would occur only in cells that have the activating pathway, but lack the inhibiting pathway. For example, transforming growth factor β (TGF-β) signals through SMAD1, phosphorylation of which by ERK inhibits nuclear translocation, and transcriptional activity (Kretzschmar et al. 1997). Therefore, activation of the MEK/ERK pathway by other growth factors attenuates TGFβ signalling.

2.4 Tissue-Specific Downstream Effectors

Tissue-specific expression of downstream effectors and transcription factors may contribute to MAPK specificity (Tan and Kim 1999). While some tissue-specific MAPK-activated transcription factors have been found, including ELK-1, SAP, NET and HSF1

(Treisman 1996), most appear to be ubiquitous. Nevertheless, there may be more tissue-specific MAPK effectors that have not been identified, and this mechanism may yet prove to be important in providing signalling specificity to the MAPK cascade.

3 Compartmentalisation of the MAPK Pathway

The kinase cascade initiated by Ras originates from transmembrane receptors that are activated by external cues. Consequently, scientists initially believed that Ras was active only at the plasma membrane and that the MAPK pathway propagated from the plasma membrane to the nucleus. More recent work has shed light on the importance of initiation of MAPK signalling from discrete subcellular domains and organelles. This compartmentalisation provides not only regulation of the MAPK pathway, but also enables cells to distinguish MAPK signalling from different sources (Mor and Philips 2006). Further spatio-temporal regulation of MAPK is provided by molecular scaffolds (see Sect. 4, below). The combination of extracellular signals, compartmentalisation and molecular scaffolds creates an intricate system used by cells to regulate a fundamental signalling pathway.

3.1 Plasma Membrane

The plasma membrane is the cellular compartment from which the MAPK cascade frequently originates. Here, transmembrane receptors are activated by factors from the extracellular environment and propagate the signal to Ras via adaptor proteins and Ras GEFs. Consequently, the plasma membrane is considered to be vital for both spatial and temporal regulation of MAPK signalling. Far from being uniform, the plasma membrane contains multiple compartments that serve as nucleation sites for signalling complexes.

3.1.1 Caveolae

Caveolae are invaginations of the membrane, rich in cholesterol, sphingomyelin and glycosphingolipids, and are stabilised by the proteins caveolin-1, -2 and -3 (Parton et al. 2006). Caveolin-1 in particular has been the focus of much research and is considered to be a tumour suppressor (Engelman et al. 1999). A large body of work has provided strong evidence for the participation of caveolae in the regulation of the MAPK pathway.

EGFRs are highly enriched in caveolae (Smart et al. 1995), and many of the early signalling events following EGF binding, including activation of the intrinsic receptor tyrosine kinase activity (Mineo et al. 1996), phosphorylation of substrates (Liu et al. 1996), recruitment of adaptors and kinases (Mineo et al. 1996) and activation of MAPK components (Liu et al. 1996), occur within caveolae. VEGFR2 is also enriched

in caveolae, potentially through association with caveolin-1 (Labrecque et al. 2003). VEGF-dependant activation of ERK1/2, but not phosphorylation of VEGFR2, is abrogated by disruption of caveolae, suggesting that while receptor function remains unaffected, propagation of the signalling cascade is disrupted (Labrecque et al. 2003).

Caveolae are abundant in adipocytes, and their numbers increase during differentiation of fibroblasts into fat cells (Scherer et al. 1994). Consequently, caveolae are thought be important for insulin signalling. Insulin regulates both metabolic and mitogenic signalling pathways through the insulin receptor and its target, insulin receptor substrate (IRS) (Taniguchi et al. 2006). The mitogenic activity of insulin is mediated through the MAPK pathway (Taniguchi et al. 2006). Insulin receptors and IRS are both localised to caveolae (Gustavsson et al. 1999; Karlsson et al. 2004). Disruption of caveolae renders cells resistant to insulin, by preventing phosphorylation of IRS by the receptor kinase (Parpal et al. 2001), and abrogates activation of ERK1/2 (Karlsson et al. 2004), suggesting caveolae are important regulatory compartments for insulin-dependant signalling, including activation of the MAPK pathway.

Neurotrophins are a well-studied class of factors that activate MAPK to promote survival and differentiation of neurons (Kalb 2005). The NGF receptors, $p75^{NTR}$ and TrkA, are localised to caveolae in PC12 cells, and NGF binding and signalling are highly concentrated in these regions (Huang et al. 1999). Analogous to the insulin receptor, disruption of caveolae does not affect TrkA autophosphorylation, but abrogates ERK phosphorylation (Peiro et al. 2000). Interestingly, EGF-dependant ERK phosphorylation is not affected, suggesting caveolae provide some degree of signal specification, regulating MAPK activation in response to specific stimuli.

As a consequence of a series of posttranslational modifications, Ras is tightly associated with membranes (reviewed in Hancock 2003). Fractionation of Rat-1 cells showed that caveolin-1, H-Ras and K-Ras are all enriched in caveolae (Mineo et al. 1996), although this localisation is not altered by EGF. Also contained in the caveolae are other components of MAPK signalling, including Grb2 and SOS, which localise to caveolae in response to EGF. However, more dramatic results were obtained when C-Raf was examined. EGF promoted the translocation of C-Raf from the cytosol to the caveolae, and C-Raf kinase activity was concentrated in the caveolae fractions (Mineo et al. 1996).

It appears, therefore, that caveolae provide platforms for efficient propagation of the MAPK cascade, from the external stimuli to intracellular components. By concentrating both transmembrane receptors, such as those for EGF, VEGF and insulin, and intracellular molecules, for example, Ras, Grb2 and SOS, caveolae serve as signalling modules.

3.1.2 Lipid Rafts

Lipid rafts are freely diffusing, stable assemblies of sphingolipids and cholesterol (reviewed in Hancock 2006). Like caveolae, lipid rafts have been proposed to act as signalling platforms, allowing components of a signalling pathway to congregate and efficiently propagate the cascade.

Evidence supports a role for lipid rafts in MAPK signalling. Ras itself does not appear to be concentrated in lipid rafts. K-Ras is predominantly associated with non-raft membranes, and lipid rafts are not required for K-Ras signalling (Carozzi et al. 2002). While GDP-bound H-Ras is partially associated with lipid rafts, GTP-bound H-Ras is completely absent from rafts (Prior et al. 2001). This raises tantalising questions as to the role played by lipid rafts during the activation/deactivation cycle of H-Ras. H-Ras may exist in a dynamic equilibrium between GDP-bound, lipid-raft-associated and GTP-bound, non-raft-associated states. Signalling from H-Ras requires intact lipid rafts (Carozzi et al. 2002), implying that these compartments are important for H-Ras function. Mutations in the hypervariable region of H-Ras that confine it to lipid rafts impede activation of C-Raf and abolish the biological function of H-Ras (Carozzi et al. 2002). Consequently, C-Raf localised to lipid rafts has a lower kinase activity than that localised outside the rafts. These data suggest that GDP-bound H-Ras transiently enters lipid rafts, but once activated, leaves the rafts and stimulates C-Raf outside of these domains.

Stimulation of the T-cell antigen receptor (TCR) initiates multiple signalling pathways, including the MAPK pathway, which are important for T cell function during immune responses (Qian and Weiss 1997). Following activation of the receptor, the adaptor protein Shc is tyrosine phosphorylated, promoting its interaction with Grb2 and SOS (Ravichandran et al. 1993) and recruiting them to the receptor. It has been known for some time that lipid rafts are required for TCR function (Montixi et al. 1998). Following ligand binding, the TCR localises to lipid rafts, where Shc becomes phosphorylated (Xavier et al. 1998). When artificially localised to plasma membranes, Shc concentrates in lipid rafts, where it becomes constitutively phosphorylated, promoting Ras/Raf/MEK/ERK signalling in the absence of activated TCR (Plyte et al. 2000). Membrane-localised Shc increases TCR-dependant activation of the transcription factor NF-AT in a Grb2-dependant manner (Plyte et al. 2000), providing strong evidence that lipid rafts are important for TCR-dependant MAPK signalling.

3.2 Endosomes

Following activation, receptors such as those for growth factors are internalised into endosomes, in a process that was originally considered to be exclusively a recycling process. However, more recent studies have demonstrated that endosomes also act as signalling platforms, allowing propagation of MAPK cascades (Hancock 2003).

Signalling components, including Shc, Grb2, SOS and Ras, have been found on endosomes containing EGFR (Pol et al. 1998). After receptor internalisation, EGF does not dissociate from the EGFR (Lai et al. 1989), which remains tyrosine phosphorylated and co-localises with GTP-Ras (Burke et al. 2001; Jiang and Sorkin 2002). Cells deficient in clathrin-mediated endocytosis also exhibit impaired activation of ERK by various receptor tyrosine kinases (Vieira et al. 1996; Kranenburg et al. 1999), suggesting endocytosis is an important step in MAPK signalling.

Interestingly, signalling from H-Ras, but not K-Ras, is dependant on efficient endocytosis (Roy et al. 2002), highlighting again the functional differences between the Ras isoforms. Showing that activation of MAPK components can occur from endosomes, and not from membrane-associated receptors prior to endocytosis, is a difficult proposition. However, an elegant study from Wang et al. (2002) demonstrated that internalised EGFR, contained in endosomes, can activate ERK.

Signalling from endosomes may provide specificity of MAPK signalling and therefore modulate cellular responses. For neuronal cells in particular, endosomes appear to provide an important platform for MAPK components in response to growth factors. These cells must interpret stimuli from multiple neurotrophins that bind to receptors at synaptic terminals (Chao 2003). The signals have to be transported along the length of axons and dendrites, some up to 1 m long, to the cell body and the nucleus. Compelling evidence suggests that endosomes provide the cells with the solution to this problem. Following NGF binding, phosphorylated TrkA receptors are found in vesicles that also contain Shc, Ras, C-Raf and ERK (Howe et al. 2001). These endosomes are transported by retrograde motion into the cell body (Howe and Mobley 2004), and accumulation of phosphorylated TrkA receptors can be found in the cell bodies of neurons (Howe and Mobley 2005). Analysis in PC12 cells implies that MAPK signalling from endosomes and the plasma membrane can differentially regulate cellular responses to stimuli. Following NGF stimulation, active TrkA receptors, contained within endosomes, are able to promote both neuronal survival and differentiation, while active TrkA receptors confined to the plasma membrane can promote only cell survival (Zhang et al. 2000).

As described above, NGF induces internalisation and retrograde transport of the TrkA receptor in neurons. However, another neurotrophin that also signals through the TrkA receptor, NT-3, does not cause internalisation (Kuruvilla et al. 2004). These two neurotrophins play divergent roles, at different times, during development of the nervous system (reviewed in Chao 2003). Therefore, these two factors provide an example of how compartment-specific signalling can produce distinct responses from the same receptor.

3.3 Golgi and Endoplasmic Reticulum

Ras proteins localise to both the Golgi and endoplasmic reticulum (ER). Differences are observed among the Ras isoforms. While both N-Ras and H-Ras localise to the plasma membrane, Golgi and the ER, K-Ras is more restricted and is found predominantly at the plasma membrane (Choy et al. 1999). With the development of markers, consisting of the Ras-binding domain of C-Raf linked to a fluorescent tag, which permit visualisation of GTP-bound Ras, active H-Ras was observed to localise at the Golgi and ER following growth-factor stimulation (Chiu et al. 2002). Interestingly, the kinetics of H-Ras activation differs among cellular compartments. At the plasma membrane and the ER, H-Ras activation is rapid, occurring within 1 min, and reversed after 20–40 min, while activation at the Golgi is delayed, taking

10 min, and persisting for 60 min (Chiu et al. 2002). Localisation to neither the Golgi nor ER is affected by inhibiting vesicular transport, suggesting that Ras is activated in situ by a diffusible factor. This premise has turned out to be correct, and the factor was identified as calcium (Bivona et al. 2003; Arozarena et al. 2004). Calcium activates distinct Ras GEFs on the Golgi (Bivona et al. 2003) and ER (Arozarena et al. 2004), resulting in activation of Ras, and thereby bypassing the typical Grb2/SOS pathway. Interestingly, calcium also activates the Ras GAP, CAPRI, at the plasma membrane (Lockyer et al. 2001), enabling differential activation/deactivation of Ras to occur in distinct cellular compartments.

A physiological role for MAPK signalling from the ER or the Golgi has been difficult to elucidate. Nevertheless, some difference in signalling between these two compartments has been demonstrated. Ras61L tethered to the Golgi is a strong activator of ERK and the Akt protein kinase (also known as protein kinase B), but weakly activates JNK. Conversely, when tethered to the ER, Ras61L strongly activates JNK, while only weakly activating ERK and Akt (Chiu et al. 2002). In Jurkat T cells, low-grade stimulation of the TCR upregulates the Ras GEF, Ras GRP1, and results in restriction of active N-Ras to the Golgi (Perez de Castro et al. 2004). The highly restricted activation suggests N-Ras signalling from the Golgi plays an important role in T-cell activation. Finally, an ER-confined Ras effector, ER-associated Ras inhibitor protein 1 (ERI1), was discovered in *Saccharomyces cerevisae* (Sobering et al. 2003). ERI1 binds to and inhibits GTP-Ras2p at the ER.

4 Molecular Scaffolds

The signalling output of the MAPK cascade is tightly controlled by both the cellular compartment from which the signal originates and the duration of the signal. Cells have developed a class of proteins that confer spatial and temporal regulation of the MAPK pathway. Molecular scaffolds bind to multiple members of the MAPK pathway, bringing them into close proximity and thereby facilitating efficient propagation of the signal (Fig. 4). Consequently, these proteins act as a signal module that can regulate not only the intensity of the signal, but also the cellular compartment from which the signal originates (Fig. 5). Therefore, scaffolds provide an intricate level of control over MAPK signalling. Originally identified in yeast (Elion 2001), several scaffolds that modulate MAPK activity have been recognised (Morrison and Davis 2003; Kolch 2005). Some of these will be discussed here.

4.1 Kinase Suppressor of Ras

Kinase suppressor of Ras (KSR) is a scaffold with a high degree of homology with C-Raf, although it does not appear to have any kinase activity (reviewed in Morrison 2001). KSR, which is one of the best characterised scaffolds in the MAPK pathway, binds to C-Raf, MEK1/2 and ERK1/2. Interestingly, MEK is constitutively associ-

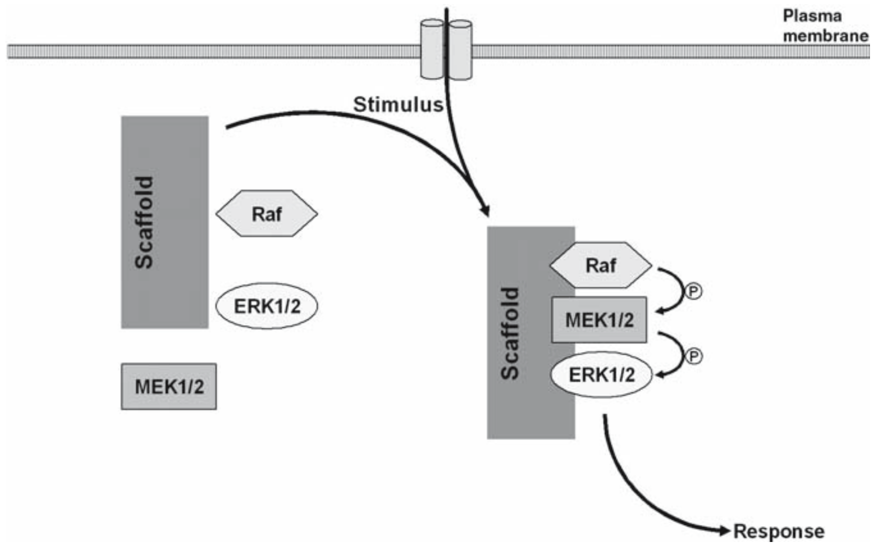

Fig. 4 Molecular scaffold for the MEK/ERK pathway. Molecular scaffolds bind to multiple components of the MAPK cascade, bringing them into close proximity, thereby facilitating signal propagation

ated to KSR, while ERK binds only in response to a stimulus. As is typical for scaffolds, optimal expression levels of KSR are required for maximal responses of MAPK to signalling cues (Kortum and Lewis 2004).

KSR seems to provide a docking platform at the plasma membrane onto which C-Raf, MEK1/2 and ERK1/2 can form a complex and allows efficient propagation of the signalling cascade (Fig. 5). In support of this notion is the finding that in quiescent cells, KSR is maintained in the cytosol through an interaction with 14-3-3 (Muller et al. 2001) and in a Triton-insoluble fraction through an interaction that "impedes mitogenic signal propagation" (IMP) (Matheny et al. 2004). Following stimulation by growth factors, KSR translocates to the plasma membrane, where it facilitates activation of MEK and ERK (Muller et al. 2001). Therefore, KSR is able to regulate the spatial activation of MEK/ERK, and presumably the cellular response. This concept is bolstered by the observation that overexpression in PC12 cells of B-KSR, a neuronal-specific isoform of KSR, switches EGF signalling from a brief proliferative signal to a sustained differentiation signal (Muller et al. 2000).

Although developmentally normal, KSR knockout mice have defects in antigen-triggered T cell proliferation (Nguyen et al. 2002) and are resistant to antibody-induced arthritis (Fusello et al. 2006). Furthermore, mouse embryonic fibroblasts from knockout mice display defects in activation of ERK by TNF-α and interleukin-1 (Fusello et al. 2006). Together, these studies strongly suggest that KSR fulfils an important role in the regulation of MAPK signalling during the immune response and inflammation. Interestingly, KSR null mice are less susceptible to Ras-mediated skin cancer (Lozano et al. 2003), identifying a role for KSR in the regulation of MAPK-mediated cell proliferation.

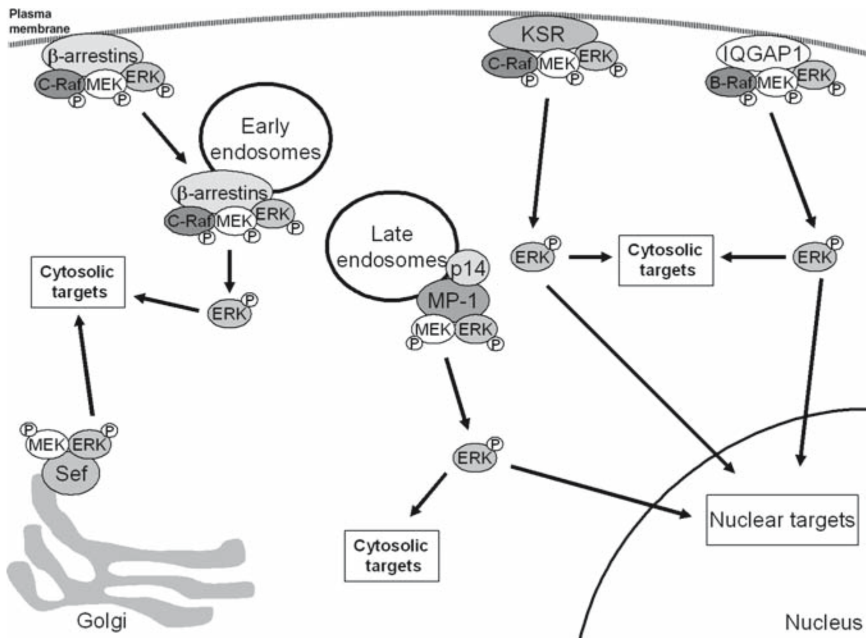

Fig. 5 Spatial regulation of MEK/ERK signalling by molecular scaffolds. Molecular scaffolds regulate the subcellular distribution of components of the MAPK pathway. Both IQGAP1 and KSR bind to Raf, MEK and ERK and are thought to localise the kinases to the plasma membrane, while MP-1 (through it interaction with p14) localises active MEK and ERK to endosomes. Following activation, ERK is free to phosphorylate cytosolic targets or translocate to the nucleus and activate nuclear targets. Like MP-1, β-arrestins localise Raf, MEK and ERK to endosomes. However, once activated ERK is prevented from translocating to the nucleus and is restricted to cytosolic targets by an unknown mechanism. Sef localises MEK and ERK to the Golgi and, like β-arrestin, prevents translocation of ERK to the nucleus, limiting it to cytosolic substrates

4.2 IQGAP1

IQGAP1 is a large, widely expressed protein that regulates many signalling pathways and cellular functions (reviewed in Briggs and Sacks 2003a; Brown and Sacks 2006). With several domains, IQGAP1 is able to bind to a broad spectrum of proteins (Brown and Sacks 2006), thereby modulating actin dynamics, microtubule dynamics, cell–cell adhesion and transcriptional regulation. Recent studies have provided strong evidence that IQGAP1 is a scaffold for the MAPK cascade. IQGAP1 binds directly to MEK1, MEK2, ERK1 and ERK2 (Fig. 5) and regulates their activation in response to EGF (Roy et al. 2004, 2005) and CD44 (Bourguignon et al. 2005). Analogous to KSR, both an increase and a decrease in the IQGAP1 expression level attenuates EGF-dependant activation of MEK and ERK, suggesting that the correct stoichiometry of IQGAP1 to MAPK components is required for efficient propagation of the cascade (Roy et al. 2004, 2005). Interestingly, while ERK associates constitutively with IQGAP1 and the binding is not sensitive

to EGF, the interaction between IQGAP1 and MEK1 increases, while that with MEK2 decreases following EGF treatment (Roy et al. 2005). This raises the possibility that IQGAP1 preferentially activates the MEK1 signalling pathway. It has been suggested that MEK1 promotes proliferation, while MEK2 promotes differentiation (Ussar and Voss 2004), and IQGAP1 may therefore regulate the cellular response to MAPK signalling. In addition, new findings reveal that IQGAP1 binds directly to B-Raf and modulates B-Raf kinase activity (Ren et al. 2007). Importantly, deletion of IQGAP1 renders B-Raf insensitive to EGF stimulation. Together these findings highlight the importance of IQGAP1 in the B-Raf/MEK/ERK signalling cascade.

Following stimulation with growth factors, the MAPK pathway regulates many cellular responses, one of which is the modulation of the cytoskeleton (Reszka et al. 1997). IQGAP1 is a well-documented regulator of the actin and microtubule cytoskeletons (Briggs and Sacks 2003b; Noritake et al. 2005), and it is tempting to speculate that IQGAP1 links MAPK signalling to cytoskeletal dynamics. In support of this idea, both IQGAP1 and ERK2 localise to microtubule-associated protein-2 in neuronal cells (Morishima-Kawashima and Kosik 1996; Li et al. 2005).

4.3 MEK Partner-1

MEK partner-1 (MP-1) is a widely expressed scaffold that promotes the association of MEK with ERK, thereby facilitating ERK phosphorylation (Schaeffer et al. 1998). Through an interaction with p14, MP-1 is localised to endosomes (Fig. 5), and a reduction in expression of either MP-1 or p14 attenuates EGF-dependant ERK activation (Teis et al. 2002). Conversely, increasing expression of either MP-1 or p14 promotes activation of ERK by EGF. Endosome localisation is crucial for MP-1 activity, as mislocalisation abrogates the positive effect of MP-1 overexpression on ERK activation (Teis et al. 2002). MP-1 binds MEK1 constitutively, but associates with ERK transiently. Following phosphorylation by MEK1, ERK is released from MP-1, and it has been proposed that MP-1 can translate a weak MEK signal into sustained ERK activation (Sharma et al. 2005).

4.4 β-Arrestins

β-Arrestins are well-known regulators of GPCR. Following activation of the receptor, β-arrestins bind to phosphorylated motifs, causing dissociation of the heterotrimeric G-protein, and targets the receptor to clathrin-coated pits (Fig. 5) (reviewed in Lefkowitz and Whalen 2004). Downstream of GPCR, β-arrestins assemble complexes of C-Raf, MEK and ERK, which accompany the receptor to early endosomes to promote efficient MAPK signalling (Tohgo et al. 2003). Importantly for compartmentalised MAPK signalling, β-arrestins prevent the translocation of ERK into the nucleus

(Fig. 5), thereby reducing phosphorylation of nuclear substrates and consequently MAPK-dependant gene expression (Tohgo et al. 2003). Instead, active ERK is maintained in the cytosol, presumably promoting phosphorylation of cytosolic targets.

4.5 Similar Expression to FGF

Similar expression to FGF (Sef) is a transmembrane protein, whose expression is under the control of fibroblast growth factor (FGF) (Furthauer et al. 2002). Sef captures active MEK/ERK complexes at the Golgi (Fig. 5), promoting ERK activation (Torii et al. 2004). Like β-arrestin, Sef prevents translocation of ERK to the nucleus, thereby restricting signalling to cytosolic substrates (Torii et al. 2004). Because of the distinct roles of MAPK signalling from the Golgi, Sef is likely to influence the cellular response to MAPK activation. Indeed, expression of Sef in PC12 cells prevents FGF and NGF-dependant differentiation (Xiong et al. 2003).

5 MAPK as Drug Targets

As described above, the MAPK cascade regulates numerous cellular functions, many of which have been implicated in disease. The ability of this pathway to regulate growth factor signalling, differentiation, proliferation and immune responses has made it an attractive target for pharmacological agents targeting cancer or inflammatory disorders. Furthermore, the diversity of the signalling networks should enable drugs to target highly specific cellular responses, thereby reducing side effects. Here we discuss the current therapies directed against the MAPK pathway, and the signalling components that provide potential future targets.

5.1 MAPK and Oncogenes

The participation of the MAPK signalling module in cancer has been the subject of intense research. Ras genes encoded by rat sarcoma virus, *v-H-Ras* and *v-K-Ras*, were among the first oncogenes discovered (DeFeo et al. 1981; Ellis et al. 1981; Ruta et al. 1986). These mutant forms of cellular Ras genes are insensitive to the activity of GAPs. Consequently, the mutant Ras proteins are constitutively GTP-bound, and therefore constitutively active. Ras is the most common mutated oncogene in human neoplasms, with 30% of all cancers harbouring a Ras mutation (Mor and Philips 2006). The Ras isoforms display distinct prevalence among different cancers. K-Ras mutations have been found in non-small-cell lung, colorectal, ovarian and pancreatic carcinomas, H-Ras in bladder, kidney and thyroid carcinomas and N-Ras in melanoma, hepatocellular carcinoma and haemtologic malignancies (Mor and Philips 2006). Mutations in N-Ras are particularly prevalent in melanoma, having

been identified in 15–30% of cases (Gray-Schopfer et al. 2007). The most common mutation is Q61L, which results in constitutively GTP-bound Ras, and therefore hyperstimulation of the MAPK cascade (Gray-Schopfer et al. 2007).

In addition to Ras, other proteins in the MAPK pathway contribute to carcinogenesis. The B-Raf mutation, V600E (originally reported as V599E, Wellbrock et al. 2004), has been identified in approximately 60% of patients with human melanoma and is the most common mutation is such cancers (Gray-Schopfer et al. 2007). The V600E mutation results in highly active B-Raf kinase, which is independent of the GTP/GDP-bound state of Ras. Consequently, the Raf/MEK/ERK cascade is hyperactive. Further mutations in B-Raf have been identified in breast, colon and ovarian cancer (Davies et al. 2002).

Many cancers display MAPK hyperactivation without any of the mutations described above, suggesting deregulation of the cascade by (an) unknown mechanism(s). While it is possible that the overactive MAPKs contribute to tumourogenesis, it is not clear whether the hyperactivity is a cause or consequence of neoplastic transformation. Increased ERK phosphorylation and expression has been found in pancreatic cancer (Tan et al. 2004), and increased ERK phosphorylation correlates with tumour progression in prostate cancer (Gioeli et al. 1999). Increased MEK phosphorylation has been identified in colon cancer (Lee et al. 2004) and in 74% of myeloblasts in acute myelogenous leukaemia (Milella et al. 2005). Finally, increased MAPK activity has also been demonstrated in breast cancer (Sivaraman et al. 1997; Coutts and Murphy 1998). The involvement of the MAPK pathway in breast cancer is complicated because of the crosstalk between MAPKs and oestrogen signalling. Treatment of breast cancer cells with estradiol activates MAPKs directly and potentially indirectly through increased production of TGFα, IGF-1 and IGFR (Santen et al. 2002). Consequently, MAPKs contribute, at least in part, to estradiol-dependant proliferation. The ability of ERK to phosphorylate and activate the oestrogen receptor provides a potential positive feedback mechanism (Kato et al. 1995). Importantly for treatment of breast cancer, pharmacologically depriving hormone-dependant breast cancer cell lines of estradiol increases MAPK activity, leading to regrowth of the tumour (Santen et al. 2002).

5.1.1 Targeting the MAPK Pathway for Cancer Therapy

Due to the overwhelming evidence for increased activation of the MAPK pathway in multiple cancers, it is not surprising that components of this signalling network have been a focus of the pharmacotherapeutic industry (Gray-Schopfer et al. 2007). The MAPK cascade can be targeted at different levels, ranging from receptors in the plasma membrane through Ras, Raf and MEK to ERK. Several compounds, such as the tyrosine kinase inhibitor, Imatinib, which inhibits the ABL, KIT and PDGFR kinases (Pui and Jeha 2007), have been developed to inhibit receptor function. These agents will not be addressed in this review. We will concentrate here on the efforts to target the intracellular components of the MAPK cascade.

5.1.2 Ras

Four farnesyl transferase inhibitors (FTIs) are in phase-III clinical trials, at the time of writing, showing positive responses for breast, pancreatic and colorectal cancers as well as leukaemia. These compounds compete with farnesyl transferases for the CAAX motif, thereby preventing addition of a farnesyl group. By this mechanism, FTIs inhibit membrane localisation of Ras (Adjei 2001) and consequently its activation by membrane-localised GEFs. The specificity of these agents has not been unequivocally established, and it is possible that these drugs also attenuate farnesylation of proteins other than Ras (Adjei 2001).

In the presence of FTIs, both K-Ras and N-Ras can be geranylgeranylated (James et al. 1996). Geranylgeranylation is a posttranslational modification catalysed by geranylgeranyltransferase type 1. A 20-carbon geranylgeranyl lipid is added to small GTPases, such as RhoA or Cdc42, to localise them to the plasma membrane. A recent study showed that deletion of geranylgeranyltransferase type 1 inhibits K-Ras-dependant cell proliferation and lung tumour formation, thereby increasing survival of mice expressing oncogenic K-Ras in lung tissue (Sjogren et al. 2007). Therefore, combined therapy, antagonising both geranylgeranyltransferases and farnesyltransferases, may be more effective than monotherapy in targeting Ras.

5.1.3 Raf

Raf kinases also provide attractive pharmacotherapeutic targets, and several C-Raf inhibitors are currently in clinical trials. ISIS5132 is a small antisense oligonucleotide that reduces C-Raf mRNA and kinase activity. Antisense oligonucleotides decrease protein production by specifically blocking the coding gene, thereby reducing the amount of the target protein in tumour cells. ISIS5132 is currently in phase-II trials for metastatic breast cancer and metastatic ovarian cancer. A second antisense oligonucleotide targeting C-Raf, LErafAON-ETU (Neopharm), is in phase-I studies for advanced solid tumours. Small molecule inhibitors of C-Raf, such as XL-281 (Exelixis), have also progressed into early clinical trials. It is possible that some of the small molecules also inhibit enzymes other than C-Raf, such as receptor tyrosine kinases (Sebolt-Leopold and Herrera 2004). The multi-kinase inhibitor, sorafenib (BAY 43-9006), which targets B-Raf, C-Raf, VEGFR and PDGFR, was approved by the US Food and Drug Administration in 2005 for treatment of adults with advanced renal cell carcinoma.

The identification of the important contribution of ^{V600E}B-Raf to the development of melanoma has stimulated the interest of many companies to target B-Raf in the chemotherapy of melanoma (Gray-Schopfer et al. 2007). A specific B-Raf inhibitor, CHIR-265, is in phase-I safety trials (Gray-Schopfer et al. 2007), while sorafenib has reached phase II. Alone sorafenib has modest activity against melanoma and is far more effective when combined with other therapies (Flaherty 2006). The reason why sorafenib monotherapy of melanoma is ineffective is not

known. More potent B-Raf drugs are required to ascertain whether B-Raf is a suitable target in melanoma (Gray-Schopfer et al. 2007). In addition to targeting B-Raf, inhibitors of C-Raf are being explored as potential chemotherapeutics for melanoma. The rationale is that C-Raf is required for proliferation of melanoma cells harbouring ^{V600E}B-Raf and also activates MEK in melanoma caused by Ras mutations (Dumaz et al. 2006).

5.1.4 MEK

Downstream from B-Raf, antagonism of MEK is being pursued as potential therapy for melanoma. Two MEK inhibitors, PD0325901 (Pfizer) and AZD6244 (Astrazeneca), attenuate in vitro proliferation, soft-agar colony formation and matrigel invasion of melanoma cell lines harbouring the ^{V600E}B-Raf mutation (Collisson et al. 2003). Interestingly, melanoma cells with V600E B-Raf are more sensitive to MEK inhibition than melanoma cells harbouring an activating Ras mutation (Solit et al. 2006), suggesting mutant Ras signals through pathways other than MEK/ERK in melanoma. Both PD0325901 and AZD6244 have recently moved to phase-II studies for advanced melanoma (Gray-Schopfer et al. 2007). Blocking MEK function is also being evaluated in other neoplasms. The MEK inhibitor XL518 (Exelixis) is in phase-I trials for patients with solid tumours.

5.1.5 ERK

Direct inhibition of ERK activity has not been widely adopted as a therapeutic approach. Instead, blocking the ability of MEK to phosphorylate ERK has been used to downregulate ERK function.

5.1.6 p38

Although the MEK/ERK pathway has been the major MAPK pathway targeted for cancer chemotherapy, inhibitors of p38 are currently also being assessed. For example, SCIO-469 (Scios) is in phase-II studies for myelodysplastic syndrome.

5.2 MAPK and Inflammatory Diseases

There is a wealth of evidence highlighting MAPK function in the regulation and function of the immune system (Alberola-Ila and Hernandez-Hoyos 2003; Ashwell 2006). Consistent with these data, the MAPK cascade has been implicated in pathogenesis of numerous inflammatory disorders. For example, the activity of the major MAPK pathways is increased in rheumatoid arthritis (Sweeney and Firestein 2006).

In psoriasis, both expression and activity of ERK are enhanced, and JNK phosphorylation is increased (Sweeney and Firestein 2006). p38 contributes to inflammatory bowel disease, allergic airway disease and asthma, and increased JNK activity has been shown in systemic lupus erythematosus (Sweeney and Firestein 2006).

5.2.1 Targeting the MAPK Pathway for Treatment of Inflammatory Diseases

Highly potent and specific p38 inhibitors block the production of inflammatory cytokines such as IL-1, TNF-α and IL-6. Consequently, such compounds are under investigation for treating inflammatory disorders, including inflammatory bowel disease, asthma and rheumatoid arthritis (Kumar et al. 2003). Antagonism of p38 in an animal model of asthma reduces both cytokine production and chemotaxis of eosinophils into the airways (Popescu 2003). Rheumatoid arthritis is an inflammatory disease of the synovial joints marked by production of pro-inflammatory cytokines. Inhibitors of p38 have been effective in animal models of rheumatoid arthritis (Kumar et al. 2003), and these drugs, such as PH-797804 (Pfizer), are in clinical trials.

In animal models, JNK inhibitors reduced recruitment of leukocytes in asthma and attenuated joint destruction in arthritis (O'Neill et al. 2006). The p38 and JNK inhibitor, CNI-1493 (Cytokine PharmaSciences), which reduces both macrophage activation and production of TNF-α, IL-1 and IL-6, entered early human studies for inflammatory bowel disease (Stokoe et al. 1994). Patients exhibited mucosal healing and reduced disease activity, lending some hope for these therapies. Currently, CNI-1493 is undergoing phase-III trials for Crohn's disease.

5.3 Targeting the MAPK Pathway in Other Diseases

While the focus of research in manipulating the MAPK cascade in disease has concentrated on cancer and inflammatory disorders, there is evidence to suggest that modulation of these kinases may prove effective in other conditions. For example, replication of human immunodeficiency virus (HIV) is attenuated in both T cells and monocytes by the p38 inhibitor, RWJ67657 (Muthumani et al. 2004). Activity of reverse transcriptase was suppressed, while HIV-induced apoptosis of T cells was blocked, suggesting that targeting the p38 MAPK pathway could provide a means to reduce HIV pathogenicity.

In a mouse animal model of myocardial injury, inhibition of both p38 and fibroblast growth factor (FGF) increased mitosis in cardiomyocytes, reduced scarring and wall thinning and improved cardiac function (Engel et al. 2006). While the p38 inhibitor alone did not improve cardiac function, it did increase cardiomyocyte mitosis. These findings raise the possibility that the p38 MAPK pathway may be targeted, potentially in combination with other treatments, to promote recovery following a heart attack. Consistent with these preclinical obser-

vations, the p38 inhibitor SB-681323 (GlaxoSmithKline) is in phase-II studies for patients with coronary heart disease.

5.4 Future Direction for Therapies Directed Against the MAPK Cascade

Although several MAPK inhibitors are currently under assessment in clinical trials, advances in developing novel therapies for treatment of cancer and inflammatory disease have been disappointing. There are problems associated with the use of conventional kinase inhibitors for targeting the MAPK pathway, including lack of specificity, which potentially contributes to adverse effects. In order to address this issue, future therapies will need to use alternative methods to regulate MAPK signalling, and several approaches are being pursued.

5.4.1 Small Molecules

Many of the MAPK inhibitors are targeted against the ATP-binding site of the kinases. The structure of the ATP-binding pocket for several of these kinases has been solved, and it is relatively straightforward to design small molecules that block access of ATP, thereby specifically inhibiting the kinase. Over the last few years, a different strategy has emerged. The objective is to modulate protein:protein interactions with small molecules. Although more challenging than conventional kinase inhibitors, advances in our knowledge and understanding of protein complexes will greatly facilitate the development of such compounds. For example, in the MAPK pathway, inhibiting the association between Ras and SOS should be an effective method to reduce the activation of Ras in response to growth factors. This concept has been established in principle. A small molecule, NSC23766, which inhibits the interaction between Rac1 and the Rac1 GEFs TrioN and Tiam1 in vivo, prevents activation of Rac1 and attenuates Rac1-dependant cell growth, colony formation and anchorage-dependant growth (Gao et al. 2004). Another approach would be to stabilise the GTPase in a non-functional complex. Again, this has been shown for a GTPase other than Ras. The antibiotic, Brefeldin A, stabilises the complex of GDP-bound Arf with Arf GEFs, thereby preventing signalling (Peyroche et al. 1999). These preclinical studies establish proof-of-principle and are likely to eventually yield agents that are suitable for clinical studies.

5.4.2 Compartment-Specific Inhibition

As described above, the compartment from which MAPK signals influences the cellular response to the stimulus. Therefore, it would be advantageous to inhibit MAPK

in a specific cellular domain, such as endosomes or the Golgi, while minimising interference of MAPK signalling from other parts of the cell. The selective targeting of MAPK signalling from a distinct compartment may modulate a discrete MAPK response, thereby antagonising signalling from a specific stimulus or from a particular upstream kinase. Importantly, by not inhibiting total cellular MAPKs, pathways that are not involved in the disease or disorder should be spared. Such therapies could provide high selectivity with potentially fewer adverse effects. At present, there is no evidence that such an approach is feasible or effective. Although conceptually appealing, a greater understanding of MAPK signalling from distinct subcellular compartments is required before such compounds can be developed.

5.4.3 Molecular Scaffolds

In the future, molecular scaffolds may provide effective therapeutic targets. Multiple scaffolds participate in the MAPK pathway, with each probably having a specific, non-overlapping function. Scaffolds are also important regulators of the localisation of many MAPK components (see Sect. 4). Pharmacological modulation of scaffolds may enable specific regulation of MAPK function, directing the cellular response towards (or away from) a particular response (e.g. cell proliferation), without attenuating global MAPK activity. Furthermore, targeting scaffolds may allow inhibition of MAPK signalling from a specific cellular compartment, providing further specificity to MAPK modulation. There is little evidence to show effective pharmaceutical targeting of scaffolds. Nevertheless, published data support the concept. For example, KSR appears to be involved in Ras-mediated cancer (see Sect. 4.1) and could, therefore, provide an effective target for tumours displaying hyperactive Ras. In addition, KSR knockout mice are less susceptible to induction of rheumatoid arthritis and exhibit defects in TNF-α and IL-1 signalling (Fusello et al. 2006), suggesting that KSR could also provide a novel target for therapeutic intervention in inflammatory diseases.

Like KSR, IQGAP1 is a scaffold in MAPK signalling and has been implicated in carcinogenesis (Briggs and Sacks 2003b; Brown and Sacks 2006). For example, IQGAP1 is upregulated by gene amplification in some diffuse types of gastric carcinoma (Sugimoto et al. 2001), and IQGAP1 protein is overexpressed in colorectal carcinoma, particularly at the invasion front (Nabeshima et al. 2002). The translocation of IQGAP1 from the cytoplasm to the cell membrane, which inhibits E-cadherin-mediated cell–cell adhesion (Li et al. 1999), correlates with E-cadherin dysfunction and tumour dedifferentiation in gastric carcinoma (Takemoto et al. 2001). In addition, a screen for genes exhibiting altered expression in a mouse model of metastatic melanoma, identified *Iqgap1*, its regulator, calmodulin and ERK as 3 of only 32 genes (from ~10,500 arrayed genes) that showed a >2.5-fold increase in expression in metastatic cells (Clark et al. 2000). Therefore, IQGAP1 and calmodulin are likely to be important in metastasis. Furthermore, IQGAP1 promotes cell migration and invasion, via direct interactions with Cdc42, Rac1, actin and calmodulin (Mataraza et al. 2003a, 2007). Importantly, overexpression of

IQGAPI in human breast epithelial cells increases formation and invasion of tumours in immunocompromised mice, while human breast carcinoma displays higher IQGAPI expression levels compared with normal breast tissue (Jadeski et al. 2008). Collectively, these data suggest IQGAP1 is involved in tumourigenesis and metastasis, and therefore may provide a novel target for therapeutic intervention.

While no chemical inhibitors of IQGAP1 have been developed, two approaches support the concept of IQGAP1 as a therapeutic target. In the first approach, transfection of a dominant negative IQGAP1 construct reduced motility and invasion of malignant human breast epithelial cells (Mataraza et al. 2003a). Moreover, dominant negative IQGAP1 reduced the motility of malignant breast cells induced by constitutively active Cdc42 (Mataraza et al. 2003a). The second strategy employed a peptide corresponding to the Cdc42-binding domain of IQGAP1, which disrupts the interaction between Cdc42 and IQGAP1 (Mataraza et al. 2003b). Treatment of cultured cells with this cell-permeable peptide inhibited protein-tyrosine phosphatase μ-mediated neurite outgrowth (Phillips-Mason et al. 2006). These approaches neither targeted MAPK signalling, nor are they amenable to oral or parenteral therapy. Nevertheless, the findings suggest that modulating IQGAP1 function may be beneficial. The peptide strategy was implemented to modulate the function of the JNK pathway scaffold, JNK interacting protein 1 (JIP1). A peptide corresponding to the JNK binding domain of JIP1, which inhibits JNK activity (Barr et al. 2004), protected neurones in a mouse model of Parkinson's disease (Xia et al. 2001).

Documentation of the participation of other MAPK scaffolds in neoplasia is less abundant, but some of it is persuasive. For example, loss of Sef expression correlates with high-grade metastatic prostate cancer (Darby et al. 2006). In addition, through an interaction with c-Src, β-arrestins modulate transactivation of EGFR by prostaglandin E_2 in colorectal carcinoma cell lines (Buchanan et al. 2006). The interaction between β-arrestin and c-Src is crucial for migration of colorectal carcinoma cells in vitro and metastasis in vivo. Thus, disruption of the normal homeostatic regulation mediated by the scaffolds may result in neoplasia. Collectively, the evidence outlined above supports the concept of developing compounds that selectively modulate the interaction of scaffolds with individual targets as a means to specifically modify MAPK activity.

6 Perspectives

It is now clear MAPK signalling is considerably more intricate than originally believed. Once considered to occur only at the plasma membrane, signalling is now known to originate from endomembranes including the Golgi, ER and endosomes, as well as from specific plasma membrane compartments. The cellular domain from which MAPK signals is an important factor in determining how the cell responds to a particular stimulus. This localisation is partly under the regulation of post-translational modifications, such as farnesylation. However, it is becoming evident that molecular scaffolds are integral to spatio-temporal regulation of signalling.

The combination of spatial, temporal and amplitude regulation, coupled with the large number of external factors known to activate the MAPKs, provides a glimpse of a complex network of pathways and how cells are able to interpret diverse signals to appropriately respond to their environment.

While many of the MAPK components have been targeted for therapy, as yet this strategy has failed to yield effective pharmacotherapeutic agents. The lack of efficacy may be due, at least in part, to the lack of selectivity when inhibiting a key signalling kinase, such as MEK, Raf or p38. Because these kinases regulate myriad cellular functions, inhibiting their activity is likely to affect multiple processes, some not linked to the pathophysiology of the disease being targeted. Therefore, new approaches are being explored to enhance specificity. Different strategies could be adopted to modulate signalling from a specific compartment. An indirect approach would be to inhibit proteins that alter the subcellular localisation of MAPKs. This concept has been shown in principle with the development of FTIs, which prevent membrane localisation of Ras. The efficacy is limited by the relative non-specificity of FTIs, which prevents Ras from anchoring into any membrane. However, as we gain further insight into the mechanisms controlling the location of the MAPKs, more specific targets may be identified. For example, inhibition of as yet unidentified chaperone proteins might prevent Ras from localising to the Golgi, but not affect localisation of Ras to the plasma membrane.

A second approach would be to develop small molecules that are, themselves, localised to specific cellular compartments. For example, by restricting a small molecule inhibitor of B-Raf to the endosome, B-Raf signalling from that compartment would be antagonised, while signalling from other compartments, such as the plasma membrane, would remain unaffected. This concept has been established in principle, with the development of fluorescent probes that localise to specific subcellular compartments and organelles (Zorov et al. 2004). This idea is further supported by the development of a specific peptide inhibitor of calmodulin, which was selectively localised to the nucleus or plasma membrane. Antagonism of calmodulin function in discrete subcellular domains differentially modulates cellular responses (Li et al. 2003; Mataraza et al. 2007). It is therefore theoretically possible to target inhibitors of MAPKs to specific cellular domains.

An alternative strategy to modulate compartmentalised MAPK signalling is to manipulate the function of protein scaffolds. These molecules regulate many aspects of the MAPK cascade and may offer an opportunity to provide the specificity required for effective therapy. Several MAPK scaffolds have been identified, and it is likely that other, as yet unidentified, molecules exist. Therefore, scaffolds provide a reservoir of potential targets, each of which controls highly specific aspects of MAPK function.

Despite the intense effort directed by scientists in both academia and industry, the progress in developing effective therapies for malignant or inflammatory disease by targeting MAPK signalling has been disappointing. While preclinical findings have been promising, toxicity and/or a poor therapeutic response has prevented widespread adoption of MAPK inhibitors as pharmacotherapeutic agents. We look forward to advances in the understanding of MAPK signalling, including the role

played by molecular scaffolds, and how this translates into the development of specific, targeted pharmacologic agents.

Acknowledgements We thank Rob Krikorian for assistance with preparation of the manuscript. Work in the authors' laboratory is funded by grants from the National Institutes of Health.

References

Adams JM, Houston H, Allen J, Lints T, Harvey R (1992) The hematopoietically expressed vav proto-oncogene shares homology with the dbl GDP-GTP exchange factor, the bcr gene and a yeast gene (CDC24) involved in cytoskeletal organization. Oncogene 7:611–618

Adjei AA (2001) Blocking oncogenic Ras signaling for cancer therapy. J Natl Cancer Inst 93:1062–1074

Alberola-Ila J, Hernandez-Hoyos G (2003) The Ras/MAPK cascade and the control of positive selection. Immunol Rev 191:79–96

Arozarena I, Matallanas D, Berciano MT, Sanz-Moreno V, Calvo F, Munoz MT, Egea G, Lafarga M, Crespo P (2004) Activation of H-Ras in the endoplasmic reticulum by the RasGRF family guanine nucleotide exchange factors. Mol Cell Biol 24:1516–1530

Ashwell JD (2006) The many paths to p38 mitogen-activated protein kinase activation in the immune system. Nat Rev Immunol 6:532–540

Barr RK, Boehm I, Attwood PV, Watt PM, Bogoyevitch MA (2004) The critical features and the mechanism of inhibition of a kinase interaction motif-based peptide inhibitor of JNK. J Biol Chem 279:36327–36338

Bivona TG, Perez De Castro I, Ahearn IM, Grana TM, Chiu VK, Lockyer PJ, Cullen PJ, Pellicer A, Cox AD, Philips MR (2003) Phospholipase Cgamma activates Ras on the Golgi apparatus by means of RasGRP1. Nature 424:694–698

Bos JL (2005) Linking Rap to cell adhesion. Curr Opin Cell Biol 17:123–128

Bourguignon LY, Gilad E, Rothman K, Peyrollier K (2005) Hyaluronan-CD44 interaction with IQGAP1 promotes Cdc42 and ERK signaling, leading to actin binding, Elk-1/estrogen receptor transcriptional activation, and ovarian cancer progression. J Biol Chem 280:11961–11972

Briggs MW, Sacks DB (2003a) IQGAP1 as signal integrator: Ca^{2+}, calmodulin, Cdc42 and the cytoskeleton. FEBS Lett 542:7–11

Briggs MW, Sacks DB (2003b) IQGAP proteins are integral components of cytoskeletal regulation. EMBO reports 4:571–574

Brown MD, Sacks DB (2006) IQGAP1 in cellular signaling: bridging the gap. Trends Cell Biol 16:242–249

Buchanan FG, Gorden DL, Matta P, Shi Q, Matrisian LM, DuBois RN (2006) Role of beta-arrestin 1 in the metastatic progression of colorectal cancer. Proc Natl Acad Sci USA 103:1492–1497

Burke P, Schooler K, Wiley HS (2001) Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking. Mol Biol Cell 12:1897–1910

Carozzi AJ, Roy S, Morrow IC, Pol A, Wyse B, Clyde-Smith J, Prior IA, Nixon SJ, Hancock JF, Parton RG (2002) Inhibition of lipid raft-dependent signaling by a dystrophy-associated mutant of caveolin-3. J Biol Chem 277:17944–17949

Chao MV (2003) Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat Rev Neurosci 4:299–309

Chiu VK, Bivona T, Hach A, Sajous JB, Silletti J, Wiener H, Johnson RL 2nd, Cox AD, Philips MR (2002) Ras signalling on the endoplasmic reticulum and the Golgi. Nat Cell Biol 4:343–350

Choy E, Chiu VK, Silletti J, Feoktistov M, Morimoto T, Michaelson D, Ivanov IE, Philips MR (1999) Endomembrane trafficking of ras: the CAAX motif targets proteins to the ER and Golgi. Cell 98:69–80

Clark EA, Golub TR, Lander ES, Hynes RO (2000) Genomic analysis of metastasis reveals an essential role for RhoC. Nature 406:532–535

Collisson EA, De A, Suzuki H, Gambhir SS, Kolodney MS (2003) Treatment of metastatic melanoma with an orally available inhibitor of the Ras-Raf-MAPK cascade. Cancer Res 63:5669–5673

Coutts AS, Murphy LC (1998) Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. Cancer Res 58:4071–4074

Darby S, Sahadevan K, Khan MM, Robson CN, Leung HY, Gnanapragasam VJ (2006) Loss of Sef (similar expression to FGF) expression is associated with high grade and metastatic prostate cancer. Oncogene 25:4122–4127

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA (2002) Mutations of the BRAF gene in human cancer. Nature 417:949–954

DeFeo D, Gonda MA, Young HA, Chang EH, Lowy DR, Scolnick EM, Ellis RW (1981) Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc Natl Acad Sci USA 78:3328–3332

Dumaz N, Hayward R, Martin J, Ogilvie L, Hedley D, Curtin JA, Bastian BC, Springer C, Marais R (2006) In melanoma, RAS mutations are accompanied by switching signaling from BRAF to CRAF and disrupted cyclic AMP signaling. Cancer Res 66:9483–9491

Elion EA (2001) The Ste5p scaffold. J Cell Sci 114:3967–3978

Ellis RW, Defeo D, Shih TY, Gonda MA, Young HA, Tsuchida N, Lowy DR, Scolnick EM (1981) The p21 src genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature 292:506–511

Engel FB, Hsieh PC, Lee RT, Keating MT (2006) FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction. Proc Natl Acad Sci USA 103:15546–15551

Engelman JA, Zhang XL, Lisanti MP (1999) Sequence and detailed organization of the human caveolin-1 and -2 genes located near the D7S522 locus (7q31.1). Methylation of a CpG island in the 5 promoter region of the caveolin-1 gene in human breast cancer cell lines. FEBS Lett 448:221–230

Flaherty KT (2006) Chemotherapy and targeted therapy combinations in advanced melanoma. Clin Cancer Res 12:2366s–2370s

Furthauer M, Lin W, Ang SL, Thisse B, Thisse C (2002) Sef is a feedback-induced antagonist of Ras/MAPK-mediated FGF signalling. Nat Cell Biol 4:170–174

Fusello AM, Mandik-Nayak L, Shih F, Lewis RE, Allen PM, Shaw AS (2006) The MAPK scaffold kinase suppressor of Ras is involved in ERK activation by stress and proinflammatory cytokines and induction of arthritis. J Immunol 177:6152–6158

Gao Y, Dickerson JB, Guo F, Zheng J, Zheng Y (2004) Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. Proc Natl Acad Sci USA 101:7618–7623

Gioeli D, Mandell JW, Petroni GR, Frierson HF Jr, Weber MJ (1999) Activation of mitogen-activated protein kinase associated with prostate cancer progression. Cancer Res 59:279–284

Gray-Schopfer V, Wellbrock C, Marais R (2007) Melanoma biology and new targeted therapy. Nature 445:851–857

Gustavsson J, Parpal S, Karlsson M, Ramsing C, Thorn H, Borg M, Lindroth M, Peterson KH, Magnusson KE, Stralfors P (1999) Localization of the insulin receptor in caveolae of adipocyte plasma membrane. FASEB J 13:1961–1971

Hancock JF (2003) Ras proteins: different signals from different locations. Nat Rev Mol Cell Biol 4:373–384

Hancock JF (2006) Lipid rafts: contentious only from simplistic standpoints. Nat Rev Mol Cell Biol 7:456–462
Hill CS, Wynne J, Treisman R (1995) The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell 81:1159–1170
Howe CL, Mobley WC (2004) Signaling endosome hypothesis: A cellular mechanism for long distance communication. J Neurobiol 58:207–216
Howe CL, Mobley WC (2005) Long-distance retrograde neurotrophic signaling. Curr Opin Neurobiol 15:40–48
Howe CL, Valletta JS, Rusnak AS, Mobley WC (2001) NGF signaling from clathrin-coated vesicles: evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway. Neuron 32:801–814
Huang CS, Zhou J, Feng AK, Lynch CC, Klumperman J, DeArmond SJ, Mobley WC (1999) Nerve growth factor signaling in caveolae-like domains at the plasma membrane. J Biol Chem 274:36707–36714
Jadeski L, Mataraza JM, Jeong HW, Li Z, Sacks DB (2008) IQGAPI stimulates proliferation and enhances tumourigenesis of human breast epithelial cells. J Biol Chem 283:1008–1017.
James G, Goldstein JL, Brown MS (1996) Resistance of K-RasBV12 proteins to farnesyltransferase inhibitors in Rat1 cells. Proc Natl Acad Sci USA 93:4454–4458
Jiang X, Sorkin A (2002) Coordinated traffic of Grb2 and Ras during epidermal growth factor receptor endocytosis visualized in living cells. Mol Biol Cell 13:1522–1535
Kalb R (2005) The protean actions of neurotrophins and their receptors on the life and death of neurons. Trends Neurosci 28:5–11
Karlsson M, Thorn H, Danielsson A, Stenkula KG, Ost A, Gustavsson J, Nystrom FH, Stralfors P (2004) Colocalization of insulin receptor and insulin receptor substrate-1 to caveolae in primary human adipocytes. Cholesterol depletion blocks insulin signalling for metabolic and mitogenic control. Eur J Biochem 271:2471–2479
Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P (1995) Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270: 1491–1494
Kolch W (2005) Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat Rev Mol Cell Biol 6:827–837
Kortum RL, Lewis RE (2004) The molecular scaffold KSR1 regulates the proliferative and oncogenic potential of cells. Mol Cell Biol 24:4407–4416
Kranenburg O, Verlaan I, Moolenaar WH (1999) Dynamin is required for the activation of mitogen-activated protein (MAP) kinase by MAP kinase kinase. J Biol Chem 274:35301–35304
Kretzschmar M, Doody J, Massague J (1997) Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. Nature 389:618–622
Kumar S, Boehm J, Lee JC (2003) p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. Nat Rev Drug Discov 2:717–726
Kuruvilla R, Zweifel LS, Glebova NO, Lonze BE, Valdez G, Ye H, Ginty DD (2004) A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. Cell 118:243–255
Labrecque L, Royal I, Surprenant DS, Patterson C, Gingras D, Beliveau R (2003) Regulation of vascular endothelial growth factor receptor-2 activity by caveolin-1 and plasma membrane cholesterol. Mol Biol Cell 14:334–347
Lai WH, Cameron PH, Doherty JJ 2nd, Posner BI, Bergeron JJ (1989) Ligand-mediated autophosphorylation activity of the epidermal growth factor receptor during internalization. J Cell Biol 109:2751–2760
Lee SH, Lee JW, Soung YH, Kim SY, Nam SW, Park WS, Kim SH, Yoo NJ, Lee JY (2004) Colorectal tumors frequently express phosphorylated mitogen-activated protein kinase. APMIS 112:233–238

Lefkowitz RJ, Whalen EJ (2004) Beta-arrestins: traffic cops of cell signaling. Curr Opin Cell Biol 16:162–168
Li Z, Kim SH, Higgins JM, Brenner MB, Sacks DB (1999) IQGAP1 and calmodulin modulate E-cadherin function. J Biol Chem 274:37885–37892
Li L, Li Z, Sacks DB (2003) Calmodulin regulates the transcriptional activity of estrogen receptors. Selective inhibition of calmodulin function in subcellular compartments. J Biol Chem 278:1195–1200
Li Z, McNulty DE, Marler KJM, Lim L, Hall C, Annan RS, Sacks DB (2005) IQGAP1 promotes neurite outgrowth in a phosphorylation-dependent manner. J Biol Chem 280: 13871–13878
Liu P, Ying Y, Ko YG, Anderson RG (1996) Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. J Biol Chem 271:10299–10303
Lockyer PJ, Kupzig S, Cullen PJ (2001) CAPRI regulates Ca(2+)-dependent inactivation of the Ras-MAPK pathway. Curr Biol 11:981–986
Lozano J, Xing R, Cai Z, Jensen HL, Trempus C, Mark W, Cannon R, Kolesnick R (2003) Deficiency of kinase suppressor of Ras1 prevents oncogenic ras signaling in mice. Cancer Res 63:4232–4238
Marais R, Wynne J, Treisman R (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell 73:381–393
Marshall CJ (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179–185
Mataraza JM, Briggs MW, Li Z, Entwistle A, Ridley AJ, Sacks DB (2003a) IQGAP1 promotes cell motility and invasion. J Biol Chem 278:41237–41245
Mataraza JM, Briggs MW, Li Z, Frank R, Sacks DB (2003b) Identification and characterization of the Cdc42-binding site of IQGAP1. Biochem Biophys Res Commun 305:315–321
Mataraza JM, Li Z, Jeong HW, Brown MD, Sacks DB (2007) Multiple proteins mediate IQGAP1-stimulated cell migration. Cell Signal 19:1857–1865
Matheny SA, Chen C, Kortum RL, Razidlo GL, Lewis RE, White MA (2004) Ras regulates assembly of mitogenic signalling complexes through the effector protein IMP. Nature 427:256–260
Milella M, Precupanu CM, Gregorj C, Ricciardi MR, Petrucci MT, Kornblau SM, Tafuri A, Andreeff M (2005) Beyond single pathway inhibition: MEK inhibitors as a platform for the development of pharmacological combinations with synergistic anti-leukemic effects. Curr Pharm Des 11:2779–2795
Mineo C, James GL, Smart EJ, Anderson RG (1996) Localization of epidermal growth factor-stimulated Ras/Raf-1 interaction to caveolae membrane. J Biol Chem 271:11930–11935
Montixi C, Langlet C, Bernard AM, Thimonier J, Dubois C, Wurbel MA, Chauvin JP, Pierres M, He HT (1998) Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. EMBO J 17:5334–5348
Mor A, Philips MR (2006) Compartmentalized Ras/MAPK signaling. Annu Rev Immunol 24:771–800
Morishima-Kawashima M, Kosik KS (1996) The pool of map kinase associated with microtubules is small but constitutively active. Mol Biol Cell 7:893–905
Morrison DK (2001) KSR: a MAPK scaffold of the Ras pathway? J Cell Sci 114:1609–1612
Morrison DK, Davis RJ (2003) Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annu Rev Cell Dev Biol 19:91–118
Muller J, Cacace AM, Lyons WE, McGill CB, Morrison DK (2000) Identification of B-KSR1, a novel brain-specific isoform of KSR1 that functions in neuronal signaling. Mol Cell Biol 20:5529–5539
Muller J, Ory S, Copeland T, Piwnica-Worms H, Morrison DK (2001) C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. Mol Cell 8:983–993
Muthumani K, Wadsworth SA, Dayes NS, Hwang DS, Choo AY, Abeysinghe HR, Siekierka JJ, Weiner DB (2004) Suppression of HIV-1 viral replication and cellular pathogenesis by a novel p38/JNK kinase inhibitor. AIDS 18:739–748

Nabeshima K, Shimao Y, Inoue T, Koono M (2002) Immunohistochemical analysis of IQGAP1 expression in human colorectal carcinomas: its overexpression in carcinomas and association with invasion fronts. Cancer Lett 176:101–109
Nguyen A, Burack WR, Stock JL, Kortum R, Chaika OV, Afkarian M, Muller WJ, Murphy KM, Morrison DK, Lewis RE, McNeish J, Shaw AS (2002) Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. Mol Cell Biol 22:3035–3045
Noritake J, Watanabe T, Sato K, Wang S, Kaibuchi K (2005) IQGAP1: a key regulator of adhesion and migration. J Cell Sci 118:2085–2092
O'Neill EJ, Day MJ, Wraith DC (2006) IL-10 is essential for disease protection following intranasal peptide administration in the C57BL/6 model of EAE. J Neuroimmunol 178:1–8
Parpal S, Karlsson M, Thorn H, Stralfors P (2001) Cholesterol depletion disrupts caveolae and insulin receptor signaling for metabolic control via insulin receptor substrate-1, but not for mitogen-activated protein kinase control. J Biol Chem 276:9670–9678
Parton RG, Hanzal-Bayer M, Hancock JF (2006) Biogenesis of caveolae: a structural model for caveolin-induced domain formation. J Cell Sci 119:787–796
Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22:153–183
Peiro S, Comella JX, Enrich C, Martin-Zanca D, Rocamora N (2000) PC12 cells have caveolae that contain TrkA. Caveolae-disrupting drugs inhibit nerve growth factor-induced, but not epidermal growth factor-induced, MAPK phosphorylation. J Biol Chem 275:37846–37852
Perez de Castro I, Bivona TG, Philips MR, Pellicer A (2004) Ras activation in Jurkat T cells following low-grade stimulation of the T-cell receptor is specific to N-Ras and occurs only on the Golgi apparatus. Mol Cell Biol 24:3485–3496
Peyroche A, Antonny B, Robineau S, Acker J, Cherfils J, Jackson CL (1999) Brefeldin A acts to stabilize an abortive ARF-GDP-Sec7 domain protein complex: involvement of specific residues of the Sec7 domain. Mol Cell 3:275–285
Phillips-Mason PJ, Gates TJ, Major DL, Sacks DB, Brady-Kalnay SM (2006) The receptor protein-tyrosine phosphatase PTPmu interacts with IQGAP1. J Biol Chem 281:4903–4910
Plyte S, Majolini MB, Pacini S, Scarpini F, Bianchini C, Lanfrancone L, Pelicci P, Baldari CT (2000) Constitutive activation of the Ras/MAP kinase pathway and enhanced TCR signaling by targeting the Shc adaptor to membrane rafts. Oncogene 19:1529–1537
Pol A, Calvo M, Enrich C (1998) Isolated endosomes from quiescent rat liver contain the signal transduction machinery. Differential distribution of activated Raf-1 and Mek in the endocytic compartment. FEBS Lett 441:34–38
Popescu FD (2003) New asthma drugs acting on gene expression. J Cell Mol Med 7:475–486
Prior IA, Harding A, Yan J, Sluimer J, Parton RG, Hancock JF (2001) GTP-dependent segregation of H-ras from lipid rafts is required for biological activity. Nat Cell Biol 3:368–375
Pui CH, Jeha S (2007) New therapeutic strategies for the treatment of acute lymphoblastic leukaemia. Nat Rev Drug Discov 6:149–165
Qian D, Weiss A (1997) T cell antigen receptor signal transduction. Curr Opin Cell Biol 9:205–212
Raman M, Cobb MH (2003) MAP kinase modules: many roads home. Curr Biol 13:R886–R888
Ravichandran KS, Lee KK, Songyang Z, Cantley LC, Burn P, Burakoff SJ (1993) Interaction of Shc with the zeta chain of the T cell receptor upon T cell activation. Science 262:902–905
Ren JG, Li Z, Sacks DB (2007) IQGAPI modulates activation of B-Rof. PNAS 104:10465–10469
Reszka AA, Bulinski JC, Krebs EG, Fischer EH (1997) Mitogen-activated protein kinase/extracellular signal-regulated kinase 2 regulates cytoskeletal organization and chemotaxis via catalytic and microtubule-specific interactions. Mol Biol Cell 8:1219–1232
Roux PP, Blenis J (2004) ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68:320–344
Roy S, Wyse B, Hancock JF (2002) H-Ras signaling and K-Ras signaling are differentially dependent on endocytosis. Mol Cell Biol 22:5128–5140

Roy M, Li Z, Sacks DB (2004) IQGAP1 binds ERK2 and modulates its activity. J Biol Chem 279:17329–17337
Roy M, Li Z, Sacks DB (2005) IQGAP1 is a scaffold for mitogen-activated protein kinase signalling. Mol Cell Biol 25:9740–9752
Ruta M, Wolford R, Dhar R, Defeo-Jones D, Ellis RW, Scolnick EM (1986) Nucleotide sequence of the two rat cellular rasH genes. Mol Cell Biol 6:1706–1710
Sacks DB (2006) The role of scaffold proteins in MEK/ERK signalling. Biochem Soc Trans 34:833–836
Santen RJ, Song RX, McPherson R, Kumar R, Adam L, Jeng MH, Yue W (2002) The role of mitogen-activated protein (MAP) kinase in breast cancer. J Steroid Biochem Mol Biol 80:239–256
Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A, Weber MJ (1998) MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science 281:1668–1671
Scherer PE, Lisanti MP, Baldini G, Sargiacomo M, Mastick CC, Lodish HF (1994) Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. J Cell Biol 127:1233–1243
Sebolt-Leopold JS, Herrera R (2004) Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat Rev Cancer 4:937–947
Sharma C, Vomastek T, Tarcsafalvi A, Catling AD, Schaeffer HJ, Eblen ST, Weber MJ (2005) MEK partner 1 (MP1): regulation of oligomerization in MAP kinase signaling. J Cell Biochem 94:708–719
Sivaraman VS, Wang H, Nuovo GJ, Malbon CC (1997) Hyperexpression of mitogen-activated protein kinase in human breast cancer. J Clin Invest 99:1478–1483
Sjogren AK, Andersson KM, Liu M, Cutts BA, Karlsson C, Wahlstrom AM, Dalin M, Weinbaum C, Casey PJ, Tarkowski A, Swolin B, Young SG, Bergo MO (2007) GGTase-I deficiency reduces tumor formation and improves survival in mice with K-RAS-induced lung cancer. J Clin Invest 117:1294–1304
Smart EJ, Ying YS, Mineo C, Anderson RG (1995) A detergent-free method for purifying caveolae membrane from tissue culture cells. Proc Natl Acad Sci USA 92:10104–10108
Sobering AK, Romeo MJ, Vay HA, Levin DE (2003) A novel Ras inhibitor, Eri1, engages yeast Ras at the endoplasmic reticulum. Mol Cell Biol 23:4983–4990
Solit DB, Garraway LA, Pratilas CA, Sawai A, Getz G, Basso A, Ye Q, Lobo JM, She Y, Osman I, Golub TR, Sebolt-Leopold J, Sellers WR, Rosen N (2006) BRAF mutation predicts sensitivity to MEK inhibition. Nature 439:358–362
Stokoe D, Macdonald SG, Cadwallader K, Symons M, Hancock JF (1994) Activation of Raf as a result of recruitment to the plasma membrane. Science 264:1463–1467
Sugimoto N, Imoto I, Fukuda Y, Kurihara N, Kuroda S, Tanigami A, Kaibuchi K, Kamiyama R, Inazawa J (2001) IQGAP1, a negative regulator of cell-cell adhesion, is upregulated by gene amplification at 15q26 in gastric cancer cell lines HSC39 and 40A. J Hum Genet 46:21–25
Sweeney SE, Firestein GS (2006) Mitogen activated protein kinase inhibitors: where are we now and where are we going? Ann Rheum Dis 65 (Suppl 3):iii83–iii88
Szuts D, Eresh S, Bienz M (1998) Functional intertwining of Dpp and EGFR signaling during Drosophila endoderm induction. Genes Dev 12:2022–2035
Takemoto H, Doki Y, Shiozaki H, Imamura H, Utsunomiya T, Miyata H, Yano M, Inoue M, Fujiwara Y, Monden M (2001) Localization of IQGAP1 is inversely correlated with intercellular adhesion mediated by e-cadherin in gastric cancers. Int J Cancer 91:783–788
Tan PB, Kim SK (1999) Signaling specificity: the RTK/RAS/MAP kinase pathway in metazoans. Trends Genet 15:145–149
Tan X, Egami H, Ishikawa S, Kurizaki T, Tamori Y, Takai E, Hirota M, Ogawa M (2004) Relationship between the expression of extracellular signal-regulated kinase 1/2 and the dissociation of pancreatic cancer cells: Involvement of ERK1/2 in the dissociation status of cancer cells. Int J Oncol 24:815–820
Taniguchi CM, Emanuelli B, Kahn CR (2006) Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol 7:85–96

Teis D, Wunderlich W, Huber LA (2002) Localization of the MP1-MAPK scaffold complex to endosomes is mediated by p14 and required for signal transduction. Dev Cell 3:803–814
Tohgo A, Choy EW, Gesty-Palmer D, Pierce KL, Laporte S, Oakley RH, Caron MG, Lefkowitz RJ, Luttrell LM (2003) The stability of the G protein-coupled receptor-beta-arrestin interaction determines the mechanism and functional consequence of ERK activation. J Biol Chem 278:6258–6267
Torii S, Kusakabe M, Yamamoto T, Maekawa M, Nishida E (2004) Sef is a spatial regulator for Ras/MAP kinase signaling. Dev Cell 7:33–44
Treisman R (1996) Regulation of transcription by MAP kinase cascades. Curr Opin Cell Biol 8:205–215
Ussar S, Voss T (2004) MEK1 and MEK2, different regulators of the G1/S transition. J Biol Chem 279:43861–43869
Vieira AV, Lamaze C, Schmid SL (1996) Control of EGF receptor signaling by clathrin-mediated endocytosis. Science 274:2086–2089
Wang Y, Pennock S, Chen X, Wang Z (2002) Endosomal signaling of epidermal growth factor receptor stimulates signal transduction pathways leading to cell survival. Mol Cell Biol 22:7279–7290
Wellbrock C, Karasarides M, Marais R (2004) The RAF proteins take centre stage. Nat Rev Mol Cell Biol 5:875–885
Xavier R, Brennan T, Li Q, McCormack C, Seed B (1998) Membrane compartmentation is required for efficient T cell activation. Immunity 8:723–732
Xia XG, Harding T, Weller M, Bieneman A, Uney JB, Schulz JB (2001) Gene transfer of the JNK interacting protein-1 protects dopaminergic neurons in the MPTP model of Parkinson's disease. Proc Natl Acad Sci USA 98:10433–10438
Xiong S, Zhao Q, Rong Z, Huang G, Huang Y, Chen P, Zhang S, Liu L, Chang Z (2003) hSef inhibits PC-12 cell differentiation by interfering with Ras-mitogen-activated protein kinase MAPK signaling. J Biol Chem 278:50273–50282
York RD, Yao H, Dillon T, Ellig CL, Eckert SP, McCleskey EW, Stork PJ (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 392:622–626
Zhang Y, Moheban DB, Conway BR, Bhattacharyya A, Segal RA (2000) Cell surface Trk receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation. J Neurosci 20:5671–5678
Zorov DB, Kobrinsky E, Juhaszova M, Sollott SJ (2004) Examining intracellular organelle function using fluorescent probes: from animalcules to quantum dots. Circ Res 95:239–252

Dynamic Protein Complexes Regulate NF-κB Signaling

E. Wegener and D. Krappmann(✉)

Contents

1 Two Routes to NF-κB 238
2 The IKK Complex: The Gatekeeper of NF-κB Pathways 241
  2.1 Structure and Activation of the IKK Complex 241
  2.2 IKKγ-Binding Peptides Counteract IKK Activation 243
  2.3 Chaperone HSP90 Regulates IKK Activity: Implications for Tumor Therapy 245
3 Protein Assemblies and Regulatory Ubiquitination Trigger Activation of the IKK Complex 246
  3.1 Dynamic Signaling Complexes Control Activation of the Canonical NF-κB Pathway 246
  3.2 Regulatory Ubiquitin Chains Connect Upstream Events to the IKK Complex 249
  3.3 IKKγ Modification Triggers NF-κB Activation in Response to DNA Damage 251
4 Outlook 253
References 254

Abstract NF-κB is a major regulator of the first-line defense against invading pathogens, antigen-specific adaptive immune responses or chemical stress. Stimulation either by extracellular ligands (e.g., inflammatory cytokines, microbial pathogens, peptide antigens) or by intracellular stressors (e.g., genotoxic drugs) initiates signal-specific pathways that all converge at the IκB kinase (IKK) complex, the gatekeeper for NF-κB activation. During recent years, considerable progress has been made in understanding the function of NF-κB in the regulation of cell growth, survival and apoptosis. In this review, we will focus on the regulation of large signaling complexes on the route to NF-κB. Recently published data demonstrate that the assembly, maintenance and activity of the IKK complex determine downstream activation of NF-κB. In addition, dynamic complexes upstream of IKK

D. Krappmann
Helmholtz Zentrum München - German Research Center for Environmental Health, Institute of Toxicology, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany
daniel.krappmann@helmholtz-muenchen.de

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.* Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

are formed in response to tumor necrosis factor (TNF), antigenic peptides or DNA-damaging agents. Clustering of signaling adaptors promotes the association and activation of ubiquitin ligases that trigger the conjugation of regulatory ubiquitin to target proteins. Ubiquitination serves as a platform to recruit the IKK complex and potentially other protein kinases to trigger IKK activation. These findings support a concept whereby protein complex assembly induces regulatory ubiquitination, which in turn recruits and activates protein kinases. Notably, the great interest in a detailed description of the mechanisms that regulate NF-κB activity stems from many observations that link dysregulated NF-κB signaling with the onset or progression of various diseases, including cancer, chronic inflammation, cardiovascular disorders and neurodegenerative diseases. Thus, the formation of large signaling clusters and regulatory ubiquitin chains represents promising targets for pharmacological intervention to modulate NF-κB signal transduction in disease.

1 Two Routes to NF-κB

Nuclear factor-kappaB (NF-κB) is an evolutionary conserved family of transcription factors with key functions in many physiological and pathological processes (Ghosh and Karin 2002; Hayden and Ghosh 2004). The mammalian NF-κB family comprises five members, namely NFKB1 (p50/p105), NFKB2 (p52/p100), p65/RelA, cRel and RelB (Fig. 1). All NF-κB proteins are characterized by an N-terminal Rel homology domain (RHD) that confers hetero- or homodimerization between Rel proteins and sequence-specific DNA binding to gene regulatory elements in the chromatin. Only p65, c-Rel and RelB contain C-terminal transactivation domains (TAD) that are required for gene induction. In resting cells, NF-κB proteins are sequestered in the cytoplasm by inhibitory molecules (IκB: inhibitor of kappaB). The family of cytosolic IκBs consists of IκBα, IκBβ, IκBε as well as the NF-κB precursors NF-κB1/p105 and NF-κB2/p100 (Fig. 1). By an intramolecular cleavage, the precursors p105 and p100 give rise to transcription factors p50 and p52, respectively. As a common feature, all IκBs share an ankyrin repeat domain (ARD) that mediates interaction with the RHD and causes cytosolic retention by shielding the nuclear localization signals (NLS) of NF-κBs.

NF-κB activation is induced by a variety of stimulatory agents that include inflammatory cytokines, microbial pathogens, antigenic peptides, mitogens, morphogens and cellular stress. All upstream specific pathways converge at the IκB kinase (IKK) complex composed of two catalytic subunits IKKα and IKKβ and a regulatory component IKKγ/NEMO (NF-κB essential modifier) (Fig. 1). The first described canonical or type-1 NF-κB pathway is activated by all known physiological agents and involves site-specific serine phosphorylation of small IκBs or p105 by IKKβ/IKKγ in the cytosol (Fig. 2) (Li and Verma 2002). Phosphorylation targets IκBs for recognition by the SCF/βTRCP ubiquitin ligase complex that catalyzes the attachment of lysine 48-linked ubiquitin chains to the IκBs. Polyubiquitinated

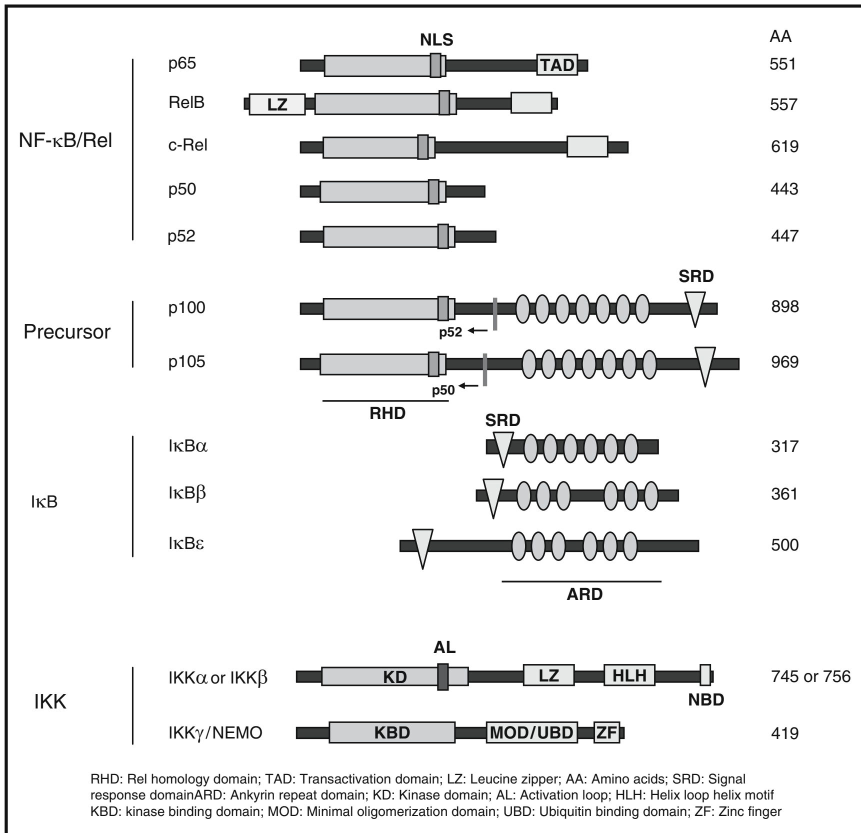

Fig. 1 Scheme of NF-κB, IκB and IKK proteins. NF-κB transcription factors process an N-terminal Rel homology domain (RHD) that mediates dimerization, nuclear localization and DNA binding as a common characteristic. The C-terminal transactivation domain (*TAD*) of p65, RelB and c-Rel is needed for the recruitment of transcriptional coactivators. p100 and p105 represent precursor molecules that are proteolytically processed to p52 and p50, respectively. Inhibitory IκB proteins interact via their akyrin repeat domains with the C-terminal part of the RHD and prevent nuclear translocation of NF-κB by masking the nuclear localization sequence (*NLS*). The signal response domain (*SRD*) contains phosphor acceptor sites that are of critical function for stimulus-dependent degradation of IκB proteins. IKKα and IKKβ are highly homologous and contain an activation loop (*AL*) within their N-terminal kinase domain (*KD*). In addition, leucine zippers and helix-loop-helix motifs are found in the C-terminus. Through the C-terminal Nemo-binding domain (*NBD*), IKKα and IKKβ interact with the structurally unrelated IKKγ/NEMO protein that is characterized by the N-terminal kinase-binding domain (KBD), a central minimal oligomerization/ubiquitin-binding domain (MOD/UBD), and a C-terminal zinc finger (*ZF*)

IκBs are subsequently degraded by the 26 S proteasome, which leads to the release and nuclear translocation of predominately p50/RelA- and p50/c-Rel-containing dimers. NF-κB activation by the canonical pathway is a rapid post-translational process that peaks within minutes and triggers induction of many immediate early

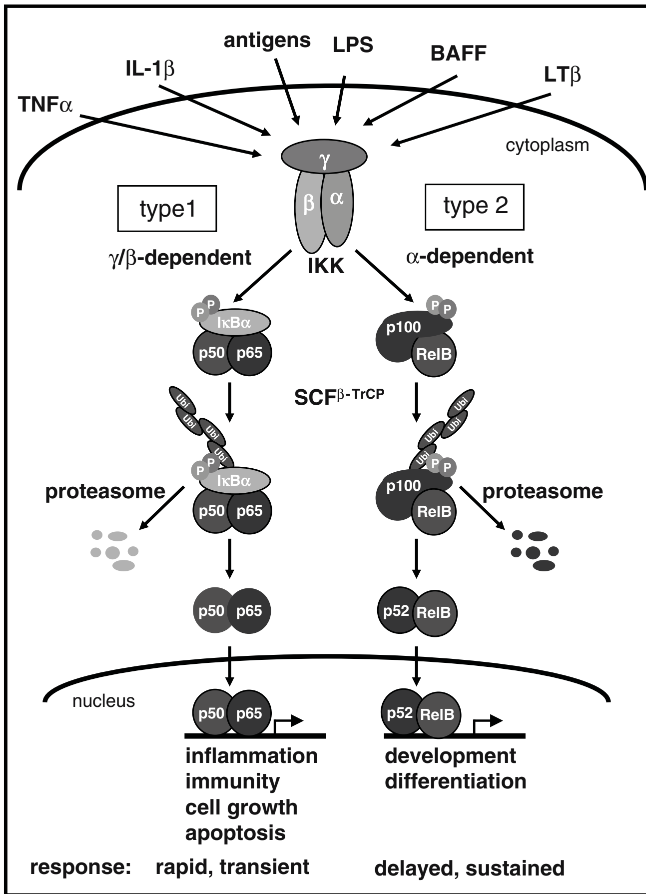

Fig. 2 Type-1 and type-2 signaling to NF-κB. Extracellular stimuli activate NF-κB either by type-1 (IKKβ/IKKγ-dependent) or type-2 (IKKα-dependent) signaling. To date, all known inducers initiate type-1 signal transduction, leading to proteosomal degradation of cytosolic IκB proteins and subsequent nuclear translocation of predominately p65/p50 dimers that control the expression of target genes involved in inflammation, immunity and apoptosis. Type-1 signaling promotes a rapid response that is temporally restricted. A subset of NF-κB inducers is a strong activator of type-2 signaling, which induces the cleavage of the p100 precursor bound to RelB. Processing of p100 results in nuclear translocation of RelB/p52 dimers that activate target genes involved in development and differentiation. In contrast to type-1 signaling, type-2 responses are delayed and provoke sustained NF-κB activation

genes involved in inflammation, immune response, apoptosis and proliferation. As a negative feedback mechanism, NF-κB induces expression of inhibitory IκBα proteins to assure the temporal restriction of canonical NF-κB activation.

A subset of tumor necrosis factor (TNF) receptor family members, e.g., B cell activating factor receptor (BAFFR), lymphotoxin β (LTβ), CD40 or receptor activator of NF-κB (RANK) are potent inducers of the novel or type-2 NF-κB

pathway (Bonizzi and Karin 2004). However, also pathogenic stimulations by lipopolysccharides (LPS), *Heliobacter pylori* or human T cell leukemia virus (HTLV) have been shown to induce type-2 signaling (Fig. 2) (Mordmuller et al. 2003; Ohmae et al. 2005; Saccani et al. 2003). Type-2 activation promotes the processing of the p100 precursor to p52 and the generation of transcriptionally active p52/RelB complexes. Processing of p100 is initiated by NIK (NF-κB inducing kinase) dependent IKKα phosphorylation of p100. Phosphorylated p100 is processed in a way similar to IκB degradation in the type-1 pathway. However, in contrast to the rapid and transient induction of type-1 NF-κB complexes, p100 processing and p52/RelB activation are delayed for several hours and provoke sustained activation. Processing of p100 requires ongoing protein synthesis, which is rather in support of a co-translational mechanism (Mordmuller et al. 2003). The sustained activity of p52/RelB induces distinct sets of target genes that are often involved in the regulation of development and differentiation. However, a considerable crosstalk between both signaling systems can be anticipated (Grech et al. 2004; Lo et al. 2006).

The great interest in mechanistic details that govern the NF-κB signaling pathways stems from the many pathological conditions associated with deregulations in the NF-κB network. Constitutive IKK/NF-κB activation is associated with a variety of different neoplasias, e.g., colon cancer, mammary tumors, leukemias and lymphomas. Due to its anti-apoptotic and pro-proliferative activity, enhanced NF-κB activity is considered to be a central factor for neoplastic transformation and an attractive target in tumor therapy (Gilmore and Herscovitch 2006; Lin and Karin 2003). Other diseases related to constitutive NF-κB activity are autoimmune diseases such as rheumatoid arthritis or inflammatory airway diseases such as asthma or COPD (chronic obstructive pulmonary disease) (Burke 2003; Caramori et al. 2004).

2 The IKK Complex: The Gatekeeper of NF-κB Pathways

The complete absence of inducible NF-κB in IKKα/IKKβ-deficient cells emphasizes the master-regulatory function of IKKs for type-1 and type-2 NF-κB activation in response to almost all known physiological stimuli. In this section we will focus on the data about the structure of the IKKs and discuss the functional implications. The biological functions of individual IKK family members have been intensively reviewed (Hacker and Karin 2006), and this section will only briefly highlight major areas in the context of structural constraints.

2.1 Structure and Activation of the IKK Complex

In the 1990s biochemical purification and protein–protein interaction screening led to the identification of the IκBα protein kinase complex, consisting of two catalytic

components (IKKα/IKK1 and IKKβ/IKK2) and a regulatory subunit (IKKγ) (Chen et al. 1996; DiDonato et al. 1997; Mercurio et al. 1997; Rothwarf and Karin 1999; Yamaoka et al. 1998). IKKα and IKKβ show 52% similarity in protein sequence (Fig. 1). Both subunits can homo- and heterodimerize through a leucine zipper motif and harbor a catalytically active kinase domain in the N-terminus that is responsible for phosphorylation of IκB proteins (Mercurio et al. 1997). IKKγ is structurally not related to IKKα and IKKβ, but indispensable for stimulus-dependent activation of the type-1 NF-κB pathway (Yamaoka et al. 1998; Zandi et al. 1998).

Despite their structural similarities, IKKα and IKKβ were shown to have differing physiological functions. IKKα-deficient mice are viable, but exhibit perturbed B cell maturation and developmental defects of secondary lymphoid organs. The NF-κB response to proinflammatory stimuli in these cells is normal or only slightly impaired (Hu et al. 1999; Li et al. 1999a; Takeda et al. 1999). In contrast, IKKβ-deficient mice die in utero because of massive hepatocyte apoptosis, a phenotype that resembles p65 deficiency in mice (Beg and Baltimore, 1996; Li et al., 1999b, c; Tanaka et al., 1999). Embryonic fibroblasts isolated from these mice show strongly impaired NF-κB activity and increased apoptosis after stimulation with pro-inflammatory cytokines or pathogenic agents. Apparently, IKKα cannot compensate for IKKβ in the type-1 signaling pathways, while developmental defects after IKKα ablation can be explained by its unique function in the type-2 NF-κB activating pathway.

Cellular IKKs form a higher order complex that migrates with an apparent molecular weight of approximately 700–900 kDA in gel filtrations (Yamaoka et al. 1998). Reconstitution using recombinant proteins revealed that the tripartite IKK core complex is only composed of the three subunits IKKα, β and γ (Krappmann et al. 2000). There is evidence that cellular IKKα and IKKβ preferentially form functional heterodimers bound to IKKγ. As evident from single knock-out cells and immunodepletion experiments, IKKα and IKKβ can also form homodimers. However, it is not clear whether homodimers are formed under physiological conditions when all three IKKs are present (Khoshnan et al. 1999; Mercurio et al. 1999). The stoichiometric relation between the catalytic and regulatory IKK components is still elusive. Studies using recombinant proteins or yeast expression systems suggest equivalent amounts of IKKα, IKKβ and IKKγ (Miller and Zandi 2001). However, in vitro cross-linking and ultracentrifugation indicated that IKKγ oligomerizes through the MOD (minimal oligomerization domain) and might form preferentially trimers or tetramers (Agou et al. 2004b; Tegethoff et al. 2003). Even though open questions remain regarding this issue, the oligomeric state of IKKγ is clearly important for activation of the IKK complex by physiological stimuli.

Phosphorylation of IKKα and IKKβ is critical for their stimulus-dependent activation. Two serine residues within the T loop of both kinase domains were mapped to be indispensable for IKK activity. Treatment of purified IKK with PP2A (phosphatase 2A) or mutation of the T loop serines to alanines prevents IKK activation, while mutation to glutamate results in constitutively active IKKα and IKKβ (Delhase

et al. 1999; DiDonato et al. 1997). It was suggested that structural changes of the complex after binding to signaling molecules allow IKK trans-autophosphorylation, thereby triggering kinase activation. Cellular stimulation leads to IKKα and IKKβ phosphorylation, but only IKKβ phospho sites are critical for signal propagation in response to pro-inflammatory stimuli, which is consistent with results from gene-deficient mice (Delhase et al. 1999). In addition, several IKK kinases that belong to the family of mitogen-activated protein kinase kinase kinases (MEKKs) [e.g., NIK, MEKK1, MEKK3 and TAK1 (TGFβ-activated kinase)] were suggested to be involved in IKK activation in response to various stimuli. In most cases the in vivo relevance is not clear because of possible compensatory effects in knock-out mice (Hatada et al. 2000). Intriguingly, regulatory ubiquitination of signaling mediators has been recognized as an essential step for IKK activation. Ubiquitin chains are thought to serve as platforms that position upstream regulatory kinases and the IKK complex to cellular substructures and thereby induce proximity and activation (see below Sect. 3.2).

A considerable gap in our knowledge about the structural determinants of the IKK complex is due to the fact that there are no crystallographic data available yet. In addition, we need to learn more about the composition of cellular IKK complexes. Complex assembly is an important issue regarding the unique functions of IKKα and IKKβ in the type-1 and type-2 NF-κB pathway, respectively. It will be important to elucidate in the future whether the different pathways are initiated from a single IKK holo-complex or whether distinct IKK complexes are connecting to distinct downstream effector pathways. Even though structural insights are limited, different studies suggest that not only the catalytic activities, but also the assembly and composition of the IKK complex represent interesting targets for therapeutic interference.

2.2 IKKγ-Binding Peptides Counteract IKK Activation

The functional association of IKK/NF-κB signaling with the development and progression of many human diseases has prompted intensive searches for small molecule inhibitors that interfere especially with the activity of IKKβ, the critical kinase for pro-inflammatory and anti-apoptotic NF-κB signaling (Karin et al. 2004). Most inhibitors are directed against the ATP-binding pocket within the kinase domain and block IKKβ in a reversible or irreversible fashion. An alternative approach has been proposed by Ghosh and coworkers that aims at the destruction of the tripartite IKK complex by interfering with IKKβ/IKKγ association (May et al. 2000). They precisely mapped the interaction surface of IKKα and IKKβ with IKKγ (Fig. 1). A short peptide sequence of about ten amino acids in the C-terminus of IKKα and IKKβ termed the NEMO-binding domain (NBD) is sufficient for mediating the interaction of both kinases with the N-terminal part of IKKγ. By fusing the amino acids 735–745 of IKKβ to a peptide sequence derived from the

Antennapedia (ANT) homeodomain that mediates membrane translocation and therefore cellular uptake of the peptide, they created a cell permeable NBD-ANT peptide (May et al., 2000). Incubation of cells with the NBD-ANT fusion peptide specifically blocks inducible activation of NF-κB in vivo by disrupting the endogenous IKK complex. In mice, phorbol ester induced ear edema, and zymosan-induced peritonitis could be prevented by injection of the NBD-ANT peptides (May et al. 2000). NBD-ANT was as effective as the anti-inflammatory steroid hormone analogue dexamethasone, providing evidence for the great therapeutic potential.

A number of reports support the effectiveness of the NBD peptide. In vivo studies in mice using cell-permeable NBD peptides confirmed that IKK signaling modulates osteoclastogenisis and inflammatory responses leading to bone erosion. Further, NBD peptides promoted an alleviation of the inflammatory response in mice susceptible to inflammatory arthritis and suppressed osteoclast formation as well as bone destruction in the joints (Clohisy et al. 2003; Dai et al. 2004). Polymorphonuclear neutrophils have a crucial role in the first line of the inflammatory defense to invading pathogens. Therefore, induction of apoptosis in neutrophils could help to resolve acute and chronic inflammation. Uptake of cell-permeable NBD peptides by primary neutrophils abolished LPS-induced activation of NF-κB (Choi et al. 2003). Further, constitutive neutrophil apoptosis is accelerated, indicating that NBD may be used as a potent inhibitor of NF-κB in neutrophils. Thus, the treatment with cell-permeable NBD peptides represents a promising approach to modulate inflammatory responses in vivo. However, its effectiveness as a potential anti-cancer drug awaits further analysis.

It was suggested that oligomerization of IKKγ is needed for IKK activation (Inohara et al. 2000; Poyet et al. 2000). Although conflicting data exist about the exact oligomerization state of IKKγ, the oligomerization is mediated by the MOD (Fig. 1) (Agou et al. 2004b; Tegethoff et al. 2003). Its physiological relevance is apparent as mutations in the C-terminus of IKKγ, including the MOD, are associated with human disease, e.g., anhydrotic ectodermal dysplasia or incontinentia pigmenti (Schmidt-Supprian et al. 2000; Vinolo et al. 2006). Resembling the NBD approach, cell-permeable fusion peptides homologous to parts of the MOD inhibit IKK activity with an IC_{50} in the μm range. Treatment leads to efficient inhibition of LPS-induced NF-κB activation and to induction of apoptosis in cell lines exhibiting constitutive NF-κB activity (Agou et al. 2004a). However, a confirmation in disease-related model systems has not yet been accomplished.

Most pre-clinical research in the NF-κB field focuses on inhibitors of enzymatic activities. Intriguingly, the data on NBD and MOD peptides reflect that interference with the integrity of the IKK complex may be a promising tool to develop potent NF-κB inhibitors. However, the limited distribution and stability of peptides constitute a major caveat to peptide-based therapies. As an alternative, small molecules mimicking the structure of inhibitory peptides may be developed, a strategy that appears promising, especially in light of the fact that a short ten-amino-acid NBD peptide is sufficient for molecular interference. The design of

such compounds might overcome the limitations, but again more structural information about the IKK complex is necessary to inspire a rational design.

2.3 Chaperone HSP90 Regulates IKK Activity: Implications for Tumor Therapy

Biochemical purification revealed that the chaperone HSP90 (heat shock protein 90) and its co-chaperone CDC37 are associated with the cellular IKK complex (Chen et al. 2002). These factors directly bind to the kinase domains of IKKα and IKKβ. Typically, HSPs assist proteins to gain their native folding after ribosomal synthesis, to conduct their intracellular disposition or to trigger their proteolytic turnover (Wegele et al. 2004). Upon environmental stress, e.g., heat shock, HSP expression increases as part of a cellular emergency program that helps to maintain protein homeostasis and cell survival. In contrast to the pleitropic effects of chaperones HSP60 and HSP70, HSP90 interacts only with a limited number of client proteins. Interestingly, in many cancer cells HSP90 is needed to maintain the malignant phenotype of the cells, probably by stabilizing mutant signaling proteins (Mosser and Morimoto 2004). Thereby, HSP90 enables oncogenic proteins to support abnormal signaling in tumor cells. HSP90 function is ATP dependent, and naturally occurring compounds such as geldanamycin (GA) or radicicol bind with high affinity to the ATP-binding pocket of HSP90, selectively blocking HSP90 activity. A growing number of synthetic derivatives of GA and radicicol with improved pharmacologic and pharmacokinetic properties are being developed as potential anti-cancer drugs (Whitesell and Lindquist 2005).

By using GA, it was shown that HSP90 and co-chaperones influence the activity of a number of signaling molecules, including steroid hormone receptors and several kinases that regulate cellular proliferation and survival, e.g., Raf, Akt, Cdk4 and IKKs (Maloney and Workman 2002). Intriguingly, GA treatment prevents HSP90/IKK co-purification, indicating that protein complexes are disrupted, when HSP90 activity is inhibited. Further, GA treatment interferes with IKK recruitment to receptor complexes at the membrane, a process known to be essential for IKK activation (Chen et al. 2002). As a result, GA-treated cells do not activate NF-κB when stimulated with TNFα, IL-1β or PMA, establishing a physiologically relevant role for the IKK/HSP90 interaction (Broemer et al. 2004; Chen et al. 2002). Moreover, constitutive IKK/NF-κB activation in Hodgkin lymphoma-derived tumor cells is inhibited with GA or radicicol treatment (Broemer et al. 2004). HSP90 apparently has a dual function. First, it assists the folding of IKKα and IKKβ. Thus, sustained GA incubation provokes a depletion of cellular IKKs. Second, HSP90 is required for maintaining an active confirmation of IKKs within the assembled IKK complex. Short-term HSP90 inhibition reduces the ability of IKK complexes to react to extracellular stimulation. It was suggested that HSP90 keeps the IKK components in a conformation that enables signaling molecules to bind and activate its kinase activity or enhance trans-autophosphorylation (Broemer

et al. 2004). Another possibility of HSP90 function could be that chaperone binding facilitates post-translational modification, such as polyubiquitination of IKKγ (see also below Sect. 3.2). Thus, HSP90 allows kinase activation via the regulation of protein–protein interactions or via ubiquitin-dependent subcellular trafficking.

Certainly, the effects of HSP90 inhibitors on tumor growth are caused by modulation of many cellular processes, and disruption of the IKK/HSP90 complex is only one part of the complex picture. However, HSP90 inhibitors apparently target IκB kinases in their inactive state. Therefore, a detailed analysis of the structural basis of HSP90 function on IKK activity might pave the way for the development of new classes of inhibitory compounds. These might influence the inactive kinase conformation in a way to prevent activation from upstream regulatory pathways.

3 Protein Assemblies and Regulatory Ubiquitination Trigger Activation of the IKK Complex

Many studies during the last decade have investigated the complex mechanisms that promote IKK/NF-κB signaling in response to cytokines, pathogens, antigens or other agents (Hayden and Ghosh 2004; Janssens and Tschopp 2006; Rawlings et al. 2006). Since most receptors and many mediators on the route to IKK activation lack intrinsic enzymatic activities, we will focus on the role of protein assemblies especially for TNFα-, antigen- or DNA-damage-induced NF-κB activation. In all instances, signaling is achieved by the clustering of adaptor molecules to the receptors or other intracellular signaling complexes. Recent results have highlighted that non-degradative, regulatory ubiquitin chains provide signaling platforms that facilitate the recruitment of signaling mediators for the various receptor systems. Further, we begin to understand the complex protein interactions and modifications that are involved in the nuclear to cytosolic (inside-out) NF-κB pathway after DNA damage, which represents a clinically relevant issue, as NF-κB-induced apoptosis protection constitutes a major obstacle for efficient anti-cancer chemotherapy.

3.1 Dynamic Signaling Complexes Control Activation of the Canonical NF-κB Pathway

3.1.1 TNFα-Induced Clustering of Receptor Complexes Promotes IKK Activation

The TNFR superfamily comprises at least 29 receptors that are bound by a great diversity of different ligands (Aggarwal 2003). TNFα, a potent activator of NF-κB, is the most widely studied ligand for this family of receptors and is involved in many

processes, including inflammation, innate and adaptive immunity or liver apoptosis. Aberrant TNFα signaling is associated with many diseases, and TNFα neutralizing antibodies are in clinical use for rheumatoid arthritis. The analysis of intracellular signaling mechanisms may uncover new opportunities for therapeutic intervention.

Binding of the trimeric TNFα ligand to TNFR1 causes receptor aggregation and allows the intracellular recruitment of the adaptor protein TRADD (TNFR-associated death domain protein) (Hsu et al. 1995). Association of TRADD provokes the recruitment of TNF receptor-associated factors 2 (TRAF2) and TRAF5 (Fig. 3). Only TRAF2/5 double knockout cells display a substantial reduction in TNFα-induced IKK/NF-κB signaling, strongly arguing for a redundant role of both adaptors (Tada et al. 2001; Yeh et al. 1997). Upon TNFα stimulation, TRAF2 interacts with RIP1 (receptor interacting protein1), another effector molecule that is essential for TNFα-induced NF-κB activation (Devin et al. 2000). Intriguingly, RIP1 comprises a serine/threonine protein kinase domain, but enzymatic

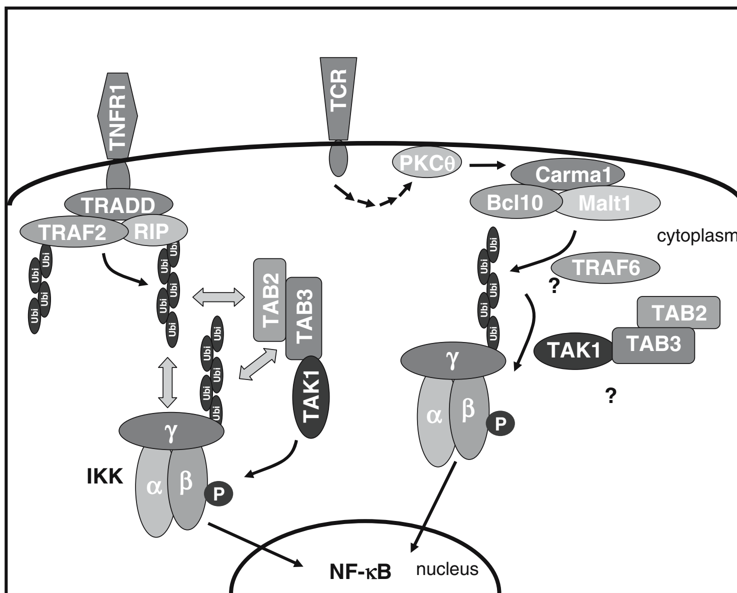

Fig. 3 Dynamic protein complexes control TNFR1- and TCR-dependent IKK activation. TNFR1 engagement leads to the recruitment of a variety of adaptor molecules, such as TRAF2 and RIP1. Regulatory K63 ubiquitination of these adaptors is essential for the formation of a signaling complex comprising TAK1 and IKKβ kinases that are associated with ubiquitin-binding proteins (TAB2/TAB3 and IKKγ, respectively). Either TAK1-dependent phosphorylation of IKKβ or proximity-induced trans-autophosphorylation of IKKβ triggers efficient IKK and subsequent NF-κB activation. TCR activation initiates a multitude of receptor proximal signaling events that conduct PKCθ-dependent CBM complex formation and IKK recruitment. The CBM complex-associated protein TRAF6 was suggested to mediate K63-linked ubiquitination of IKKγ, a prerequisite for efficient IKK activation after TCR engagement. The role of TAK1 kinase activity in TCR-mediated IKK activation is not fully understood

activity is not required for IKK activation, suggesting that RIP1 may rather function as a molecular scaffold (Hsu et al. 1996; Ting et al. 1996). In agreement with this hypothesis, RIP1 can also directly associate with IKKγ and thereby recruit IKK complexes to TNFR1 independent of TRAF2 (Zhang et al. 2000). TRAF2 in turn interacts directly to the leucine zipper regions of IKKα and IKKβ, but IKK recruitment in the absence of RIP1 is not sufficient to trigger IKK activation (Devin et al. 2001). These results demonstrate that the function of RIP1 goes beyond the mere requirement of a scaffold. Recent data showing that RIP1 is a target for regulatory ubiquitination, which in turn facilitates the recruitment of IKKγ and potentially other signaling mediators, may provide an explanation for these previous observations (see Sect. 3.2). The exact mechanism of IKK activation is still unresolved. IKK activation could be caused by the recruitment of one or several redundant IKK kinases, e.g., TAK1, MEKK1 or MEKK3. Alternatively, IKKγ recruitment to the TNFR1 could induce proximity of IKK complexes and subsequent autophosphorylation of IKKs (Delhase et al. 1999).

3.1.2 Carma1-Bcl10-Malt1 Complexes Direct Antigenic Stimulation to IKK

Antigen recognition by B cell or T cell receptors (BCR or TCR, respectively) on lymphocytes is crucial for initiating adaptive immune responses. NF-κB activation after antigen engagement is necessary for survival, proliferation, cytokine production and effector cell differentiation of the lymphocytes (Schulze-Luehrmann and Ghosh 2006). Potential mechanisms for interference could be exploited for fighting autoimmune diseases, graft rejection after transplantation or certain types of malignant lymphomas that display dysfunctions in antigen-driven signaling cascades.

Antigen binding to the TCR or BCR initiates highly homologous proximal signaling events in T and B lymphocytes that involve activation of tyrosine kinases, recruitment of adaptors and phospholipases (Schulze-Luehrmann and Ghosh, 2006). Most of these processes are required for all downstream events. The phospholipase Cγ (PLCγ)-mediated generation of second messenger diacylglycerol (DAG) leads to the activation of protein kinase C (PKCθ in T cells and PKCβ in B cells). PKC activation bridges receptor proximal events to the IKK/NF-κB pathway (Sun et al. 2000). Gene disruption in mice revealed that three gene products are essential for coupling PKCs to the activation of the IKK complex in B and T lymphocytes. These include Carma1 [caspase recruitment domain (CARD) membrane-associated guanylated kinase1, also known as CARD11], Bcl10 (B cell lymphoma 10) and Malt1 (mucosa-associated lymphoid tissue1) (Egawa et al. 2003; Hara et al. 2003; Ruefli-Brasse et al. 2003; Ruland et al. 2001, 2003). Carma1 has been identified as the direct substrate of PKCθ and PKCβ after antigen stimulation in lymphocytes (Matsumoto et al. 2005; Sommer et al. 2005). PKC phosphorylation of Carma1 in the central linker region induces a conformational change that influences the accessibility of the N-terminal CARD region. CARDs are found in many proteins involved in apoptosis and immune reactions, and CARDs function as

protein–protein interaction surfaces. Preassembled cytosolic Bcl10-Malt1 heteromers are recruited to Carma1 via heterotypic interaction between the CARDs of Carma1 and Bcl10, leading to the assembly of the Carma1-Bcl10-Malt1 (CBM) signalosome (Fig. 3) (Wegener et al. 2006). With PDK1 (3-phosphoinositide dependent kinase1), Caspase8 and TRAF6, other components are integrated into the CBM complex, and depletion of these proteins impairs NF-κB activation (Lee et al. 2005; Su et al. 2005; Sun et al. 2004). How these molecules cooperate in the activation of the IKK complex is still unclear. However, similar to TNF receptor complexes, Bcl10, Malt1 and TRAF6 induce regulatory ubiquitination, which seems to constitute the link to IKK activation (see below Sect. 3.2).

The physiological relevance of CBM complex assembly is supported by recent findings showing that negative regulatory mechanisms lead to the disassembly of the CBM complex and thereby interfere with downstream signaling pathways. After TCR engagement, and simultaneously to CBM complex formation, Bcl10 is phosphorylated at several serine residues in the C-terminal part of the protein (Wegener et al. 2006). C-terminal Bcl10 phosphorylation induces a reduction of the affinity between Bcl10 and Malt1, leading to a remodeling of the CBM complex. Remodeling results in repressive modulation of TCR-dependent signal transduction towards NF-κB, constituting a negative regulatory mechanism. Intriguingly, IKKβ acts as the Bcl10 kinase, providing evidence for an IKK-dependent negative feedback loop that balances NF-κB signaling. In addition, under persistent stimulation conditions, Bcl10 is degraded, which leads to the collapse of the CBM complex (Hu et al. 2006; Scharschmidt et al. 2004). An alternative IKK-dependent N-terminal phosphorylation event involving Bcl10 was suggested to serve as the degradation signal (Lobry et al. 2007). In the absence of Bcl10, signaling to NF-κB is terminated, which might ensure the transient induction of NF-κB activity after TCR stimulation. These results emphasize that signaling complexes integrate positive and negative regulatory processes. The elucidation of negative regulation that impinges on the CBM signalosomes might be used for novel therapeutic strategies that exploit the existence of cell-intrinsic feedback controls.

3.2 Regulatory Ubiquitin Chains Connect Upstream Events to the IKK Complex

Ubiquitin is an evolutionary conserved protein of 76 amino acids that is covalently conjugated to substrate proteins. Ubiquitin attachment is best known for its prominent role in targeting cytosolic proteins for proteasomal degradation. The C-terminus of ubiquitin is attached to ε-amino-groups of lysine residues on substrate proteins, a process that involves a three-step enzymatic cascade consisting of an ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) (Pickart 2001). The E3 ligase is responsible for substrate recognition and thus determines specificity. For targeting proteins to the proteosomal pathway, poly-ubiquitin chains need to be attached to the substrates, which are

generally linked via internal lysine 48 (K48) residues on ubiquitin. However, many proteins are modified by the attachment of K63-linked ubiquitin chains, and usually this linkage does not support protein degradation. For NF-κB signaling, a number of different studies suggests that K63-linked ubiquitin chains provide the missing link between upstream signaling events and IKK activation (Chen 2005; Krappmann and Scheidereit 2005).

Importantly, it was observed that TRAF proteins acting as intracellular adaptors for many physiological stimuli contain RING domains found in many E3 ligases. In cooperation with the E2 enzyme Ubc13/Uev1a, TRAF2 and TRAF6 preferentially catalyze the formation of K63-linked ubiquitin chains to target proteins (Deng et al. 2000; Shi and Kehrl 2003). TNFR1 trimerization induces oligomerization and trans-autoubiquitination of TRAF2, which further stimulates TRAF2 ubiquitin ligase activity (Wertz et al. 2004). Subsequently, TRAF2 induces the attachment of K63-linked poly-ubiquitin chains to RIP1 (Fig. 3) (Ea et al. 2006; Li et al. 2006; Wu et al. 2006a). RIP1 ubiquitination greatly enhances the affinity of RIP1 and IKKγ. This interaction is mediated by an ubiquitin-binding domain (UBD) on IKKγ that selectively associates with K63-linked ubiquitin chains (Wu et al. 2006a). Thus, IKKγ acts as a sensor for polyubiquitin chains after TNFα stimulation. Mutation of the potential ubiquitin acceptor lysine on RIP1 (K377) abolishes the ability of RIP1 to mediate IKK/NF-κB signaling after TNFα treatment (Ea et al. 2006; Li et al. 2006).

How do K63-linked ubiquitin chains support IKK activation? Several studies suggest a role for TAK1 as an IKK-activating kinase because of its ability to phosphorylate IKKβ directly in the activation loop (Sato et al. 2005; Wang et al. 2001). Importantly, just like IKKγ, the TAK1-binding (TAB) proteins TAB2 and TAB3 display high affinity to K63 ubiquitin chains on RIP1 (Kanayama et al. 2004; Wu et al. 2006a). Thus, RIP1 ubiquitination recruits TAB2/TAB3/TAK1 and the IKK complex to the TNFR1. IKKβ is then phosphorylated by TAK1, which evokes IKK activation (Wang et al. 2001). Congruently, TAK1 gene silencing and deficiency impair TNFα-induced IKK/NF-κB signaling (Sato et al. 2005; Shim et al. 2005; Takaesu et al. 2003). Intriguingly, IKKγ itself is also modified with K63-conjugated ubiquitin chains, suggesting IKKγ is not only a sensor of ubiquitin chains, but is also a substrate, and this may contribute to the recruitment of known and unknown ubiquitin binding proteins (Kanayama et al. 2004).

Studies on the regulation of TCR-induced NF-κB activation provide additional evidence for a prominent role of ubiquitination in the NF-κB pathway. The Bcl10-Malt1 signaling module was shown to induce IKKγ ubiquitination (Fig. 3), suggesting that regulatory ubiquitination could promote IKK activation upon antigenic stimulation (Zhou et al. 2004). It was proposed that Malt1 contains an intrinsic E3 ligase activity. Alternatively, TRAF6 was shown to enhance IKKγ ubiquitination, and TRAF6 interacts either directly or through Caspase8 with Malt1 (Bidere et al. 2006; Sun et al. 2004). Ubiquitin-binding TAK1/TAB complexes were suggested to serve as an IKK kinase upon T cell activation, and siRNA experiments support a function of TRAF2, TRAF6 and TAK1 in IKK activation in a human T-cell line (Sun et al. 2004). However, conditional genetic deletion of TAK1 in CD4 positive T cells results in defective NF-κB activation only in mature

thymocytes, but not in effector T cells, indicating that TCR-mediated regulation of NF-κB differs among T-cell subpopulations (Wan et al. 2006).

The identification of deubiquitinating enzymes that preferentially disassemble K63-conjugated ubiquitin chains supports the concept of a regulatory function of ubiquitination. Genetic ablation of the zinc-finger protein A20 results in sustained NF-κB activation after TNFα stimulation, which causes an inflammatory phenotype (Lee et al. 2000). A20 interferes with IKK/NF-κB signal transduction via two modes of action that both target RIP1 (Wertz et al. 2004). Primarily, an N-terminal deubiquitinase (DUB) activity of A20 removes K63 ubiquitin chains from RIP1. Secondly, an ubiquitin ligase activity in the C-terminus of A20 conjugates K48-linked ubiquitin chains to RIP1, which is subsequently degraded by the proteosome. The removal of regulatory ubiquitination as well as the attachment of degradative ubiquitin chains contributes to the downregulation of TNFα-induced IKK activity (Wertz et al. 2004). With CYLD (cylindromatosis) another DUB has been identified that balances NF-κB activation. Mutations in CYLD were initially found as a predisposition for the development of benign tumors of hair follicles and salivary glands in humans (Bignell et al. 2000). CYLD was identified in systematic screenings searching for DUBs that interfere with NF-κB activation (Brummelkamp et al. 2003; Kovalenko et al. 2003; Trompouki et al. 2003). Biochemical analysis suggested that CYLD specifically removes K63 ubiquitin chains from TRAF2, TRAF6 and IKKγ. The generation of several CYLD-deficient mice led to conflicting results concerning the physiological relevance of its DUB activity in NF-κB signaling (Massoumi et al. 2006; Reiley et al. 2006; Zhang et al. 2006). However, recently published data on myeloid cells isolated from CYLD-deficient mice implicate that CYLD is involved in NF-κB de-activation after TNF stimulation in vivo (Zhang et al. 2006).

Even though not all players have been identified and many questions regarding the exact mechanistic details remain to be resolved, the studies provide compelling evidence that K63 polyubiquitination serves as a platform for the tight association of protein kinases on the route to IKK/NF-κB activation. New classes of pharmacological inhibitors can be envisioned that either target ubiquitin ligases involved in the formation of K63-linked ubiquitin chains or disrupt the interaction of ubiquitin chains and the respective UBD. Especially for the latter, it will be very informative to resolve the structure of ubiquitin-UBD interfaces.

3.3 *IKKγ Modification Triggers NF-κB Activation in Response to DNA Damage*

DNA-damaging agents belong to the most widely used anti-cancer drugs. Depending on the extent of damage, the cells are either arrested in the cell cycle to allow DNA repair or the cells undergo apoptosis. The tumor suppressor p53 is a primary sensor of genotoxic stress and induces cell-cycle arrest or apoptosis (Vousden 2002). A major obstacle to an efficient anti-cancer therapy is the activation of NF-κB-mediated

pro-survival signaling by DNA-damaging agents. There is a complex crosstalk between p53 and NF-κB that involves antagonistic as well as cooperative effects in the induction of apoptosis (Perkins 2007). Owing to its potent anti-apoptotic activity, NF-κB contributes to acquired chemoresistance. Analysis of the exact mechanisms of NF-κB activation after genotoxic stress highlights that distinct signaling complexes are involved in this inside-out (nuclear to cytosolic) pathway.

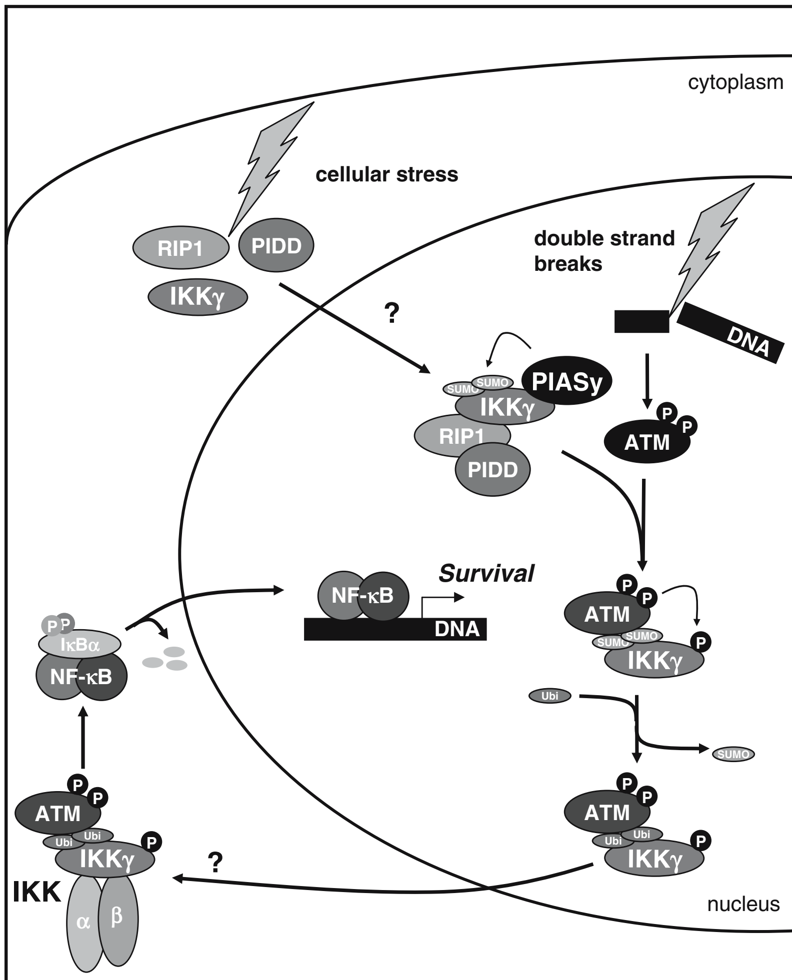

Fig. 4 Nuclear to cytoplasmic NF-κB signaling in response to genotoxic stress. Genotoxic and cellular stresses initiate the formation of a nuclear protein complex containing PIDD, RIP1, sumoylated IKKγ and active ATM. Phosphorylation of IKKγ by ATM leads to an exchange of Sumo to mono-ubiquitin on IKKγ. Ubiquitinated IKKγ and associated ATM are exported to the cytoplasm where the proteins reassemble with the cytoplasmic IKK complex and mediate IKK activation by a so far unknown mechanism. Subsequent nuclear translocation of NF-κB induces a pro-survival gene program

Topoisomerase inhibitors etoposide (Eto) or camptothecin (Cpt) exhibit cytostatic effects by inducing DNA double-strand breaks and therefore are potent inducers of NF-κB activity (Fig. 4). The actual sensor of DNA double-stranded breaks is unknown. The primary cellular transducer for DNA damage is the ATM (ataxia telangiectasia mutated) kinase. ATM-deficient cells from patients with the genomic instability ataxia telangiectasia are blocked in NF-κB activation in response genotoxic stress agents, such as γ-ionizing radiation (IR) or Cpt (Li et al. 2001; Piret et al. 1999). Upon Eto or Cpt treatment, IKKγ enters the nucleus where it associates with PIDD (p53-induced death domain) and RIP1, both proteins having essential functions in the control of NF-κB activation in response to DNA damage (Hur et al. 2003; Janssens and Tschopp 2006). Further, IKKγ is retained in the nucleus by site-directed sumoylation mediated by PIASy (protein inhibitor of activated STATy) that acts as the SUMO (small ubiquitin-like modifier) ligase (Mabb et al. 2006; Wu et al. 2006b). However, the exact mechanism for genotoxic-stress-induced nuclear uptake of IKKγ, PIDD and RIP1 is unknown. Intriguingly, activated ATM kinase preferentially associates with SUMO-conjugated IKKγ. ATM kinase then phosphorylates IKKγ, inducing an exchange of SUMO to mono-ubiquitin-conjugated IKKγ, which is exported together with ATM kinase from the nucleus (Wu et al. 2006b). In the cytosol, mono-ubiquitinated IKKγ promotes assembly of the ATM-IKK complex that induces IκB degradation, nuclear translocation of NF-κB and induction of an anti-apoptotic gene program.

To date, many questions still remain unresolved concerning genotoxic-stress-induced NF-κB activation, such as, e.g.: where is the inducible IKKγ-PIDD-RIP1 complex formed, how are these proteins targeted to the nucleus, what is the exact mechanism that mediates the switch from SUMO-IKKγ to ubiquitin-IKKγ, and what is the ubiquitin ligase for IKKγ? Further, it is completely unresolved how ATM-IKKγ activates cytosolic IKK complex and whether a distinct IKK complex exists for mediating DNA damage responses. However, these novel insights reveal that dynamic protein complexes are required for inducing an anti-apoptotic gene program in response to genotoxic stress. Future studies must determine whether pharmacological inhibitors can be used to selectively interfere with these complexes to identify substances that counteract acquired chemoresistance.

4 Outlook

Considering the large body of evidence for an involvement of IKK/NF-κB in inflammation, degenerative diseases, auto-immunity and cancer, signaling molecules participating in NF-κB activation are promising targets for pharmacological intervention. Conventional approaches for pharmacological intervention generally focus on modulating enzyme activity, and in the case of NF-κB, a variety of specific IKKβ inhibitors have been identified, and some of them are in clinical trials (Karin et al. 2004). However, the summarized data suggest that not only inhibition of kinase activity is a promising strategy. The inhibitory NBD peptide or

GA derivatives point in a novel direction, because they target the integrity or assembly of the IKK complex. The structure of the ten-amino-acid NBD peptide might be used for a rational design of small chemical compounds that interfere with IKK formation.

Many other potentially dynamic protein complexes are transmitting stimulus specific pathways to IKK activation. A serious obstacle to pharmacological interference with protein–protein interactions in scaffolding complexes is that, unlike enzymatic active sites, protein interfaces are often large and lack deeper indentations, which impedes effective binding of small inhibitory compounds (Berg 2003). Therefore, detailed structural information is needed to identify compact and centralized protein regions contributing key interactions that are in particular suitable for pharmacological intervention. Especially, resolving the structures of regulatory ubiquitin chains bound to UBD proteins could give novel hints for a pharmacologically relevant protein–protein interaction. Further, much more attention should be given to pharmacological inhibitors of ubiquitin ligases such as TRAF6. Even though these compounds target enzymatic activities, the blockade would disturb regulatory ubiquitination and thereby the generation of platforms for the recruitment of protein kinases. Finally, numerous negative regulatory feedback pathways exist that lead to the disruption of protein complexes, as for instance the CBM complex after T-cell activation. Activation of these cell-intrinsic negative regulatory mechanisms may in fact be more effective than the blockade of positive activating processes.

References

Aggarwal BB (2003) Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 3:745–756

Agou F, Courtois G, Chiaravalli J, Baleux F, Coic YM, Traincard F, Israel A, Veron M (2004a) Inhibition of NF-kappa B activation by peptides targeting NF-kappa B essential modulator (nemo) oligomerization. J Biol Chem 279:54248–54257

Agou F, Traincard F, Vinolo E, Courtois G, Yamaoka S, Israel A, Veron M (2004b) The trimerization domain of NEMO is composed of the interacting C-terminal CC2 and LZ coiled-coil subdomains. J Biol Chem 279:27861–27869

Beg AA, Baltimore D (1996) An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 274:782–784

Berg T (2003) Modulation of protein-protein interactions with small organic molecules. Angew Chem Int Ed Engl 42:2462–2481

Bidere N, Snow AL, Sakai K, Zheng L, Lenardo MJ (2006) Caspase-8 regulation by direct interaction with TRAF6 in T cell receptor-induced NF-kappaB activation. Curr Biol 16:1666–1671

Bignell GR, Warren W, Seal S, Takahashi M, Rapley E, Barfoot R, Green H, Brown C, Biggs PJ, Lakhani SR, Jones C, Hansen J, Blair E, Hofmann B, Siebert R, Turner G, Evans DG, Schrander-Stumpel C, Beemer FA, van Den Ouweland A, Halley D, Delpech B, Cleveland MG, Leigh I, Leisti J, Rasmussen S (2000) Identification of the familial cylindromatosis tumour-suppressor gene. Nat Genet 25:160–165

Bonizzi G, Karin M (2004) The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol 25:280–288

Broemer M, Krappmann D, Scheidereit C (2004) Requirement of Hsp90 activity for IkappaB kinase (IKK) biosynthesis and for constitutive and inducible IKK and NF-kappaB activation. Oncogene 23:5378–5386
Brummelkamp TR, Nijman SM, Dirac AM, Bernards R (2003) Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature 424:797–801
Burke JR (2003) Targeting I kappa B kinase for the treatment of inflammatory and other disorders. Curr Opin Drug Discov Devel 6:720–728
Caramori G, Adcock IM, Ito K (2004) Anti-inflammatory inhibitors of IkappaB kinase in asthma and COPD. Curr Opin Investig Drugs 5:1141–1147
Chen ZJ (2005) Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol 7:758–765
Chen ZJ, Parent L, Maniatis T (1996) Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination-dependent protein kinase activity. Cell 84:853–862
Chen G, Cao P, Goeddel DV (2002) TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. Mol Cell 9:401–410
Choi M, Rolle S, Wellner M, Cardoso MC, Scheidereit C, Luft FC, Kettritz R (2003) Inhibition of NF-kappaB by a TAT-NEMO-binding domain peptide accelerates constitutive apoptosis and abrogates LPS-delayed neutrophil apoptosis. Blood 102:2259–2267
Clohisy JC, Roy BC, Biondo C, Frazier E, Willis D, Teitelbaum SL, Abu-Amer Y (2003) Direct inhibition of NF-kappa B blocks bone erosion associated with inflammatory arthritis. J Immunol 171:5547–5553
Dai S, Hirayama T, Abbas S, Abu-Amer Y (2004) The IkappaB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks osteoclastogenesis and bone erosion in inflammatory arthritis. J Biol Chem 279:37219–37222
Delhase M, Hayakawa M, Chen Y, Karin M (1999) Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. Science 284:309–313
Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell 103:351–361
Devin A, Cook A, Lin Y, Rodriguez Y, Kelliher M, Liu Z (2000) The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. Immunity 12:419–429
Devin A, Lin Y, Yamaoka S, Li Z, Karin M, Liu Z (2001) The alpha and beta subunits of IkappaB kinase (IKK) mediate TRAF2-dependent IKK recruitment to tumor necrosis factor (TNF) receptor 1 in response to TNF. Mol Cell Biol 21:3986–3994
DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M (1997) A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature 388:548–554
Ea CK, Deng L, Xia ZP, Pineda G, Chen ZJ (2006) Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol Cell 22:245–257
Egawa T, Albrecht B, Favier B, Sunshine MJ, Mirchandani K, O'Brien W, Thome M, Littman DR (2003) Requirement for CARMA1 in antigen receptor-induced NF-kappa B activation and lymphocyte proliferation. Curr Biol 13:1252–1258
Ghosh S, Karin M (2002) Missing pieces in the NF-kappaB puzzle. Cell 109:S81–S96
Gilmore TD, Herscovitch M (2006) Inhibitors of NF-kappaB signaling: 785 and counting. Oncogene 25:6887–6899
Grech AP, Amesbury M, Chan T, Gardam S, Basten A, Brink R (2004) TRAF2 differentially regulates the canonical and noncanonical pathways of NF-kappaB activation in mature B cells. Immunity 21:629–642
Hacker H, Karin M (2006) Regulation and function of IKK and IKK-related kinases. Sci STKE 2006:re13
Hara H, Wada T, Bakal C, Kozieradzki I, Suzuki S, Suzuki N, Nghiem M, Griffiths EK, Krawczyk C, Bauer B, D'Acquisto F, Ghosh S, Yeh WC, Baier G, Rottapel R, Penninger JM (2003) The MAGUK family protein CARD11 is essential for lymphocyte activation. Immunity 18:763–775
Hatada EN, Krappmann D, Scheidereit C (2000) NF-kappaB and the innate immune response. Curr Opin Immunol 12:52–58

Hayden MS, Ghosh S (2004) Signaling to NF-kappaB. Genes Dev 18:2195–2224
Hsu H, Shu HB, Pan MG, Goeddel DV (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84:299–308
Hsu H, Xiong J, Goeddel DV (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell 81:495–504
Hu Y, Baud V, Delhase M, Zhang P, Deerinck T, Ellisman M, Johnson R, Karin M (1999) Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. Science 284:316–320
Hu S, Du MQ, Park SM, Alcivar A, Qu L, Gupta S, Tang J, Baens M, Ye H, Lee TH, Marynen P, Riley JL, Yang X (2006) cIAP2 is a ubiquitin protein ligase for BCL10 and is dysregulated in mucosa-associated lymphoid tissue lymphomas. J Clin Invest 116:174–181
Hur GM, Lewis J, Yang Q, Lin Y, Nakano H, Nedospasov S, Liu ZG (2003) The death domain kinase RIP has an essential role in DNA damage-induced NF-kappa B activation. Genes Dev 17:873–882
Inohara N, Koseki T, Lin J, del Peso L, Lucas PC, Chen FF, Ogura Y, Nunez G (2000) An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. J Biol Chem 275:27823–27831
Janssens S, Tschopp J (2006) Signals from within: the DNA-damage-induced NF-kappaB response. Cell Death Differ 13:773–784
Kanayama A, Seth RB, Sun L, Ea CK, Hong M, Shaito A, Chiu YH, Deng L, Chen ZJ (2004) TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Mol Cell 15:535–548
Karin M, Yamamoto Y, Wang QM (2004) The IKK NF-kappa B system: a treasure trove for drug development. Nat Rev Drug Discov 3:17–26
Khoshnan A, Kempiak SJ, Bennett BL, Bae D, Xu W, Manning AM, June CH, Nel AE (1999) Primary human CD4+ T cells contain heterogeneous I kappa B kinase complexes: role in activation of the IL-2 promoter. J Immunol 163:5444–5452
Kovalenko A, Chable-Bessia C, Cantarella G, Israel A, Wallach D, Courtois G (2003) The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. Nature 424:801–805
Krappmann D, Hatada EN, Tegethoff S, Li J, Klippel A, Giese K, Baeuerle PA, Scheidereit C (2000) The I kappa B kinase (IKK) complex is tripartite and contains IKK gamma but not IKAP as a regular component. J Biol Chem 275:29779–29787
Krappmann D, Scheidereit C (2005) A pervasive role of ubiquitin conjugation in activation and termination of IkappaB kinase pathways. EMBO Rep 6:321–326
Lee EG, Boone DL, Chai S, Libby SL, Chien M, Lodolce JP, Ma A (2000) Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science 289:2350–2354
Lee KY, D'Acquisto F, Hayden MS, Shim JH, Ghosh S (2005) PDK1 nucleates T cell receptor-induced signaling complex for NF-kappaB activation. Science 308:114–118
Li Q, Lu Q, Hwang JY, Buscher D, Lee KF, Izpisua-Belmonte JC, Verma IM (1999a) IKK1-deficient mice exhibit abnormal development of skin and skeleton. Genes Dev 13:1322–1328
Li Q, Van Antwerp D, Mercurio F, Lee KF, Verma IM (1999b) Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. Science 284:321–325
Li ZW, Chu W, Hu Y, Delhase M, Deerinck T, Ellisman M, Johnson R, Karin M (1999c) The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis. J Exp Med 189:1839–1845
Li N, Banin S, Ouyang H, Li GC, Courtois G, Shiloh Y, Karin M, Rotman G (2001) ATM is required for IkappaB kinase (IKKk) activation in response to DNA double strand breaks. J Biol Chem 276:8898–8903
Li Q, Verma IM (2002) NF-kappaB regulation in the immune system. Nat Rev Immunol 2:725–734
Li H, Kobayashi M, Blonska M, You Y, Lin X (2006) Ubiquitination of RIP is required for tumor necrosis factor alpha-induced NF-kappaB activation. J Biol Chem 281:13636–13643

Lin A, Karin M (2003) NF-kappaB in cancer: a marked target. Semin Cancer Biol 13:107–114
Lo JC, Basak S, James ES, Quiambo RS, Kinsella MC, Alegre ML, Weih F, Franzoso G, Hoffmann A, Fu YX (2006) Coordination between NF-kappaB family members p50 and p52 is essential for mediating LTbetaR signals in the development and organization of secondary lymphoid tissues. Blood 107:1048–1055
Lobry C, Lopez T, Israel A, Weil R (2007) Negative feedback loop in T cell activation through IkappaB kinase-induced phosphorylation and degradation of Bcl10. Proc Natl Acad Sci USA 104:908–913
Mabb AM, Wuerzberger-Davis SM, Miyamoto S (2006) PIASy mediates NEMO sumoylation and NF-kappaB activation in response to genotoxic stress. Nat Cell Biol 8:986–993
Maloney A, Workman P (2002) HSP90 as a new therapeutic target for cancer therapy: the story unfolds. Expert Opin Biol Ther 2:3–24
Massoumi R, Chmielarska K, Hennecke K, Pfeifer A, Fassler R (2006) Cyld inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. Cell 125:665–677
Matsumoto R, Wang D, Blonska M, Li H, Kobayashi M, Pappu B, Chen Y, Wang D, Lin X (2005) Phosphorylation of CARMA1 plays a critical role in T cell receptor-mediated NF-kappaB activation. Immunity 23:575–585
May MJ, D'Acquisto F, Madge LA, Glockner J, Pober JS, Ghosh S (2000) Selective inhibition of NF-kappaB activation by a peptide that blocks the interaction of NEMO with the IkappaB kinase complex. Science 289:1550–1554
Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A, Rao A (1997) IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. Science 278:860–866
Mercurio F, Murray BW, Shevchenko A, Bennett BL, Young DB, Li JW, Pascual G, Motiwala A, Zhu H, Mann M, Manning AM (1999) IkappaB kinase (IKK)-associated protein 1:a common component of the heterogeneous IKK complex. Mol Cell Biol 19:1526–1538
Miller BS, Zandi E (2001) Complete reconstitution of human IkappaB kinase (IKK) complex in yeast. Assessment of its stoichiometry and the role of IKKgamma on the complex activity in the absence of stimulation. J Biol Chem 276:36320–36326
Mordmuller B, Krappmann D, Esen M, Wegener E, Scheidereit C (2003) Lymphotoxin and lipopolysaccharide induce NF-kappaB-p52 generation by a co-translational mechanism. EMBO Rep 4:82–87
Mosser DD, Morimoto RI (2004) Molecular chaperones and the stress of oncogenesis. Oncogene 23:2907–2918
Ohmae T, Hirata Y, Maeda S, Shibata W, Yanai A, Ogura K, Yoshida H, Kawabe T, Omata M (2005) Helicobacter pylori activates NF-kappaB via the alternative pathway in B lymphocytes. J Immunol 175:7162–7169
Perkins ND (2007) Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol 8:49–62
Pickart CM (2001) Mechanisms underlying ubiquitination. Annu Rev Biochem 70:503–533
Piret B, Schoonbroodt S, Piette J (1999) The ATM protein is required for sustained activation of NF-kappaB following DNA damage. Oncogene 18:2261–2271
Poyet JL, Srinivasula SM, Lin JH, Fernandes-Alnemri T, Yamaoka S, Tsichlis PN, Alnemri ES (2000) Activation of the Ikappa B kinases by RIP via IKKgamma/NEMO-mediated oligomerization. J Biol Chem 275:37966–37977
Rawlings DJ, Sommer K, Moreno-Garcia ME (2006) The CARMA1 signalosome links the signalling machinery of adaptive and innate immunity in lymphocytes. Nat Rev Immunol 6:799–812
Reiley WW, Zhang M, Jin W, Losiewicz M, Donohue KB, Norbury CC, Sun SC (2006) Regulation of T cell development by the deubiquitinating enzyme CYLD. Nat Immunol 7:411–417
Rothwarf DM, Karin M (1999) The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus. Sci STKE 1999:RE1
Ruefli-Brasse AA, French DM, Dixit VM (2003) Regulation of NF-kappaB-dependent lymphocyte activation and development by paracaspase. Science 302:1581–1584

Ruland J, Duncan GS, Elia A, del Barco Barrantes I, Nguyen L, Plyte S, Millar DG, Bouchard D, Wakeham A, Ohashi PS, Mak TW (2001) Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. Cell 104:33–42
Ruland J, Duncan GS, Wakeham A, Mak TW (2003) Differential requirement for Malt1 in T and B cell antigen receptor signaling. Immunity 19:749–758
Saccani S, Pantano S, Natoli G (2003) Modulation of NF-kappaB activity by exchange of dimers. Mol Cell 11:1563–1574
Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, Matsumoto K, Takeuchi O, Akira S (2005) Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol 6:1087–1095
Scharschmidt E, Wegener E, Heissmeyer V, Rao A, Krappmann D (2004) Degradation of Bcl10 induced by T-cell activation negatively regulates NF-kappa B signaling. Mol Cell Biol 24:3860–3873
Schmidt-Supprian M, Bloch W, Courtois G, Addicks K, Israel A, Rajewsky K, Pasparakis M (2000) NEMO/IKK gamma-deficient mice model incontinentia pigmenti. Mol Cell 5:981–992
Schulze-Luehrmann J, Ghosh S (2006) Antigen-receptor signaling to nuclear factor kappa B. Immunity 25:701–715
Shi CS, Kehrl JH (2003) Tumor necrosis factor (TNF)-induced germinal center kinase-related (GCKR) and stress-activated protein kinase (SAPK) activation depends upon the E2/E3 complex Ubc13-Uev1A/TNF receptor-associated factor 2 (TRAF2). J Biol Chem 278:15429–15434
Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, Lee KY, Bussey C, Steckel M, Tanaka N, Yamada G, Akira S, Matsumoto K, Ghosh S (2005) TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev 19:2668–2681
Sommer K, Guo B, Pomerantz JL, Bandaranayake AD, Moreno-Garcia ME, Ovechkina YL, Rawlings DJ (2005) Phosphorylation of the CARMA1 linker controls NF-kappaB activation. Immunity 23:561–574
Su H, Bidere N, Zheng L, Cubre A, Sakai K, Dale J, Salmena L, Hakem R, Straus S, Lenardo M (2005) Requirement for caspase-8 in NF-kappaB activation by antigen receptor. Science 307:1465–1468
Sun Z, Arendt CW, Ellmeier W, Schaeffer EM, Sunshine MJ, Gandhi L, Annes J, Petrzilka D, Kupfer A, Schwartzberg PL, Littman DR (2000) PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. Nature 404:402–407
Sun L, Deng L, Ea CK, Xia ZP, Chen ZJ (2004) The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. Mol Cell 14:289–301
Tada K, Okazaki T, Sakon S, Kobarai T, Kurosawa K, Yamaoka S, Hashimoto H, Mak TW, Yagita H, Okumura K, Yeh WC, Nakano H (2001) Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death. J Biol Chem 276:36530–36534
Takaesu G, Surabhi RM, Park KJ, Ninomiya-Tsuji J, Matsumoto K, Gaynor RB (2003) TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway. J Mol Biol 326:105–115
Takeda K, Takeuchi O, Tsujimura T, Itami S, Adachi O, Kawai T, Sanjo H, Yoshikawa K, Terada N, Akira S (1999) Limb and skin abnormalities in mice lacking IKKalpha. Science 284:313–316
Tanaka M, Fuentes ME, Yamaguchi K, Durnin MH, Dalrymple SA, Hardy KL, Goeddel DV (1999) Embryonic lethality, liver degeneration, and impaired NF-kappa B activation in IKK-beta-deficient mice. Immunity 10:421–429
Tegethoff S, Behlke J, Scheidereit C (2003) Tetrameric oligomerization of IkappaB kinase gamma (IKKgamma) is obligatory for IKK complex activity and NF-kappaB activation. Mol Cell Biol 23:2029–2041
Ting AT, Pimentel-Muinos FX, Seed B (1996) RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. EMBO J 15:6189–6196

Trompouki E, Hatzivassiliou E, Tsichritzis T, Farmer H, Ashworth A, Mosialos G (2003) CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. Nature 424:793–796
Vinolo E, Sebban H, Chaffotte A, Israel A, Courtois G, Veron M, Agou F (2006) A point mutation in NEMO associated with anhidrotic ectodermal dysplasia with immunodeficiency pathology results in destabilization of the oligomer and reduces lipopolysaccharide- and tumor necrosis factor-mediated NF-kappa B activation. J Biol Chem 281:6334–6348
Vousden KH (2002) Switching from life to death: the Miz-ing link between Myc and p53. Cancer Cell 2:351–352
Wan YY, Chi H, Xie M, Schneider MD, Flavell RA (2006) The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. Nat Immunol 7:851–858
Wang C, Deng L, Hong M, Akkaraju GR, Inoue J, Chen ZJ (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 412:346–351
Wegele H, Muller L, Buchner J (2004) Hsp70 and Hsp90 – a relay team for protein folding. Rev Physiol Biochem Pharmacol 151:1–44
Wegener E, Oeckinghaus A, Papadopoulou N, Lavitas L, Schmidt-Supprian M, Ferch U, Mak TW, Ruland J, Heissmeyer V, Krappmann D (2006) Essential role for IkappaB kinase beta in remodeling Carma1-Bcl10-Malt1 complexes upon T cell activation. Mol Cell 23:13–23
Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A, Koonin EV, Dixit VM (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature 430:694–699
Whitesell L, Lindquist SL (2005) HSP90 and the chaperoning of cancer. Nat Rev Cancer 5:761–772
Wu CJ, Conze DB, Li T, Srinivasula SM, Ashwell JD (2006a) NEMO is a sensor of Lys 63-linked polyubiquitination and functions in NF-kappaB activation. Nat Cell Biol 8:398–406
Wu ZH, Shi Y, Tibbetts RS, Miyamoto S (2006b) Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli. Science 311:1141–1146
Yamaoka S, Courtois G, Bessia C, Whiteside ST, Weil R, Agou F, Kirk HE, Kay RJ, Israel A (1998) Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. Cell 93:1231–1240
Yeh WC, Shahinian A, Speiser D, Kraunus J, Billia F, Wakeham A, de la Pompa JL, Ferrick D, Hum B, Iscove N, Ohashi P, Rothe M, Goeddel DV, Mak TW (1997) Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity 7:715–725
Zandi E, Chen Y, Karin M (1998) Direct phosphorylation of IkappaB by IKKalpha and IKKbeta: discrimination between free and NF-kappaB-bound substrate. Science 281:1360–1363
Zhang SQ, Kovalenko A, Cantarella G, Wallach D (2000) Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKgamma) upon receptor stimulation. Immunity 12:301–311
Zhang J, Stirling B, Temmerman ST, Ma CA, Fuss IJ, Derry JM, Jain A (2006) Impaired regulation of NF-kappaB and increased susceptibility to colitis-associated tumorigenesis in CYLD-deficient mice. J Clin Invest 116:3042–3049
Zhou H, Wertz I, O'Rourke K, Ultsch M, Seshagiri S, Eby M, Xiao W, Dixit VM (2004) Bcl10 activates the NF-kappaB pathway through ubiquitination of NEMO. Nature 427:167–171

An Oncogenic Hub: β-Catenin as a Molecular Target for Cancer Therapeutics

K.-I. Takemaru(✉), M. Ohmitsu, and F.-Q. Li

Contents

1 Introduction 262
2 The Wnt/β-Catenin Signaling Pathway 262
3 Regulation of Nuclear β-Catenin Activity 264
3.1 Structural Features of β-Catenin 264
3.2 Target Gene Activation by β-Catenin 265
3.3 Co-Activators for β-Catenin 266
3.4 β-Catenin Antagonists 268
4 Wnt/β-Catenin Signaling in Cancer 269
5 Therapeutic Interventions Targeting β-Catenin Signaling Activity 271
5.1 Existing Drugs That Suppress β-Catenin Activity 272
5.2 Small-Molecule Inhibitors 272
5.3 Other Potential Therapeutic Targets in β-Catenin Signaling 274
6 Conclusions and Perspective 275
References 276

Abstract The Wnt/β-catenin signaling pathway plays diverse roles in embryonic development and in maintenance of organs and tissues in adults. Activation of this signaling cascade inhibits degradation of the pivotal component β-catenin, which in turn stimulates transcription of downstream target genes. Over the past two decades, intensive worldwide investigations have yielded considerable progress toward understanding the cellular and molecular mechanisms of Wnt signaling and its involvement in the pathogenesis of a range of human diseases. Remarkably, β-catenin signaling is aberrantly activated in greater than 70% of colorectal cancers and to a lesser extent in other tumor types, promoting cancer cell proliferation, survival and migration. Accordingly, β-catenin has gained recognition as an enticing molecular target for cancer therapeutics. Disruption of protein–protein interactions essential for β-catenin activity holds immense promise for the development of novel anti-cancer drugs. In this review, we focus on the regulation of β-catenin-dependent transcriptional activation and discuss potential therapeutic opportunities to block this signaling pathway in cancer.

K.-I. Takemaru
Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, NY 11794, USA
takemaru@pharm.stonybrook.edu

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,

© Springer-Verlag Berlin Heidelberg 2008

1 Introduction

β-Catenin (Armadillo in *Drosophila*) is a multifunctional protein involved in both cell–cell adhesion and Wnt signaling. As an essential co-activator downstream of Wnt signaling, β-catenin regulates many biological processes essential for proper embryonic development and adult homeostasis. Once abnormally activated in adult tissues, however, β-catenin could contribute to the onset of tumorigenesis or accelerate tumor growth. In recent years, a variety of novel β-catenin interactors has been identified, highlighting β-catenin's role as the hub of a very complex network of protein–protein interactions that fine-tune its transcriptional activity. These molecular interactions, therefore, represent attractive targets for pharmacological intervention. Here, we provide an overview of β-catenin signaling in tumor formation, summarize recent advances in pharmacological strategies to inhibit β-catenin transcriptional output and explore new opportunities for drug discovery in targeting oncogenic β-catenin.

2 The Wnt/β-Catenin Signaling Pathway

Intracellular signaling by the Wnt family of secreted cysteine-rich glycoproteins plays critical roles in normal embryonic development and adult homeostasis (Cadigan and Nusse 1997; Miller 2002; Wodarz and Nusse 1998). This pathway is highly conserved among the animal kingdom. There are 19 members of the Wnt gene family in mammals, which are expressed in a spatially and temporally restricted manner in embryos and in adults. Mutational analyses of Wnt genes in mice have demonstrated diverse roles in axis formation, brain development, pattern formation and organogenesis (Uusitalo et al. 1999; van Amerongen and Berns 2006). More recently, dysregulation of Wnt signaling has been linked to a range of human diseases, especially cancer (Clevers 2006b; Moon et al. 2004; Nusse 2005).

These Wnts signal through distinct intracellular pathways that are broadly categorized as either "canonical" or "non-canonical" Wnt pathways. The best understood canonical pathway utilizes nuclear β-catenin as an ultimate effector, leading to changes in gene expression that regulates cell proliferation, differentiation and survival, etc. In contrast, non-canonical pathways signal via a β-catenin-independent mechanism, generally resulting in changes in cell polarity and movement (Katoh 2005; Kohn and Moon 2005; Veeman et al. 2003).

A simplified model of the Wnt/β-catenin pathway is represented in Fig. 1. β-Catenin is the key component in this cascade, acting as a transcriptional co-activator. In the absence of a Wnt ligand, the cytosolic pool of β-catenin is continuously degraded by the action of a large protein complex called the "destruction complex" (Kimelman and Xu 2006). In this complex, the tumor suppressors Axin and adenomatous polyposis coli (APC) function as scaffold proteins that facilitate sequential phosphorylation of β-catenin at the N-terminus by casein kinase Iα (CKIα) and

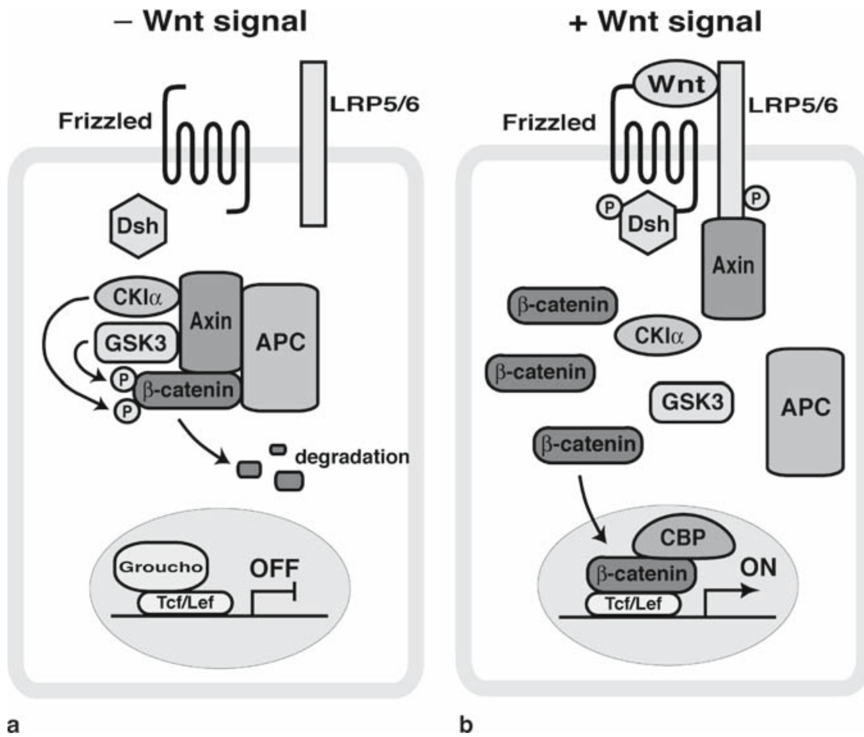

Fig. 1 The canonical Wnt/β-catenin signaling pathway. **(a)** In the absence of a Wnt signal, β-catenin is held by the scaffold proteins APC and Axin in the destruction complex, and sequentially phosphorylated by the kinases CKIα and GSK3. Consequently, β-catenin is constantly degraded by the ubiquitin-proteasome pathway. In the nucleus, Tcf/Lef transcription factors actively repress target gene expression by recruiting transcriptional co-repressors, such as Gro/TLE. **(b)** Wnt binding to Frizzled receptors and LRP5/6 co-receptors induces Frizzled-Dsh complex formation and the sequential phosphorylation of LRP5/6 by GSK3 and CKIγ, promoting recruitment of Axin to the plasma membrane and inactivation of the destruction complex. Stabilized β-catenin then translocates into the nucleus and forms a complex with Tcf/Lef factors by displacing Gro/TLE proteins. β-Catenin stimulates expression of Wnt target genes by recruiting various co-activators including CBP

glycogen synthase kinase 3 (GSK3) (Liu et al. 2002; Polakis 2002). Phosphorylated β-catenin is then recognized by the E3 ubiquitin ligase receptor βTrCP and targeted for ubiquitin-mediated proteasomal degradation. As a result, β-catenin protein levels are kept low without Wnt stimulation. In the nucleus, the DNA-binding T-cell factor/lymphoid enhancer factor (Tcf/Lef) proteins actively repress target genes through recruitment of transcriptional co-repressors such as Groucho (Gro)/TLE and C-terminal-binding protein (CtBP) (Arce et al. 2006; Brannon et al. 1999; Cavallo et al. 1998; Hoppler and Kavanagh 2007; Roose et al. 1998).

Extracellularly, the activity of Wnts is modulated by a variety of secreted antagonists, including Wnt-inhibitory factors (WIFs) (Hsieh et al. 1999) and secreted frizzled-related proteins (sFRPs) (Hoang et al. 1996). When not docked to these inhibitors, Wnts interact with the seven transmembrane frizzled (Fz) receptors and the low-density lipoprotein receptor-related protein (LRP) co-receptors LRP5 and LRP6 (Cadigan and Liu 2006; Wang et al. 2006). This results in phosphorylation and activation of the cytoplasmic protein dishevelled (Dsh), which is rapidly recruited to the plasma membrane via interaction with the intracellular tail of Fz (Malbon and Wang 2006). Activation of Dsh blocks GSK3 activity. In parallel, Wnt stimulation induces sequential phosphorylation of the LRP5/6 intracellular domain by GSK3 and CKIγ (Davidson et al. 2005; Zeng et al. 2005). The hyperphosphorylated LRP5/6 directly interacts with Axin, and Axin is then degraded by an unknown mechanism (Tolwinski and Wieschaus 2004b). As a result of the above events, β-catenin degradation is inhibited, thereby leading to accumulation of β-catenin in the cytoplasm. Subsequently, stabilized β-catenin translocates into the nucleus and binds to Tcf/Lef transcription factors to activate Wnt target genes (Stadeli et al. 2006; Willert and Jones 2006). β-Catenin protein levels are also regulated by Siah, a p53-inducible E3 ubiquitin ligase component that acts independently of GSK3 to recruit APC to target β-catenin for proteasomal degradation (Liu et al. 2001; Matsuzawa and Reed 2001).

3 Regulation of Nuclear β-Catenin Activity

3.1 Structural Features of β-Catenin

β-Catenin has two key cellular functions (Takemaru 2006). In cell adhesion, β-catenin binds type-I cadherins to mediate actin filament assembly via α-catenin at the plasma membrane (Gates and Peifer 2005; Huber and Weis 2001; Nelson and Nusse 2004; Pokutta and Weis 2000). In Wnt signaling, β-catenin acts as a co-activator through its interaction with an array of transcription factors and co-factors to regulate expression of target genes (Stadeli et al. 2006; Willert and Jones 2006).

Human or mouse β-catenin is 781 residues long and consists of a central structural core of 12 Armadillo (Arm) repeats flanked by unique N- and C-termini (Peifer et al. 1994) (Fig. 2). The central Arm repeat region is highly conserved across species. The crystal structure of this region reveals that each repeat consists of three α-helices, which pack tightly to form a highly elongated structure with a positively charged groove (Huber et al. 1997). Of note, many β-catenin-binding partners bind along the positively charged groove. Compared to the highly conserved Arm repeats, N- and C-terminal tails are diverged between *Drosophila* and vertebrate homologs. The structures of the N- and C-terminal domains are currently unknown and may not form a rigid structure by themselves. Nevertheless, these terminal domains have been shown to control a subset of protein–protein interactions

N-Terminal Activation Domain (NTAD)
C-Terminal Activation Domain (CTAD)
S/T
1 2 3 4 5 6 7 8 9 10 11 12

β-catenin Interactors	References
α-catenin	Pokutta & Weis 2000
E-cadherin	Huber & Weis 2001
APC	Ha et al. 2004 Xing et al. 2004
Axin	Xing et al. 2003
Tcf/Lef	van de Wetering et al.1997 Graham et al. 2000 Graham et al. 2001
TBP	Hecht et al.1999
Lgs	Kramps et al.2002 Holfmans & Basler 2004 Sampletro et al.2006
Pontin	Bauer et al. 1998
Brg1	Barker et al. 1998
CBP/p300	Hecht et al. 2000 Takemaru & Moon 2000
CoCoA	Yang et al. 2006
TRRAP, ISWI, MLL	Slerra et al. 2006
Parafibromin	Mosimann et al. 2006
MED12	Kim et al. 2006
ICAT	Tago et al. 2000 Daniels & Wels 2002 Graham et al. 2002
Chibby	Takemaru et al. 2003

Fig. 2 Schematic representation of β-catenin structure and its minimum domains required for protein–protein interactions with various partners. β-Catenin protein consists of 12 imperfect Armadillo (Arm) repeats (R1-12) flanked by N- and C-terminal tails. S/T represents serine/threonine residues phosphorylated by CKIα and GSK3. Two transcriptional activation domains are indicated by *gray bars*. *Black lines* denote interaction domains within β-catenin for the indicated binding partners

(Castano et al. 2002; Choi et al. 2006; Solanas et al. 2004). Several β-catenin interactors require these domains for their binding as discussed further below.

3.2 Target Gene Activation by β-Catenin

Once in the nucleus, β-catenin forms a stable complex with Tcf/Lef family transcription factors to stimulate gene expression (Stadeli et al. 2006; Willert and Jones 2006). The vertebrate genome encodes four Tcf/Lef proteins (Tcf1, Tcf3, Tcf4 and Lef1), which contain the highly conserved high-mobility group (HMG) box as a DNA-binding domain (Arce et al. 2006; Hoppler and Kavanagh 2007). In the absence of nuclear β-catenin, Tcf/Lef factors are bound to the co-repressors Gro/TLE and CtBP, actively suppressing target gene transcription. Both Gro/TLE and CtBP interact with histone deacetylases in order to silence transcription by altering local chromatin structure (Courey and Jia 2001).

β-Catenin binds to Tcf/Lef by displacing Gro/TLE (Daniels and Weis 2005). This allows β-catenin to recruit a variety of co-activators necessary to exert its transcriptional potential. Earlier studies have demonstrated that β-catenin harbors two transactivation domains, N-terminal activation domain (NTAD) and C-terminal activation domain (CTAD) (Hecht et al. 1999; Hsu et al. 1998; van de Wetering et al. 1997) (Fig. 2). Each of these domains on its own potently stimulates gene expression. In particular, the CTAD alone has been shown to be sufficient both for signaling and for oncogenic transformation (Aoki et al. 1999; Vleminckx et al. 1999).

A considerable number of direct target genes of the Tcf/β-catenin complex has been identified in various model systems, including c-Myc (He et al. 1998), cyclin D1 (Tetsu and McCormick 1999), MMP7 (matrilysin) (Crawford et al. 1999) and Nr-CAM (Conacci-Sorrell et al. 2002). For a comprehensive list of Wnt target genes, we direct the readers to the Wnt Homepage (http://www.stanford.edu/~rnusse/wntwindow.html). Also worthy of note, β-catenin modulates expression of several components of the Wnt pathway, such as an Axin homolog Axin2 (conductin) (Jho et al. 2002; Lustig et al. 2002), βTrCP (Spiegelman et al. 2000) and Tcf/Lef (Hovanes et al. 2001; Roose et al. 1999), thereby creating a negative or positive feedback loop. Generally, cellular responses to Wnt signals vary among different cell types, and many Wnt target genes are, in fact, regulated in a cell-type-specific fashion.

3.3 Co-Activators for β-Catenin

β-Catenin exerts its activation potential through communication with components of the basal transcription machinery and recruitment of co-activators. On the other hand, the activity of β-catenin can be counteracted by its antagonists. Interaction domains of β-catenin for those binding partners are illustrated in Fig. 2.

The first β-catenin transcriptional co-factor discovered was the TATA-binding protein (TBP) (Hecht et al. 1999). TBP has been shown to bind three different regions of β-catenin overlapping with the NTAD and CTAD. Although TBP binding to these regions correlates with their activation ability, it remains to be determined whether these interactions are functionally significant in an *in vivo* setting.

The closely related p300 and CREB-binding protein (CBP) are known to function as transcriptional co-activators by linking a variety of transcription factors to the basal transcription machinery or by altering local chromatin structure through their intrinsic or associated histone acetyltransferase activities (Goodman and Smolik 2000). p300 and CBP have been shown to interact with multiple regions of β-catenin, including the CTAD to enhance transcriptional activity (Hecht et al. 2000; Miyagishi et al. 2000; Sun et al. 2000; Takemaru and Moon 2000). In marked contrast, *Drosophila* CBP (dCBP) negatively regulates the Wingless (Wg; *Drosophila* Wnt) signaling pathway (Waltzer and Bienz 1998). dCBP binds to dTcf and acetylates a conserved lysine in the N-terminal Armadillo-binding domain, which weakens the interaction between dTcf and Armadillo. At present, it is not clear if

CBP/p300 could, in some context, similarly elicit a repressive effect on transcription of β-catenin target genes in vertebrates.

Brahma-related gene 1 (Brg1) is a component of mammalian SWI/SNF and Rsc chromatin-remodeling complexes, which utilizes the energy of ATP hydrolysis to mobilize nucleosomes and remodel chromatin to facilitate transcription (Roberts and Orkin 2004). Brg1 binds β-catenin through Arm repeats 7–12 (R7-12) (Barker et al. 2001). It synergizes with β-catenin to stimulate gene expression in mammalian cultured cells and also genetically interacts with Armadillo in *Drosophila*.

Two closely related proteins, Pontin/Tip49 and Reptin/Tip48, are members of a highly conserved family with ATPase and helicase activities (Kanemaki et al. 1997, 1999). Pontin and Reptin can form homo- and hetero-dimers. They both bind TBP and are found in multimeric complexes believed to be important for chromatin remodeling and transcription. They have been shown to interact with β-catenin R2-5 and play opposing roles in β-catenin-mediated transcriptional activation (Bauer et al. 1998, 2000). Pontin potentiates activation by β-catenin, whereas Reptin represses it (Bauer et al. 1998; Rottbauer et al. 2002). By modulating Wnt/β-catenin signaling, these two proteins control wing development in *Drosophila* (Bauer et al. 1998) and heart growth in zebrafish (Rottbauer et al. 2002). The activity of Pontin appears to be required for β-catenin-mediated neoplastic transformation *in vitro* (Feng et al. 2003).

Nuclear β-catenin activity is positively regulated by Legless (Lgs) and Pygopus (Pygo). Lgs and Pygo were identified in genetic screens for modifiers for the Wg pathway in fly (Belenkaya et al. 2002; Kramps et al. 2002; Parker et al. 2002; Thompson et al. 2002). *Drosophila* mutants for these genes display phenotypes highly similar to that of Wg loss-of-function, and ectopic expression of Pygo and/or Lgs stimulates Tcf/β-catenin activity in mammalian cultured cells. Lgs directly binds to β-catenin R1-4 and serves as an adapter molecule to recruit Pygo to target gene promoters (Hoffmans and Basler 2004; Kramps et al. 2002; Sampietro et al. 2006). Pygo harbors a PHD finger that is often found in chromatin remodeling factors. Pygo is exclusively localized in the nucleus and has been proposed to function in the nuclear import/retention of β-catenin (Townsley et al. 2004). Additionally, Pygo and Lgs directly contribute to the ability of β-catenin to enhance gene transcription (Hoffmans et al. 2005). The human ortholog of Lgs, Bcl9, was originally identified as an oncoprotein associated with precursor B-cell acute lymphoblastic leukemia (ALL), which exhibits the chromosomal translocation t(1;14)(q21;q32) (Willis et al. 1998). Although genetic analyses in fly clearly demonstrate that Lgs and Pygo are segment polarity gene products required for Wg signaling events, the biological functions of their vertebrate orthologs remain enigmatic.

More recently, a genetic screen in *Drosophila* identified Parafibromin/Hyrax as a novel β-catenin binding partner (Mosimann et al. 2006). The polymerase-associated factor 1 (Paf1) complex, which contains parafibromin, interacts with the C-terminal domain of the large subunit of RNA polymerase II (Pol II) and plays a key role in transcription elongation and RNA processing (Rozenblatt-Rosen et al. 2005; Yart et al. 2005). Parafibromin forms a stable complex with β-catenin through R12-C and is essential for activation of Wnt/Wg signaling (Mosimann et al. 2006).

The transactivation potential by parafibromin depends on the recruitment of Pygo to β-catenin, and in agreement with this, Bcl9 and Pygo are present in the parafibromin/β-catenin complex. Parafibromin is the protein product of the tumor suppressor gene HRPT2 mutated in hyperparathyroism-jaw tumor (HPT-JT) syndrome (Carpten et al. 2002). HPT-JT syndrome is a rare autosomal dominant multi-tumor syndrome characterized by hyperparathyroidism due to parathyroid tumors (Wang et al. 2005). However, it remains unknown if alterations in the Wnt/β-catenin pathway directly contribute to the pathogenesis of this syndrome.

In vitro studies, using a reconstituted transcription system, have shown that β-catenin potently enhances binding and transactivation by Tcf/Lef proteins on chromatin templates (Tutter et al. 2001). In this system, the C-terminal region of β-catenin (R11-C) behaves as a dominant-negative inhibitor that specifically represses transcription by the Tcf/β-catenin complex. This is consistent with the previous notion that the C-terminal region of β-catenin acts as a transactivation domain via recruitment of co-activators. More recent biochemical studies have identified additional factors that associate with the CTAD to stimulate β-catenin-dependent transcription, including the coiled-coil co-activator CocoA (R10-C) (Yang et al. 2006), the mediator subunit Med12 (R12-C) (Kim et al. 2006) and subunits of multiple chromatin remodeling complexes, such as the transformation/transcription-domain-associated protein (TRAP), the imitation switch nucleosome-remodeling ATPase (ISWI) and the trithorax-family mixed-lineage-leukemia (MLL1/MLL2) SET-type proteins (R11-C) (Sierra et al. 2006). Further studies will be required to validate the physiological relevance of these interactions.

3.4 β-Catenin Antagonists

In addition to the co-activators discussed above, β-catenin signaling is also subjected to regulation by multiple inhibitors.

ICAT (inhibitor of β-catenin and Tcf4) is a 9-kD protein found only in vertebrates (Tago et al. 2000). ICAT binds to R10-12 of β-catenin and interferes with the formation of the Tcf/β-catenin complex, resulting in inhibition of target gene activation. The crystallographic studies of ICAT bound to the Arm repeat region of β-catenin display that an N-terminal helical domain of ICAT binds to the groove formed by R10-12, and an extended C-terminal tail binds to R5-9 in a manner similar to that of Tcf/Lef transcription factors, thereby disrupting Tcf/β-catenin interactions (Daniels and Weis 2002; Graham et al. 2002). In colorectal or hepatocellular tumor cells harboring mutations in APC, Axin or β-catenin, forced expression of ICAT inhibits proliferation by inducing G2 arrest followed by cell death (Sekiya et al. 2002). Consistent with its inhibitory effect on β-catenin activity, ICAT expression is abundant in well-differentiated intestinal villus cells, while absent in crypt stem/progenitor cells where β-catenin signaling is active (Gottardi and Gumbiner 2004). In colorectal cancers, however, no ICAT mutations have been detected. Instead, ICAT expression appears to be elevated

compared to adjacent non-cancerous tissues (Koyama et al. 2002). To the contrary, ICAT expression levels were found to be markedly reduced in malignant melanomas (Reifenberger et al. 2002).

Chibby (Cby) is a small nuclear protein that is highly conserved throughout evolution (Takemaru et al. 2003). Cby interacts with the CTAD of β-catenin and competes with Tcf/Lef proteins for binding to β-catenin, leading to inhibition of Wnt/β-catenin target gene activation. Cby may also displace β-catenin co-activators such as CBP/p300 and parafibromin since they all interact with the CTAD. Furthermore, reduction of Cby protein levels by RNA interference (RNAi) in *Drosophila* embryos results in hyperactivation of the Wg pathway, demonstrating that Cby negatively regulates β-catenin activity *in vivo* (Greaves 2003; Takemaru et al. 2003; Tolwinski and Wieschaus 2004a). In murine embryonic stem cells, Cby facilitates cardiomyocyte differentiation (Singh et al. 2007), consistent with the known inhibitory role of the canonical Wnt pathway in heart formation. The human Cby gene is located at chromosome 22q12-q13, which is frequently lost in certain types of tumors (Gad et al. 2004; Schuierer et al. 2006). However, recent studies found no evidence for mutations or altered expression of the Cby gene in colorectal cancer and Wilms' tumors (nephroblastoma), although Cby expression has been reported to be significantly down-regulated in colon carcinoma cell lines (Gad et al. 2004; Schuierer et al. 2006; Zirn et al. 2005).

It is worth noting that β-catenin has been shown to serve as a co-activator or, in some cases, a co-repressor for a wide variety of DNA-binding transcription factors, including members of the nuclear hormone receptor family and HMG-box-containing Sox proteins (Mulholland et al. 2005; Sinner et al. 2004). For example, the vitamin A, vitamin D and androgen receptors physically interact with β-catenin in a ligand-dependent fashion to potentiate activation of their target genes, while suppressing expression of Tcf/β-catenin-dependent genes (Easwaran et al. 1999; Shah et al. 2006; Truica et al. 2000). Thus, it is apparent that the cross-regulation of the Wnt/β-catenin pathway and these transcription factors affects a diverse array of biological processes and may have important clinical implications.

4 Wnt/β-Catenin Signaling in Cancer

Given the critical and pleiotropic roles of Wnts, it is not surprising that perturbations in Wnt signaling have been implicated in a range of human disorders, especially cancer (Clevers 2006b; Logan and Nusse 2004; Moon et al. 2004). Activating mutations in the canonical Wnt pathway ultimately lead to stabilization and nuclear accumulation of β-catenin. Nuclear β-catenin is the hallmark of activated canonical Wnt signaling, and its nuclear accumulation can be clearly observed in cancer cells (Bienz and Clevers 2003). By binding to Tcf/Lef transcription factors, hyperactive β-catenin turns on a genetic program sufficient to initiate development of a multitude of different tumor types, primarily of gastrointestinal origin (Giles et al. 2003; Lustig and Behrens 2003; Polakis 2000).

A series of elegant genetic studies in mice has established that Wnt/β-catenin signaling plays a fundamental role in controlling normal epithelial physiology of the intestine, most notably self-renewal of crypt stem cells (Clarke 2006; Pinto and Clevers 2005; Reya and Clevers 2005). Several Wnts and Wnt receptors are indeed expressed in various compartments of the intestine (Gregorieff et al. 2005). Among four Tcf/Lef family members, Tcf4 is prominently expressed throughout life in the intestinal epithelium. Strikingly, in neonatal mice lacking Tcf4, the differentiated villus epithelium appears normal, but the crypt stem-cell compartment in the small intestine is mostly depleted (Korinek et al. 1998). Therefore, it appears that Tcf4 mediates transformation of gut epithelial cells upon β-catenin activation. Viral or transgenic expression of Dickkopf 1 (Dkk1), a secreted Wnt antagonist, in this tissue results in markedly reduced proliferation of intestinal crypts, further supporting the crucial role of canonical Wnt signaling in regulating the maintenance of crypt stem/progenitor cells (Kuhnert et al. 2004; Pinto et al. 2003). In contrast, inducible inactivation of APC in the adult mouse intestine leads to rapid nuclear accumulation of β-catenin and repopulation of villi by crypt progenitor-like cells that fail to migrate and differentiate (Sansom et al. 2004). In addition to maintaining the proliferative crypt compartment, this signaling pathway promotes maturation of Paneth cells localized at the base of crypts (van Es et al. 2005). These observations highlight the physiological importance of the Wnt/β-catenin pathway in intestinal development and homeostasis.

Wnt/β-catenin signaling was initially linked to cancer formation when the APC tumor suppressor was found to be mutated in inherited familial adenomatous polyposis (FAP) (Kinzler et al. 1991; Kinzler and Vogelstein 1996; Nishisho et al. 1991) and sporadic colorectal tumors (Kinzler and Vogelstein 1996; Korinek et al. 1997; Morin et al. 1997). FAP patients inherit one mutant APC allele and acquire a somatic mutation in the second APC allele at low frequency in their intestinal epithelial cells. These patients typically develop hundreds to thousands of colorectal adenomas, and some of those eventually progress to malignant adenocarcinomas. These APC mutations typically yield truncated APC proteins that are no longer able to degrade β-catenin, followed by accumulation of β-catenin in the nucleus (Korinek et al. 1997; Morin et al. 1997). This results in inappropriate constitutive activation of β-catenin target genes, promoting the formation of benign adenoma or polyps. Of note, around 70% of all colorectal cancers have homozygous mutations in APC (Miyaki et al. 1994; Miyoshi et al. 1992; Powell et al. 1992). Mouse models for FAP harboring similar APC truncations, such as APC^{Min} (for multiple intestinal neoplasia), develop multiple intestinal polyps predominantly in the small intestine (Moser et al. 1990; Su et al. 1992; Taketo 2006). Unfortunately, most APC mutant animals die at the age of 4–5 months due to anemia resulting from the heavy tumor load in the small intestine even before adenomatous polyps progress into metastatic adenocarcinomas. Nonetheless, these mouse models have become invaluable and powerful tools for studying the underlying mechanisms of colorectal tumorigenesis.

Approximately 10% of colorectal tumors carry activating mutations in the β-catenin gene (Miller et al. 1999; Polakis 2000). These mutations typically affect the

N-terminal region of β-catenin containing the highly conserved serine (S)/threonine (T) residues, which are sequentially phosphorylated by CKIα (S45) and then by GSK3 (S33, S37 and T41) for proteasomal degradation (Liu et al. 2002; Polakis 2002). Consequently, β-catenin becomes refractory to degradation and its nuclear levels rise, resulting in activation of target genes by the Tcf/β-catenin complex. More compelling evidence for the involvement of β-catenin signaling in intestinal tumor formation came from transgenic mouse models that conditionally express a stabilized form of β-catenin (Harada et al. 1999). When activated in the intestinal epithelium, these mice develop a large number of adenomatous polyps highly similar to those in APC mutant mice, demonstrating that activation of β-catenin signaling is sufficient for tumor formation. Moreover, frequent mutations in β-catenin have been detected in a wide range of tumor types, including malignant melanomas (Rubinfeld et al. 1997), hepatocellular carcinomas (de La Coste et al. 1998; Miyoshi et al. 1998), ovarian carcinomas (Gamallo et al. 1999; Palacios and Gamallo 1998) and Wilms' tumors (Koesters et al. 1999; Maiti et al. 2000).

Besides APC and β-catenin, mutations in the scaffold protein Axin and its close homolog Axin2 (Behrens et al. 1998) have been reported in several types of cancers, including colorectal tumors and hepatocellular carcinomas (Giles et al. 2003; Polakis 2000; Segditsas and Tomlinson 2006). In addition to these mutations in intracellular signaling components, multiple tumor types display loss of expression of the secreted Wnt antagonists sFRPs and WIF1 due to epigenetic silencing by hypermethylation (Mazieres et al. 2004; Rubin et al. 2006; Suzuki et al. 2004). Regardless of which component is misregulated, the common outcome resulting from these mutations is the stabilization of the key player β-catenin and the subsequent formation of nuclear Tcf/β-catenin complexes, causing uncontrolled transcriptional activation, the hallmark of cancer cells.

The Wnt/β-catenin pathway is considered to be crucial not only for cancer initiation, but also for cancer progression. Inhibition of β-catenin signaling by a dominant negative form of Tcfs that lack β-catenin-binding domain results in a rapid G1 arrest in colorectal cancer cell lines (Tetsu and McCormick 1999; van de Wetering et al. 2002). Furthermore, loss of β-catenin decreases tumor growth rates in colorectal carcinoma xenograft models (Green et al. 2001; Kim et al. 2002). These findings suggest that the high proliferative capacity of late-stage colon cancer cells remains heavily dependent on β-catenin signaling activity.

5 Therapeutic Interventions Targeting β-Catenin Signaling Activity

The persistent presence of stabilized nuclear β-catenin in a broad spectrum of human cancers makes it an attractive target for cancer therapeutics. Despite the fact that there has been great interest in discovering drugs that effectively block β-catenin activity over the past decade, the development of such therapeutics is still in its infancy. The number of novel molecular interactions identified in this signaling

pathway discussed above offers new opportunities for pharmacological intervention. Outlined below are current drug discovery and development efforts aimed at inhibiting aberrant Wnt/β-catenin signaling in cancer.

5.1 Existing Drugs That Suppress β-Catenin Activity

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, are widely used for the treatment of inflammation, pain and fever. The beneficial effects of NSAIDs are mainly attributed to inhibition of cyclooxygenase-2 (COX-2) involved in prostaglandin biosynthesis. In recent years, numerous epidemiological and clinical studies have shown that regular use of these compounds reduces the incidence and severity of various human cancers, including colorectal cancer (Giovannucci et al. 1995; Jolly et al. 2002; Koehne and Dubois 2004; Thun et al. 1991). NSAIDs exert their anti-neoplastic effects through a number of mechanisms, including suppression of tumor cell proliferation, angiogenesis and metastasis, as well as induction of apoptosis (Koehne and Dubois 2004; Williams et al. 1997). Several lines of evidence indicate that inhibition of elevated COX-2 activity in tumors contributes to their anti-cancer activity (Oshima et al. 1996; Sonoshita et al. 2001). Their precise mechanism of action, however, seems to be fairly complex. Recent reports have suggested that COX-2-independent routes may also explain the anti-cancer effects of NSAIDs (Grosch et al. 2006).

Cumulative data indicate that the anti-tumor action of NSAIDs is mediated in part through suppression of the canonical Wnt pathway. This was first proposed when NSAID treatment was found to drastically decrease the overall number and size of adenomatous polyps in patients with FAP (Giardiello et al. 1993; Phillips et al. 2002) as well as in FAP mouse models (Torrance et al. 2000; Yang et al. 2003). Mechanistically, these drugs appear to inhibit Wnt/β-catenin signaling at multiple levels, including induction of β-catenin degradation (Bos et al. 2006; Dihlmann et al. 2003) and disruption of the Tcf/β-catenin complex (Hawcroft et al. 2002; Nath et al. 2003). A more recent report suggests that prostaglandin E_2 stimulates colon cancer cell growth via a G-protein-dependent pathway that ultimately leads to inactivation of GSK3, thereby elevating β-catenin levels (Castellone et al. 2005; Clevers 2006a). Further studies will be required to define the mechanistic aspects of NSAID action in the treatment of colorectal cancer.

5.2 Small-Molecule Inhibitors

In the nucleus, the formation of the Tcf-β-catenin complex is a prerequisite for target gene activation. Targeted interference with this complex formation in colon cancer cells has been shown to effectively abrogate activation of target genes and to inhibit their growth *in vitro* (Tetsu and McCormick 1999; van de Wetering et al. 2002).

Accordingly, small compounds that can specifically disrupt the Tcf-β-catenin complex *in vivo* hold immense potential for the treatment of Wnt-driven tumors.

Earlier crystallographic studies of Tcf-β-catenin complexes demonstrate that the N-terminal acidic domain of Tcf proteins binds, with a high affinity (K_d = ~10 nM), to an extended region encompassing R3-10 of β-catenin (Graham et al. 2000, 2001). However, this basic groove of the β-catenin Arm repeat domain also interacts with other molecules. For instance, comparison of crystal structures of β-catenin bound to E-cadherin, APC or Axin reveals that these ligands bind along the basic groove of β-catenin in a very similar manner to Tcf proteins (Ha et al. 2004; Huber and Weis 2001; Xing et al. 2003, 2004). These observations raise concern that any small molecule capable of disrupting the Tcf-β-catenin complex would potentially interfere with β-catenin-cadherins, β-catenin-APC and/or β-catenin-Axin interactions. Interaction with E-cadherin is essential for cell–cell adhesion, while complex formation with APC or Axin controls β-catenin levels in normal tissues. Disruption of such interactions is likely to have undesirable consequences. Hence, small-molecule inhibitors must selectively interfere with the Tcf-β-catenin complex without disrupting other essential interactions of β-catenin.

The first small-molecule disruptors of the Tcf-β-catenin complex were successfully identified using a high-throughput immunoenzymatic detection of the protein–protein interaction (Lepourcelet et al. 2004). Among 7,000 purified natural compounds, eight displayed reproducible, dose-dependent inhibition of the Tcf-β-catenin interaction with IC_{50} < 10 μM. Two most potent compounds (PKF115-584 and CGP049090; Fig. 3) consistently scored as inhibitors of the Tcf-β-catenin interaction in nearly every secondary assay, including disruption of Tcf-β-catenin complexes *in vitro*, and inhibition of colon cancer cell proliferation, β-catenin-dependent reporter activity and β-catenin-mediated axis duplication in *Xenopus* embryos. Significantly, these compounds share a common core chemical structure, implying that they exert their effects in a similar mechanism. However, the precise molecular mechanisms by which these active compounds block the interaction between Tcf and β-catenin are unclear. Unfortunately, these small molecules also interfere with APC-β-catenin interactions, limiting their therapeutic potential as anticancer drugs (Lepourcelet et al. 2004). Nevertheless, this pioneer work underscores the

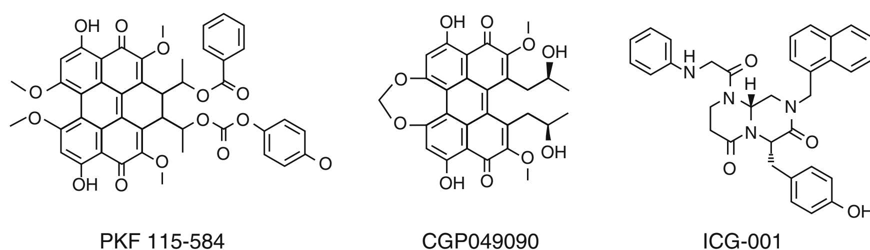

Fig. 3 Chemical structures of small-molecule inhibitors of β-catenin signaling. RKF115-584 and CGP049090 abrogate the Tcf/β-catenin interaction, while ICG-001 interferes with β-catenin binding to CBP, leading to inhibition of β-catenin signaling

feasibility of targeting protein–protein interactions among the essential components of oncogenic Wnt/β-catenin signaling in cancer drug discovery.

Given the high-affinity and dynamic binding of Tcf proteins to β-catenin, and the fact that substantially overlapping regions in the basic groove of β-catenin interact with multiple molecules, screening or designing inhibitors that are highly specific for the Tcf-β-catenin interaction may face a major challenge. Additionally, one might envision that disrupting binding of β-catenin to essential transcriptional co-activators represents potential opportunities for small-molecule-mediated intervention of β-catenin activity in tumor cells.

Another class of drugs with inhibitory effects on β-catenin activity has been isolated by a high-throughput cell-based assay and found to disrupt the interaction of β-catenin with CBP (Eguchi et al. 2005; Emami et al. 2004). SW480 colon cancer cells express truncated APC and hence show elevated basal activities of the β-catenin reporter TOPFLASH, which contains three optimal Tcf/Lef-binding sites driving luciferase expression (Korinek et al. 1997). Among 5,000 structurally divergent synthetic compounds, three closely related small molecules were found to display a dose-dependent inhibition of TOPFLASH activity in SW480 cells, with an IC_{50} of 3 μM, without significantly affecting the mutant reporter FOPFLASH. The lead compound ICG-001 (Fig. 3), with a molecular weight of 548, selectively binds to the N-terminal portion of CBP and interferes with its interaction with β-catenin. Curiously, this compound also binds to the corresponding region of the CBP homolog p300, but does not seem to disrupt its association with β-catenin. Through a microarray analysis, ICG-001 treatment of SW480 cells was found to inhibit expression of a subset of β-catenin target genes including cyclin D1 and the anti-apoptotic gene survivin (Ma et al. 2005). ICG-001 also exhibited selective growth inhibitory effects and cytotoxicity in colon carcinoma cells with activated β-catenin, but not in normal colonic epithelial cells *in vitro*. More importantly, treatment with this compound dramatically decreases tumor growth *in vivo* in both APC^{Min} and SW620 xenograft mouse models of cancer (Emami et al. 2004).

5.3 Other Potential Therapeutic Targets in β-Catenin Signaling

Other β-catenin co-activators such as TBP, Bcl9, Pontin, Brg1, CoCoA, TRAP, ISWI, MLL, parafibromin and Med12 are alternative targets for future cancer therapeutics. Eventually, most of these co-activators are predicted to bind β-catenin at regions distinct from those for the interaction with cadherins, APC or Axin (Fig. 2). Small-molecule-mediated inhibition of their interaction with β-catenin is, therefore, expected to be more selective. The crystal structure of a β-catenin-Bcl9-Tcf triple complex reveals that the β-catenin binding site of Bcl9 is distinct from that of the majority of β-catenin-interacting partners, highlighting the great potential of this target for pharmacological intervention (Sampietro et al. 2006). Aside from directly interfering with the transactivation potential of β-catenin, other candidate

approaches include modulation of subcellular localization of β-catenin, induction of β-catenin degradation, as well as small-interfering RNA (siRNA) approaches to silence β-catenin expression (Cong et al. 2003; Su et al. 2003; van de Wetering et al. 2003; Verma et al. 2003). Tcf/β-catenin target genes such as cyclin D1 and c-Myc also provide alternative opportunities for therapeutic interventions in human cancers, as these genes are most likely to directly contribute to cancer formation and progression. Finally, targeting more upstream signaling components in the canonical Wnt pathway might be of therapeutic value depending upon which component is dysregulated in a disease state (He et al. 2005; You et al. 2004).

6 Conclusions and Perspective

It has become increasingly clear that perturbations in Wnt signaling are involved in human diseases. In particular, a wealth of evidence implicates that chronic activation of β-catenin signaling is a common feature in nearly all colorectal tumors as well as in other tumor types. Therefore, small molecules that modulate this signaling cascade hold great promise for treating a range of cancers and potentially other diseases associated with aberrant Wnt signaling. Recent advances in understanding the molecular mechanisms of β-catenin transactivation provide new and exciting opportunities for therapeutic intervention. In addition to disrupting the Tcf/β-catenin complex in order to effectively block target gene activation, interactions between β-catenin and essential co-activators are attractive targets that certainly deserve intensive pharmacological searches.

A significant number of obstacles remain to be overcome before small-molecule β-catenin inhibitors become a reality. First of all, drugs developed to inhibit the Tcf/β-catenin interaction must be highly selective without affecting other β-catenin complexes essential for normal cell function. Second, drug development or design targeting β-catenin co-activators may similarly encounter specificity problems, as the majority of these co-activators are most likely to participate in multiple signaling pathways. Better understanding of the function of these co-activators and their co-crystal structure with β-catenin will help to assess their true potential as therapeutic targets. Another critical issue is whether adults can tolerate Wnt pathway blockade since β-catenin signaling activity is crucial for normal stem cell renewal, tissue regeneration and repair (Beachy et al. 2004; Pinto and Clevers 2005; Reya and Clevers 2005). Persistent inhibition of this signaling activity may have detrimental side effects. On the other hand, currently available drugs such as NSAIDs have clearly shown promise in the treatment of colorectal tumors resulting from activated β-catenin in humans. Since long-term NSAID therapy is associated with potentially life-threatening adverse effects including gastrointestinal bleeding and kidney impairment, the development of safer and more effective NSAID derivatives is warranted to minimize these severe unwanted effects.

Although there are a number of formidable obstacles to success, the next several years are likely to be fruitful and exciting, providing new lead compounds targeting the Wnt/β-catenin pathway as cancer therapeutics.

References

Aoki M, Hecht A, Kruse U, Kemler R, Vogt PK (1999) Nuclear endpoint of Wnt signaling: neoplastic transformation induced by transactivating lymphoid-enhancing factor 1. Proc Natl Acad Sci USA 96:139–144

Arce L, Yokoyama NN, Waterman ML (2006) Diversity of LEF/TCF action in development and disease. Oncogene 25:7492–7504

Barker N, Hurlstone A, Musisi H, Miles A, Bienz M, Clevers H (2001) The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation. EMBO J 20:4935–4943

Bauer A, Huber O, Kemler R (1998) Pontin52, an interaction partner of beta-catenin, binds to the TATA box binding protein. Proc Natl Acad Sci USA 95:14787–14792

Bauer A, Chauvet S, Huber O, Usseglio F, Rothbacher U, Aragnol D, Kemler R, Pradel J (2000) Pontin52 and reptin52 function as antagonistic regulators of beta-catenin signalling activity. EMBO J 19:6121–6130

Beachy PA, Karhadkar SS, Berman DM (2004) Tissue repair and stem cell renewal in carcinogenesis. Nature 432:324–331

Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D, Birchmeier W (1998) Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science 280:596–599

Belenkaya TY, Han C, Standley HJ, Lin X, Houston DW, Heasman J, Lin X (2002) pygopus Encodes a nuclear protein essential for wingless/Wnt signaling. Development 129:4089–4101

Bienz M, Clevers H (2003) Armadillo/beta-catenin signals in the nucleus – proof beyond a reasonable doubt? Nat Cell Biol 5:179–182

Bos CL, Kodach LL, van den Brink GR, Diks SH, van Santen MM, Richel DJ, Peppelenbosch MP, Hardwick JC (2006) Effect of aspirin on the Wnt/beta-catenin pathway is mediated via protein phosphatase 2A. Oncogene 25:6447–6456

Brannon M, Brown JD, Bates R, Kimelman D, Moon RT (1999) XCtBP is a XTcf-3 co-repressor with roles throughout Xenopus development. Development 126:3159–3170

Cadigan KM, Liu YI (2006) Wnt signaling: complexity at the surface. J Cell Sci 119:395–402

Cadigan KM, Nusse R (1997) Wnt signaling: a common theme in animal development. Genes Dev 11:3286–3305

Carpten JD, Robbins CM, Villablanca A, Forsberg L, Presciuttini S, Bailey-Wilson J, Simonds WF, Gillanders EM, Kennedy AM, Chen JD, Agarwal SK, Sood R, Jones MP, Moses TY, Haven C, Petillo D, Leotlela PD, Harding B, Cameron D, Pannett AA, Hoog A, Heath H, 3rd, James-Newton LA, Robinson B, Zarbo RJ, Cavaco BM, Wassif W, Perrier ND, Rosen IB, Kristoffersson U, Turnpenny PD, Farnebo LO, Besser GM, Jackson CE, Morreau H, Trent JM, Thakker RV, Marx SJ, Teh BT, Larsson C, Hobbs MR (2002) HRPT2, encoding parafibromin, is mutated in hyperparathyroidism-jaw tumor syndrome. Nat Genet 32:676–680

Castano J, Raurell I, Piedra JA, Miravet S, Dunach M, Garcia de Herreros A (2002) Beta-catenin N- and C-terminal tails modulate the coordinated binding of adherens junction proteins to beta-catenin. J Biol Chem 277:31541–31550

Castellone MD, Teramoto H, Williams BO, Druey KM, Gutkind JS (2005) Prostaglandin E2 promotes colon cancer cell growth through a Gs-axin-beta-catenin signaling axis. Science 310:1504–1510

Cavallo RA, Cox RT, Moline MM, Roose J, Polevoy GA, Clevers H, Peifer M, Bejsovec A (1998) Drosophila Tcf and Groucho interact to repress Wingless signalling activity. Nature 395:604–608

Choi HJ, Huber AH, Weis WI (2006) Thermodynamics of beta-catenin-ligand interactions: the roles of the N- and C-terminal tails in modulating binding affinity. J Biol Chem 281:1027–1038

Clarke AR (2006) Wnt signalling in the mouse intestine. Oncogene 25:7512–7521

Clevers H (2006a) Colon cancer – understanding how NSAIDs work. N Engl J Med 354:761–773

Clevers H (2006b) Wnt/beta-catenin signaling in development and disease. Cell 127:469–480
Conacci-Sorrell ME, Ben-Yedidia T, Shtutman M, Feinstein E, Einat P, Ben-Ze'ev A (2002) Nr-CAM is a target gene of the beta-catenin/LEF-1 pathway in melanoma and colon cancer and its expression enhances motility and confers tumorigenesis. Genes Dev 16:2058–2072
Cong F, Zhang J, Pao W, Zhou P, Varmus H (2003) A protein knockdown strategy to study the function of beta-catenin in tumorigenesis. BMC Mol Biol 4:10
Courey AJ, Jia S (2001) Transcriptional repression: the long and the short of it. Genes Dev 15:2786–2796
Crawford HC, Fingleton BM, Rudolph-Owen LA, Goss KJ, Rubinfeld B, Polakis P, Matrisian LM (1999) The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. Oncogene 18:2883–2891
Daniels DL, Weis WI (2002) ICAT inhibits beta-catenin binding to Tcf/Lef-family transcription factors and the general coactivator p300 using independent structural modules. Mol Cell 10:573–584
Daniels DL, Weis WI (2005) Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. Nat Struct Mol Biol 12:364–371
Davidson G, Wu W, Shen J, Bilic J, Fenger U, Stannek P, Glinka A, Niehrs C (2005) Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. Nature 438:867–872
de La Coste A, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, Fabre M, Chelly J, Beldjord C, Kahn A, Perret C (1998) Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. Proc Natl Acad Sci USA 95: 8847–8851
Dihlmann S, Klein S, Doeberitz Mv MK (2003) Reduction of beta-catenin/T-cell transcription factor signaling by aspirin and indomethacin is caused by an increased stabilization of phosphorylated beta-catenin. Mol Cancer Ther 2:509–516
Easwaran V, Pishvaian M, Salimuddin, Byers S (1999) Cross-regulation of beta-catenin-LEF/TCF and retinoid signaling pathways. Curr Biol 9:1415–1418
Eguchi M, Nguyen C, Lee SC, Kahn M (2005) ICG-001, a novel small molecule regulator of TCF/beta-catenin transcription. Med Chem 1:467–472
Emami KH, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M, Moon RT, Teo JL, Kim HY, Moon SH, Ha JR, Kahn M (2004) A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. Proc Natl Acad Sci USA 101:12682–12687
Feng Y, Lee N, Fearon ER (2003) TIP49 regulates beta-catenin-mediated neoplastic transformation and T-cell factor target gene induction via effects on chromatin remodeling. Cancer Res 63:8726–8734
Gad S, Teboul D, Lievre A, Goasguen N, Berger A, Beaune P, Laurent-Puig P (2004) Is the gene encoding Chibby implicated as a tumour suppressor in colorectal cancer ? BMC Cancer 4:31
Gamallo C, Palacios J, Moreno G, Calvo de Mora J, Suarez A, Armas A (1999) Beta-catenin expression pattern in stage I and II ovarian carcinomas: relationship with beta-catenin gene mutations, clinicopathological features, and clinical outcome. Am J Pathol 155:527–536
Gates J, Peifer M (2005) Can 1,000 reviews be wrong? Actin, alpha-Catenin, and adherens junctions. Cell 123:769–772
Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR, Offerhaus GJ (1993) Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N Engl J Med 328:1313–1316
Giles RH, van Es JH, Clevers H (2003) Caught up in a Wnt storm: Wnt signaling in cancer. Biochim Biophys Acta 1653:1–24
Giovannucci E, Egan KM, Hunter DJ, Stampfer MJ, Colditz GA, Willett WC, Speizer FE (1995) Aspirin and the risk of colorectal cancer in women. N Engl J Med 333:609–614
Goodman RH, Smolik S (2000) CBP/p300 in cell growth, transformation, and development. Genes Dev 14:1553–1577
Gottardi CJ, Gumbiner BM (2004) Role for ICAT in beta-catenin-dependent nuclear signaling and cadherin functions. Am J Physiol Cell Physiol 286:C747–C756

Graham TA, Weaver C, Mao F, Kimelman D, Xu W (2000) Crystal structure of a beta-catenin/Tcf complex. Cell 103:885–896

Graham TA, Ferkey DM, Mao F, Kimelman D, Xu W (2001) Tcf4 can specifically recognize beta-catenin using alternative conformations. Nat Struct Biol 8:1048–1052

Graham TA, Clements WK, Kimelman D, Xu W (2002) The crystal structure of the beta-catenin/ICAT complex reveals the inhibitory mechanism of ICAT. Mol Cell 10:563–571

Greaves S (2003) Small changes in Wnt signalling. Nat Cell Biol 5:387

Green DW, Roh H, Pippin JA, Drebin JA (2001) Beta-catenin antisense treatment decreases beta-catenin expression and tumor growth rate in colon carcinoma xenografts. J Surg Res 101:16–20

Gregorieff A, Pinto D, Begthel H, Destree O, Kielman M, Clevers H (2005) Expression pattern of Wnt signaling components in the adult intestine. Gastroenterology 129:626–638

Grosch S, Maier TJ, Schiffmann S, Geisslinger G (2006) Cyclooxygenase-2 (COX-2)-independent anticarcinogenic effects of selective COX-2 inhibitors. J Natl Cancer Inst 98:736–747

Ha NC, Tonozuka T, Stamos JL, Choi HJ, Weis WI (2004) Mechanism of phosphorylation-dependent binding of APC to beta-catenin and its role in beta-catenin degradation. Mol Cell 15:511–521

Harada N, Tamai Y, Ishikawa T, Sauer B, Takaku K, Oshima M, Taketo MM (1999) Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. EMBO J 18:5931–5942

Hawcroft G, D'Amico M, Albanese C, Markham AF, Pestell RG, Hull MA (2002) Indomethacin induces differential expression of beta-catenin, gamma-catenin and T-cell factor target genes in human colorectal cancer cells. Carcinogenesis 23:107–114

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW (1998) Identification of c-MYC as a target of the APC pathway. Science 281:1509–1512

He B, Reguart N, You L, Mazieres J, Xu Z, Lee AY, Mikami I, McCormick F, Jablons DM (2005) Blockade of Wnt-1 signaling induces apoptosis in human colorectal cancer cells containing downstream mutations. Oncogene 24:3054–3058

Hecht A, Litterst CM, Huber O, Kemler R (1999) Functional characterization of multiple transactivating elements in beta-catenin, some of which interact with the TATA-binding protein in vitro. J Biol Chem 274:18017–18025

Hecht A, Vleminckx K, Stemmler MP, van Roy F, Kemler R (2000) The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. EMBO J 19:1839–1850

Hoang B, Moos M Jr, Vukicevic S, Luyten FP (1996) Primary structure and tissue distribution of FRZB, a novel protein related to Drosophila frizzled, suggest a role in skeletal morphogenesis. J Biol Chem 271:26131–26137

Hoffmans R, Basler K (2004) Identification and in vivo role of the Armadillo-Legless interaction. Development 131:4393–4400

Hoffmans R, Stadeli R, Basler K (2005) Pygopus and legless provide essential transcriptional coactivator functions to armadillo/beta-catenin. Curr Biol 15:1207–1211

Hoppler S, Kavanagh CL (2007) Wnt signalling: variety at the core. J Cell Sci 120:385–393

Hovanes K, Li TW, Munguia JE, Truong T, Milovanovic T, Lawrence Marsh J, Holcombe RF, Waterman ML (2001) Beta-catenin-sensitive isoforms of lymphoid enhancer factor-1 are selectively expressed in colon cancer. Nat Genet 28:53–57

Hsieh JC, Kodjabachian L, Rebbert ML, Rattner A, Smallwood PM, Samos CH, Nusse R, Dawid IB, Nathans J (1999) A new secreted protein that binds to Wnt proteins and inhibits their activities. Nature 398:431–496

Hsu SC, Galceran J, Grosschedl R (1998) Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin. Mol Cell Biol 18:4807–4818

Huber AH, Weis WI (2001) The structure of the beta-catenin/E-cadherin complex and the molecular basis of diverse ligand recognition by beta-catenin. Cell 105:391–402

Huber AH, Nelson WJ, Weis WI (1997) Three-dimensional structure of the armadillo repeat region of beta-catenin. Cell 90:871–882

Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F (2002) Wnt/beta-catenin/Tcf signaling induces the transcription of axin2, a negative regulator of the signaling pathway. Mol Cell Biol 22:1172–1183

Jolly K, Cheng KK, Langman MJ (2002) NSAIDs and gastrointestinal cancer prevention. Drugs 62:945–956

Kanemaki M, Makino Y, Yoshida T, Kishimoto T, Koga A, Yamamoto K, Yamamoto M, Moncollin V, Egly JM, Muramatsu M, Tamura T (1997) Molecular cloning of a rat 49-kDa TBP-interacting protein (TIP49) that is highly homologous to the bacterial RuvB. Biochem Biophys Res Commun 235:64–68

Kanemaki M, Kurokawa Y, Matsu-ura T, Makino Y, Masani A, Okazaki K, Morishita T, Tamura TA (1999) TIP49b, a new RuvB-like DNA helicase, is included in a complex together with another RuvB-like DNA helicase, TIP49a. J Biol Chem 274:22437–22444

Katoh M (2005) WNT/PCP signaling pathway and human cancer (review). Oncol Rep 14:1583–1588

Kim JS, Crooks H, Foxworth A, Waldman T (2002) Proof-of-principle: oncogenic beta-catenin is a valid molecular target for the development of pharmacological inhibitors. Mol Cancer Ther 1:1355–1359

Kim S, Xu X, Hecht A, Boyer TG (2006) Mediator is a transducer of Wnt/beta-catenin signaling. J Biol Chem 281:14066–14075

Kimelman D, Xu W (2006) Beta-catenin destruction complex: insights and questions from a structural perspective. Oncogene 25:7482–7491

Kinzler KW, Vogelstein B (1996) Lessons from hereditary colorectal cancer. Cell 87:159–170

Kinzler KW, Nilbert MC, Su LK, Vogelstein B, Bryan TM, Levy DB, Smith KJ, Preisinger AC, Hedge P, McKechnie D, et al. (1991) Identification of FAP locus genes from chromosome 5q21. Science 253:661–665

Koehne CH, Dubois RN (2004) COX-2 inhibition and colorectal cancer. Semin Oncol 31:12–21

Koesters R, Ridder R, Kopp-Schneider A, Betts D, Adams V, Niggli F, Briner J, von Knebel Doeberitz M (1999) Mutational activation of the beta-catenin proto-oncogene is a common event in the development of Wilms' tumors. Cancer Res 59:3880–3882

Kohn AD, Moon RT (2005) Wnt and calcium signaling: beta-catenin-independent pathways. Cell Calcium 38:439–446

Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H (1997) Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC–/– colon carcinoma. Science 275:1784–1787

Korinek V, Barker N, Moerer P, van Donselaar E, Huls G, Peters PJ, Clevers H (1998) Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet 19:379–383

Koyama T, Tago K, Nakamura T, Ohwada S, Morishita Y, Yokota J, Akiyama T (2002) Mutation and expression of the beta-catenin-interacting protein ICAT in human colorectal tumors. Jpn J Clin Oncol 32:358–362

Kramps T, Peter O, Brunner E, Nellen D, Froesch B, Chatterjee S, Murone M, Zullig S, Basler K (2002) Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. Cell 109:47–60

Kuhnert F, Davis CR, Wang HT, Chu P, Lee M, Yuan J, Nusse R, Kuo CJ (2004) Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. Proc Natl Acad Sci USA 101:266–271

Lepourcelet M, Chen YN, France DS, Wang H, Crews P, Petersen F, Bruseo C, Wood AW, Shivdasani RA (2004) Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. Cancer Cell 5:91–102

Liu J, Stevens J, Rote CA, Yost HJ, Hu Y, Neufeld KL, White RL, Matsunami N (2001) Siah-1 mediates a novel beta-catenin degradation pathway linking p53 to the adenomatous polyposis coli protein. Mol Cell 7:927–936

Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X, He X (2002) Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 108:837–847

Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol 20:781–810
Lustig B, Behrens J (2003) The Wnt signaling pathway and its role in tumor development. J Cancer Res Clin Oncol 129:199–221
Lustig B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, van de Wetering M, Clevers H, Schlag PM, Birchmeier W, Behrens J (2002) Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol 22:1184–1193
Ma H, Nguyen C, Lee KS, Kahn M (2005) Differential roles for the coactivators CBP and p300 on TCF/beta-catenin-mediated survivin gene expression. Oncogene 24:3619–3631
Maiti S, Alam R, Amos CI, Huff V (2000) Frequent association of beta-catenin and WT1 mutations in Wilms tumors. Cancer Res 60:6288–6292
Malbon CC, Wang HY (2006) Dishevelled: a mobile scaffold catalyzing development. Curr Top Dev Biol 72:153–166
Matsuzawa SI, Reed JC (2001) Siah-1, SIP, and Ebi collaborate in a novel pathway for beta-catenin degradation linked to p53 responses. Mol Cell 7:915–926
Mazieres J, He B, You L, Xu Z, Lee AY, Mikami I, Reguart N, Rosell R, McCormick F, Jablons DM (2004) Wnt inhibitory factor-1 is silenced by promoter hypermethylation in human lung cancer. Cancer Res 64:4717–4720
Miller JR (2002) The Wnts. Genome Biol 3: REVIEWS3001
Miller JR, Hocking AM, Brown JD, Moon RT (1999) Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca2+ pathways. Oncogene 18:7860–7872
Miyagishi M, Fujii R, Hatta M, Yoshida E, Araya N, Nagafuchi A, Ishihara S, Nakajima T, Fukamizu A (2000) Regulation of Lef-mediated transcription and p53-dependent pathway by associating beta-catenin with CBP/p300. J Biol Chem 275:35170–35175
Miyaki M, Konishi M, Kikuchi-Yanoshita R, Enomoto M, Igari T, Tanaka K, Muraoka M, Takahashi H, Amada Y, Fukayama M, et al (1994) Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors. Cancer Res 54:3011–3020
Miyoshi Y, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, Aoki T, Miki Y, Mori T, Nakamura Y (1992) Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Hum Mol Genet 1:229–233
Miyoshi Y, Iwao K, Nagasawa Y, Aihara T, Sasaki Y, Imaoka S, Murata M, Shimano T, Nakamura Y (1998) Activation of the beta-catenin gene in primary hepatocellular carcinomas by somatic alterations involving exon 3. Cancer Res 58:2524–2527
Moon RT, Kohn AD, De Ferrari GV, Kaykas A (2004) WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet 5:691–701
Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW (1997) Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 275:1787–1790
Moser AR, Pitot HC, Dove WF (1990) A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science 247:322–324
Mosimann C, Hausmann G, Basler K (2006) Parafibromin/Hyrax activates Wnt/Wg target gene transcription by direct association with beta-catenin/Armadillo. Cell 125:327–341
Mulholland DJ, Dedhar S, Coetzee GA, Nelson CC (2005) Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? Endocr Rev 26:898–915
Nath N, Kashfi K, Chen J, Rigas B (2003) Nitric oxide-donating aspirin inhibits beta-catenin/T cell factor (TCF) signaling in SW480 colon cancer cells by disrupting the nuclear beta-catenin-TCF association. Proc Natl Acad Sci USA 100:12584–12589
Nelson WJ, Nusse R (2004) Convergence of Wnt, beta-catenin, and cadherin pathways. Science 303:1483–1487
Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Horii A, Koyama K, Utsunomiya J, Baba S, Hedge P (1991) Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science 253:665–669
Nusse R (2005) Wnt signaling in disease and in development. Cell Res 15:28–32
Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, Taketo MM (1996) Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 87:803–809

Palacios J, Gamallo C (1998) Mutations in the beta-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. Cancer Res 58:1344–1347

Parker DS, Jemison J, Cadigan KM (2002) Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. Development 129:2565–2576

Peifer M, Berg S, Reynolds AB (1994) A repeating amino acid motif shared by proteins with diverse cellular roles. Cell 76:789–791

Phillips RK, Wallace MH, Lynch PM, Hawk E, Gordon GB, Saunders BP, Wakabayashi N, Shen Y, Zimmerman S, Godio L, Rodrigues-Bigas M, Su LK, Sherman J, Kelloff G, Levin B, Steinbach G (2002) A randomised, double blind, placebo controlled study of celecoxib, a selective cyclooxygenase 2 inhibitor, on duodenal polyposis in familial adenomatous polyposis. Gut 50:857–860

Pinto D, Clevers H (2005) Wnt, stem cells and cancer in the intestine. Biol Cell 97:185–196

Pinto D, Gregorieff A, Begthel H, Clevers H (2003) Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes Dev 17:1709–1713

Pokutta S, Weis WI (2000) Structure of the dimerization and beta-catenin-binding region of alpha-catenin. Mol Cell 5:533–543

Polakis P (2000) Wnt signaling and cancer. Genes Dev 14:1837–1851

Polakis P (2002) Casein kinase 1: a Wnt'er of disconnect. Curr Biol 12:R499–R501

Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW (1992) APC mutations occur early during colorectal tumorigenesis. Nature 359:235–237

Reifenberger J, Knobbe CB, Wolter M, Blaschke B, Schulte KW, Pietsch T, Ruzicka T, Reifenberger G (2002) Molecular genetic analysis of malignant melanomas for aberrations of the WNT signaling pathway genes CTNNB1, APC, ICAT and BTRC. Int J Cancer 100:549–556

Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. Nature 434:843–850

Roberts CW, Orkin SH (2004) The SWI/SNF complex – chromatin and cancer. Nat Rev Cancer 4:133–142

Roose J, Molenaar M, Peterson J, Hurenkamp J, Brantjes H, Moerer P, van de Wetering M, Destree O, Clevers H (1998) The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature 395:608–612

Roose J, Huls G, van Beest M, Moerer P, van der Horn K, Goldschmeding R, Logtenberg T, Clevers H (1999) Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. Science 285:1923–1926

Rottbauer W, Saurin AJ, Lickert H, Shen X, Burns CG, Wo ZG, Kemler R, Kingston R, Wu C, Fishman M (2002) Reptin and pontin antagonistically regulate heart growth in zebrafish embryos. Cell 111:661–672

Rozenblatt-Rosen O, Hughes CM, Nannepaga SJ, Shanmugam KS, Copeland TD, Guszczynski T, Resau JH, Meyerson M (2005) The parafibromin tumor suppressor protein is part of a human Paf1 complex. Mol Cell Biol 25:612–620

Rubin JS, Barshishat-Kupper M, Feroze-Merzoug F, Xi ZF (2006) Secreted WNT antagonists as tumor suppressors: pro and con. Front Biosci 11:2093–2105

Rubinfeld B, Robbins P, El-Gamil M, Albert I, Porfiri E, Polakis P (1997) Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science 275:1790–1792

Sampietro J, Dahlberg CL, Cho US, Hinds TR, Kimelman D, Xu W (2006) Crystal structure of a beta-catenin/BCL9/Tcf4 complex. Mol Cell 24:293–300

Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Batlle E, Simon-Assmann P, Clevers H, Nathke IS, Clarke AR, Winton DJ (2004) Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev 18:1385–1390

Schuierer MM, Graf E, Takemaru K, Dietmaier W, Bosserhoff AK (2006) Reduced expression of beta-catenin inhibitor Chibby in colon carcinoma cell lines. World J Gastroenterol 12:1529–1535

Segditsas S, Tomlinson I (2006) Colorectal cancer and genetic alterations in the Wnt pathway. Oncogene 25:7531–7537

Sekiya T, Nakamura T, Kazuki Y, Oshimura M, Kohu K, Tago K, Ohwada S, Akiyama T (2002) Overexpression of Icat induces G(2) arrest and cell death in tumor cell mutants for adenomatous polyposis coli, beta-catenin, or Axin. Cancer Res 62:3322–3326

Shah S, Islam MN, Dakshanamurthy S, Rizvi I, Rao M, Herrell R, Zinser G, Valrance M, Aranda A, Moras D, Norman A, Welsh J, Byers SW (2006) The molecular basis of vitamin D receptor and beta-catenin crossregulation. Mol Cell 21:799–809

Sierra J, Yoshida T, Joazeiro CA, Jones KA (2006) The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. Genes Dev 20:586–600

Singh AM, Li FQ, Hamazaki T, Kasahara H, Takemaru K, Terada N (2007) Chibby, an antagonist of the Wnt/beta-catenin pathway, facilitates cardiomyocyte differentiation of murine embryonic stem cells. Circulation 115:617–626

Sinner D, Rankin S, Lee M, Zorn AM (2004) Sox17 and beta-catenin cooperate to regulate the transcription of endodermal genes. Development 131:3069–3080

Solanas G, Miravet S, Casagolda D, Castano J, Raurell I, Corrionero A, de Herreros AG, Dunach M (2004) beta-Catenin and plakoglobin N- and C-tails determine ligand specificity. J Biol Chem 279:49849–49856

Sonoshita M, Takaku K, Sasaki N, Sugimoto Y, Ushikubi F, Narumiya S, Oshima M, Taketo MM (2001) Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc(Delta 716) knockout mice. Nat Med 7:1048–1051

Spiegelman VS, Slaga TJ, Pagano M, Minamoto T, Ronai Z, Fuchs SY (2000) Wnt/beta-catenin signaling induces the expression and activity of betaTrCP ubiquitin ligase receptor. Mol Cell 5:877–882

Stadeli R, Hoffmans R, Basler K (2006) Transcription under the control of nuclear Arm/beta-catenin. Curr Biol 16:R378–R385

Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, Gould KA, Dove WF (1992) Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science 256:668–670

Su Y, Ishikawa S, Kojima M, Liu B (2003) Eradication of pathogenic beta-catenin by Skp1/Cullin/F box ubiquitination machinery. Proc Natl Acad Sci USA 100:12729–12734

Sun Y, Kolligs FT, Hottiger MO, Mosavin R, Fearon ER, Nabel GJ (2000) Regulation of beta – catenin transformation by the p300 transcriptional coactivator. Proc Natl Acad Sci USA 97:12613–12618

Suzuki H, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Chen WD, Pretlow TP, Yang B, Akiyama Y, Van Engeland M, Toyota M, Tokino T, Hinoda Y, Imai K, Herman JG, Baylin SB (2004) Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet 36:417–422

Tago K, Nakamura T, Nishita M, Hyodo J, Nagai S, Murata Y, Adachi S, Ohwada S, Morishita Y, Shibuya H, Akiyama T (2000) Inhibition of Wnt signaling by ICAT, a novel beta-catenin-interacting protein. Genes Dev 14:1741–1749

Takemaru K-I (2006) beta-Catenin. AfCS-Nature Molecule Pages (doi:10.1038/mp.a000506.01)

Takemaru KI, Moon RT (2000) The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression. J Cell Biol 149:249–254

Takemaru K, Yamaguchi S, Lee YS, Zhang Y, Carthew RW, Moon RT (2003) Chibby, a nuclear beta-catenin-associated antagonist of the Wnt/Wingless pathway. Nature 422:905–909

Taketo MM (2006) Mouse models of gastrointestinal tumors. Cancer Sci 97:355–361

Tetsu O, McCormick F (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398:422–426

Thompson B, Townsley F, Rosin-Arbesfeld R, Musisi H, Bienz M (2002) A new nuclear component of the Wnt signalling pathway. Nat Cell Biol 4:367–373

Thun MJ, Namboodiri MM, Heath CW Jr (1991) Aspirin use and reduced risk of fatal colon cancer. N Engl J Med 325:1593–1596

Tolwinski NS, Wieschaus E (2004a) A nuclear function for armadillo/beta-catenin. PLoS Biol 2: E95

Tolwinski NS, Wieschaus E (2004b) Rethinking WNT signaling. Trends Genet 20:177–181

Torrance CJ, Jackson PE, Montgomery E, Kinzler KW, Vogelstein B, Wissner A, Nunes M, Frost P, Discafani CM (2000) Combinatorial chemoprevention of intestinal neoplasia. Nat Med 6:1024–1028

Townsley FM, Cliffe A, Bienz M (2004) Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function. Nat Cell Biol 6:626–633

Truica CI, Byers S, Gelmann EP (2000) Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res 60:4709–4713

Tutter AV, Fryer CJ, Jones KA (2001) Chromatin-specific regulation of LEF-1-beta-catenin transcription activation and inhibition in vitro. Genes Dev 15:3342–3354

Uusitalo M, Heikkila M, Vainio S (1999) Molecular genetic studies of Wnt signaling in the mouse. Exp Cell Res 253:336–348

van Amerongen R, Berns A (2006) Knockout mouse models to study Wnt signal transduction. Trends Genet 22:678–689

van de Wetering M, Cavallo R, Dooijes D, van Beest M, van Es J, Loureiro J, Ypma A, Hursh D, Jones T, Bejsovec A, Peifer M, Mortin M, Clevers H (1997) Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell 88:789–799

van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H (2002) The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 111:241–250

van de Wetering M, Oving I, Muncan V, Pon Fong MT, Brantjes H, van Leenen D, Holstege FC, Brummelkamp TR, Agami R, Clevers H (2003) Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep 4:609–615

van Es JH, Jay P, Gregorieff A, van Gijn ME, Jonkheer S, Hatzis P, Thiele A, van den Born M, Begthel H, Brabletz T, Taketo MM, Clevers H (2005) Wnt signalling induces maturation of Paneth cells in intestinal crypts. Nat Cell Biol 7:381–386

Veeman MT, Axelrod JD, Moon RT (2003) A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev Cell 5:367–377

Verma UN, Surabhi RM, Schmaltieg A, Becerra C, Gaynor RB (2003) Small interfering RNAs directed against beta-catenin inhibit the in vitro and in vivo growth of colon cancer cells. Clin Cancer Res 9:1291–1300

Vleminckx K, Kemler R, Hecht A (1999) The C-terminal transactivation domain of beta-catenin is necessary and sufficient for signaling by the LEF-1/beta-catenin complex in Xenopus laevis. Mech Dev 81:65–74

Waltzer L, Bienz M (1998) Drosophila CBP represses the transcription factor TCF to antagonize Wingless signalling. Nature 395:521–525

Wang HY, Liu T, Malbon CC (2006) Structure-function analysis of Frizzleds. Cell Signal 18:934–941

Wang PF, Tan MH, Zhang C, Morreau H, Teh BT (2005) HRPT2, a tumor suppressor gene for hyperparathyroidism-jaw tumor syndrome. Horm Metab Res 37:380–383

Willert K, Jones KA (2006) Wnt signaling: is the party in the nucleus? Genes Dev 20:1394–1404

Williams CS, Smalley W, DuBois RN (1997) Aspirin use and potential mechanisms for colorectal cancer prevention. J Clin Invest 100:1325–1329

Willis TG, Zalcberg IR, Coignet LJ, Wlodarska I, Stul M, Jadayel DM, Bastard C, Treleaven JG, Catovsky D, Silva ML, Dyer MJ (1998) Molecular cloning of translocation t(1;14)(q21;q32) defines a novel gene (BCL9) at chromosome 1q21. Blood 91:1873–1881

Wodarz A, Nusse R (1998) Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol 14:59–88

Xing Y, Clements WK, Kimelman D, Xu W (2003) Crystal structure of a beta-catenin/axin complex suggests a mechanism for the beta-catenin destruction complex. Genes Dev 17:2753–2764

Xing Y, Clements WK, Le Trong I, Hinds TR, Stenkamp R, Kimelman D, Xu W (2004) Crystal structure of a beta-catenin/APC complex reveals a critical role for APC phosphorylation in APC function. Mol Cell 15:523–533

Yang K, Fan K, Kurihara N, Shinozaki H, Rigas B, Augenlicht L, Kopelovich L, Edelmann W, Kucherlapati R, Lipkin M (2003) Regional response leading to tumorigenesis after sulindac in small and large intestine of mice with Apc mutations. Carcinogenesis 24:605–611

Yang CK, Kim JH, Li H, Stallcup MR (2006) Differential use of functional domains by coiled-coil coactivator in its synergistic coactivator function with beta-catenin or GRIP1. J Biol Chem 281:3389–3397

Yart A, Gstaiger M, Wirbelauer C, Pecnik M, Anastasiou D, Hess D, Krek W (2005) The HRPT2 tumor suppressor gene product parafibromin associates with human PAF1 and RNA polymerase II. Mol Cell Biol 25:5052–5060

You L, He B, Xu Z, Uematsu K, Mazieres J, Fujii N, Mikami I, Reguart N, McIntosh JK, Kashani-Sabet M, McCormick F, Jablons DM (2004) An anti-Wnt-2 monoclonal antibody induces apoptosis in malignant melanoma cells and inhibits tumor growth. Cancer Res 64:5385–5389

Zeng X, Tamai K, Doble B, Li S, Huang H, Habas R, Okamura H, Woodgett J, He X (2005) A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. Nature 438:873–877

Zirn B, Wittmann S, Graf N, Gessler M (2005) Chibby, a novel antagonist of the Wnt pathway, is not involved in Wilms tumor development. Cancer Lett 220:115–120

A Toolkit for Real-Time Detection of cAMP: Insights into Compartmentalized Signaling

M. Berrera, G. Dodoni, S. Monterisi, V. Pertegato, I. Zamparo, and M. Zaccolo(✉)

Contents

1 Introduction 286
2 Cyclic AMP Signaling and Specificity of Response 287
3 Detection of cAMP: Accumulation Assays versus Real-Time Monitoring 288
4 cAMP Measurement in Living Cells: CNG Channels 289
5 cAMP Measurements in Living Cells Using FRET-Based Technology 290
5.1 PKA-Based Sensors 290
5.2 Epac-Based Sensors 293
5.3 Sensors Based on Single cAMP-Binding Domains 295
6 Conclusions 296
References 297

Abstract The study of cAMP signaling has received a renewed impulse since the recognition that a key aspect of this pathway is the tight spatial control of signal propagation. The study of the mechanism that regulates cAMP signaling in space and time has prompted the development of new methodological approaches to detect cAMP in intact cells. Over the last decades, techniques to assess cAMP concentration with high spatial and temporal resolution in living cells have been elaborated that are based on fluorescent molecules and the phenomenon of fluorescence resonance energy transfer (FRET). A FRET-based indicator of cAMP concentration is typically a protein, including two fluorophores that are linked to a cAMP-binding domain. Binding of cAMP causes a change in the protein conformation and, as a consequence, in the distance between the fluorophores, thus altering the energy transfer between them. Several FRET indicators have been developed, differing in their affinity for cAMP, kinetic features and intracellular targeting. Such indicators enable the measurement of cAMP fluctuations as they happen in the complex intracellular environment and are proving to be effective tools to dissect compartmentalized cAMP signaling.

M. Zaccolo
Dulbecco Telethon Institute, Venetian Institute of Molecular Medicine, via Orus 2, 35100 Padova, Italy and Faculty of Biomedical and Life Sciences, University of Glasgow, University Avenue, G12 8QQ, Glasgow, UK
m.zaccolo@bio.gla.ac.uk

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.* Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

Abbreviations: AC, Adenylyl cyclase; AKAP, A-kinase anchoring protein; cAMP, 3′–5′-Cyclic adenosine monophosphate; C, Catalytic subunit of PKA; CFP, Cyan fluorescent protein; CNG, Cyclic-nucleotide-gated channel; DD, dimerization/docking domain; DEP, Disheveled, Egl-10 and Pleckstrin homology domain; Epac, Exchange protein directly activated by cAMP; FRET, Fluorescence resonance energy transfer; GEF, Guanine nucleotide exchange factor; GFP, Green fluorescent protein; HCN, Hyperpolarization-activated cyclic nucleotide modulated channel; IBMX, 3-Isobutyl-1-methylxanthine; IS, Inhibitory site of PKA; *mp,* Myristoylation and palmitoylation sequence; *nls,* Nuclear localization sequence; NE, Norepinephrine; PDE, Phosphodiesterase; PGE1, Prostaglandin E 1; PKA, Protein kinase A; R, Regulatory subunit of PKA; Rol, Rolipram; YFP, Yellow fluorescent protein

1 Introduction

In the cAMP-dependent pathway, an extracellular signal ("first" messenger: neurotransmitter, hormone or light) activates a cell surface receptor coupled to a trimeric G-protein that, after dissociations of its three subunits, modulates the activity of adenylyl cyclases (ACs), the enzymes that synthesize cAMP. At any given time, the intracellular concentration of cAMP results from the balance between synthesis and degradation. The catabolism of cAMP is mediated by phosphodiesterases (PDEs), a superfamily of more than 90 enzymes that differ for their specific localization and regulatory mechanisms (Bender and Beavo 2006). Upon synthesis, cAMP rapidly diffuses into the cytosol to activate downstream effector proteins, i.e., the cAMP-dependent serine–threonine protein kinase A (PKA), guanine-nucleotide exchange proteins (Epacs) and cyclic nucleotide-gated (CNG) ion channels.

PKA is a tetramer consisting of two catalytic (C) subunits held in an inactive conformation by a regulatory (R) subunit dimer. R subunits comprise a dimerization/docking (DD) domain at the N-terminus, responsible for docking of R subunits to specific intracellular sites via high-affinity binding to A-kinase anchoring proteins (AKAPs), a family of multiscaffolding proteins that anchor PKA to specific intracellular locations (Wong and Scott 2004). The DD domain is followed by a flexible linker that docks to the C subunit active site in the holoenzyme and acts as an inhibitory site (IS). The inhibitory site is followed by two tandem cAMP-binding domains. The holoenzyme dissociates when cAMP binds to the R subunits, thereby releasing the active C subunits. Two PKA subtypes are known: the type-I holoenzyme contains RI subunits and is primarily soluble, whereas the type-II holoenzyme contains RII subunits and is associated with particulate subcellular fractions (Francis and Corbin 1999).

Epacs are guanine nucleotide exchange factors (GEFs) for Rap1 and Rap2 (de Rooij et al. 2003). Rap GTPases cycle between an inactive GDP-bound and an

active GTP-bound state, with GEFs mediating the exchange of GDP for GTP. The N-terminus of Epac1 proteins consists of a regulatory domain containing a cAMP-binding site similar to those present in the R subunit of PKA and, in addition, a Disheveled, Egl-10 and pleckstrin homology (DEP) domain that mediates membrane attachment (de Rooij et al. 1998; Rehmann et al. 2003). The C-terminal region consists of a catalytic domain that is characteristic of exchange factors for the Ras family of GTPases. At low cAMP levels, Epac folds into an inactive conformation, and steric hindrance prevents the binding of Rap. When cAMP binds to the protein, Epac unfolds, allowing Rap binding (Rehmann et al. 2003).

CNG channels are cation channels that have been characterized in rod and cone photoreceptors and in olfactory sensory neurons of vertebrates (Finn et al. 1996). These channels are homo- or hetero-tetrameric with a core region in each subunit including six transmembrane helices. On the intracellular side, the so-called C-linker domain couples the gating transmembrane part to a cyclic nucleotide-binding domain, which has been shown to be more sensitive to cGMP than cAMP. By controlling the flux of ions through the membrane, these channels play a critical role in the regulation of the transmembrane potential and of the concentration of ionic species, such as Ca^{2+}. As opposed to their cognate hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels, which are opened by transmembrane hyperpolarization and whose activity is only modulated by cyclic nucleotides, CNG channels are poorly voltage dependent.

2 Cyclic AMP Signaling and Specificity of Response

Although cAMP activates a limited number of effectors, it is known to modulate a wide variety of biological processes, from metabolism and gene expression, cell division and migration, exocytosis and insulin secretion to immune response, memory formation and cardiac contraction. In order to explain how a single intracellular second messenger, cAMP, can modulate such different cellular events in a specific manner, it has been suggested that parallel and spatially segregated cAMP signaling pathways coexist within the cell (Steinberg and Brunton 2001; Zaccolo et al. 2002; Tasken and Aandahl 2004). According to this model, cAMP signaling relies on the organization of macromolecular complexes in which the receptors, the effectors, the targets and the modulators are in close proximity to each other, thus granting velocity and specificity of signaling. A key role in the organization of such PKA signaling units is played by AKAPs that act as signaling domain nucleators by anchoring PKA in close proximity to specific targets and modulators (Wong and Scott 2004). An example of how the cAMP signal is transduced through a multimeric signaling system is the activation of the L-type Ca^{2+} channel upon β_2-adrenergic receptor stimulation in hippocampal neurons. In these cells, a multiprotein assembly comprising the β_2-adrenergic receptor, the adenylyl cyclase, a heterotrimeric G protein, the AKAP MAP2B, PKA, the PKA-regulated $Ca_v1.2$ L-type Ca^{2+} channel and the phosphatase PP2A was identified (Davare et al.

2001). Activation of the β_2-adrenergic receptor was shown to lead to selective activation of the $Ca_v1.2$ channel included within the complex, thus indicating that the spatial confinement of the signaling molecules restricts signal propagation and results in local effector activation (Davare et al. 2001).

If PKA signaling specificity relies upon spatial segregation of discrete signaling units, then such individual pools of PKA must be independently activated, and this requires that cAMP is made available in discrete compartments. However, cAMP is generally considered to be freely diffusible in the cytosol, with a calculated diffusion constant of ~150–700 $\mu m^2\ s^{-1}$ (Kasai and Petersen 1994; Bacskai et al. 1993; Nikolaev et al. 2005), making it difficult to envisage how restricted domains of cAMP can be generated. Over 25 years ago, the dogma of unrestricted diffusion of cAMP was challenged, and the hypothesis of spatially restricted domains of cAMP was formulated to explain experimental data obtained in cardiac myocytes, where β-adrenergic effects correlate with activation of a particulate, membrane-bound fraction of PKA, whereas elevation of cAMP due to prostaglandin 1 is mainly in the cytosol and without functional consequences (Brunton et al. 1981; Steinberg and Brunton 2001). More recent data confirmed that spatial heterogeneity of cAMP does indeed exist inside a cell. Whole-cell patch-clamp recordings of the Ca^{2+} current (I_{Ca}) in frog heart cells have shown that a local stimulus of the β-adrenergic receptor causes activation of local I_{Ca}, whereas local application of the AC activator forskolin induces a generalized activation (Jurevicius and Fischmeister 1996), indicating that β-adrenergic receptors are functionally coupled to nearby Ca^{2+} channels via local elevations of cAMP.

These conclusions were still based on rather indirect evidence. In fact, the analysis of cAMP fluctuations inside the cell has been limited, until very recently, by the lack of appropriate tools. In the last few years, new approaches have been developed that have significantly contributed to establishing that cAMP is not free to diffuse inside the cell, but rather it signals through the generation of intracellular gradients with distinct temporal and spatial patterns (Zaccolo et al. 2002).

3 Detection of cAMP: Accumulation Assays versus Real-Time Monitoring

Traditionally, detection of cAMP is based on accumulation assays. A radioimmunoassay-based method to asses directly cAMP concentration from tissue and cell preparations was introduced in the late 1960s (Steiner et al. 1969). The general principle behind such types of accumulation assays is that changes in intracellular cAMP are detected by the competition between cAMP in the sample and a labeled form of cAMP for binding to an anti-cAMP antibody. With several variations, this method is still the most widely used to date. Several protocols are now available based on this simple approach, exploiting a whole plethora of technologies, from radiometric to enzymatic and with application for high-throughput screening (Williams 2004). Although such methods can be highly sensitive (Golla and

Seethala 2002) and remain essential research tools, their limitation derives from the necessity of fixing and/or breaking apart the sample and extracting the second messenger from the cells. In doing so, any information relating to the spatial organization of the cAMP signal is lost. In addition, cAMP accumulation assays are restricted to assessing the second messenger concentration in steady-state conditions or changes in a timescale of minutes. Therefore, their time resolution is rather poor. Moreover, only average information relative to total cAMP changes in a cell population is attainable, whereas the concentration of free cAMP in the individual cell is more physiologically relevant.

Thus, the conventional way of assessing cAMP concentration is clearly inadequate to respond to the request of high spatial and temporal resolution necessary to investigate compartmentalized signaling. An important step forward in the study of the spatial and temporal aspects of cAMP signaling came from the development of real-time detection approaches. Such methodologies allow to measure accurately quantitative and dynamic parameters of the cAMP signaling networks. In addition, they provide unprecedented resolution both in time and in space, reporting cAMP changes as they happen in the complex intracellular environment.

4 cAMP Measurement in Living Cells: CNG Channels

One approach for real-time detection of cAMP changes is based on olfactory CNG cation channels (Rich et al. 2000). Increase in cAMP concentration leads to fast gating of the channels, and cAMP changes are monitored typically by imaging of the fluorescent Ca^{2+} indicator fura-2 or by electrophysiological recordings of Ca^{2+} currents. The use of wild-type CNG channels to detect changes in cAMP concentration shows several limitations. First, wild-type channels have a lower affinity for cAMP than for cGMP (Dhallan et al. 1990; Rich et al. 2001b). Second, CNG channels can also be opened directly by nitric oxide (Broillet 2000). Third, the opening of these channels is strongly inhibited upon Ca^{2+}-calmodulin binding (Liu et al. 1994). To surmount some of these limitations, the properties of the wild-type CNG channels have been modified (Rich et al. 2001a). To reduce the sensitivity to cGMP and increase the sensitivity to cAMP, two mutations, C460W and E583M, have been introduced, whereas the regulation of the channel by Ca^{2+}-calmodulin has been removed by deletion of residues 61–90. Thus, the channel Δ61–90/C460W/E583M is virtually insensitive to cGMP and sensitive to low cAMP concentrations (Rich et al. 2001a). Although these improvements have extended the application of the CNG-based probes, some limitations persist. Firstly, changes in cAMP concentration can be detected only at the plasma membrane, where the probe is located, and the cAMP signal cannot be revealed in other compartments. Secondly, overexpression of the channel and the consequent increase in Ca^{2+} influx may affect the intracellular concentration of cAMP by acting on Ca^{2+}-sensitive ACs or PDEs. As a consequence, the concentration of cAMP reported by the CNG-based sensor may be non-physiological.

5 cAMP Measurements in Living Cells Using FRET-Based Technology

A different strategy to detect cAMP in living cells exploits fluorescence resonance energy transfer (FRET). In this approach, FRET is used to measure the conformational change that occurs upon cAMP binding to cAMP-binding domains that have been appropriately engineered for this purpose. FRET is a quantum-mechanical event that occurs when two fluorophores are placed in close proximity to each other (<100 Å), provided that the emission spectrum of the fluorophore that acts as the "donor" overlaps the excitation spectrum of the "acceptor" fluorophore. Under these circumstances, part of the vibrational energy of the excited state of the "donor" is transferred to the "acceptor" that emits at its own wavelength. The efficiency of this process (E) depends on the distance (R) between donor and acceptor with an inverse sixth power law, as described by the Förster's equation: $E=1/(1+(R/R_0)^6)$ (Förster 1948), where R_0 is the distance at which half of the energy is transferred. According to the Förster's equation, the doubling of the distance between the two fluorophores, for example, from R_0 to $2R_0$, decreases the efficiency of transfer from $E=50\%$ to $E=1.5\%$. Therefore, FRET provides a very sensitive measure of intermolecular distance. If the two fluorophores are held together by proteins that undergo a conformational change upon binding to cAMP, FRET can be used to measure cAMP concentration.

5.1 PKA-Based Sensors

Tsien and co-workers (Adams et al. 1991) devised the first application of the FRET technology for measuring cAMP concentration in living cells. The sensor they generated, termed FlCRhR, is based on PKA, the R and catalytic C subunits of which were labeled with rhodamine and fluorescein, respectively. In the absence of cAMP, R and C subunits interact to form the holoenzyme R_2C_2 and the two fluorophores come close enough for FRET to occur. When cAMP binds to the R subunits, the C subunits dissociate, thereby disrupting FRET. Although this approach allowed, for the first time, imaging of cAMP fluctuations in a living sample, it is affected by major limitations and technical difficulties, such as the necessity to microinject a large amount of the probe (µM concentrations), the aggregation of the labeled subunits and the non-specific interactions of labeled subunits with cellular structures (Goaillard et al. 2001).

An evolution of the FlCRhR prototype led to the generation of a sensor (R-CFP/C-YFP) in which the C and R subunits of PKA are fused to the yellow (YFP) and cyan (CFP) mutants of GFP, respectively (Lissandron et al. 2005; Zaccolo et al. 2000) (Fig. 1).

cAMP binding to the R subunit generates a conformational change that reduces the affinity of the R subunit for the C subunit, leading to dissociation of the holotetramer. At the low cAMP concentration of a resting cell, most of the R and C subunits are associated, and the fused GFPs are close and in the correct orientation

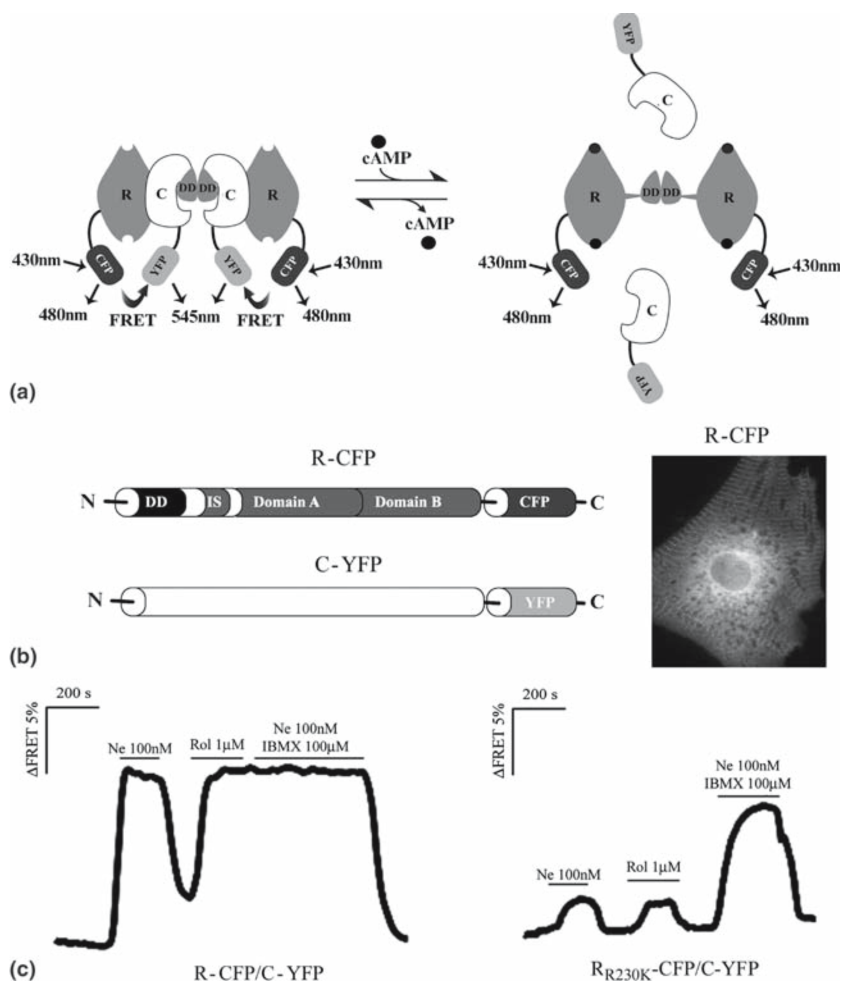

Fig. 1 PKA-based sensor. **(a)** Schematic representation of the mechanism of action of the PKA-based FRET sensor R-CFP/C-YFP. **(b)** Schematic structure of the R-CFP and C-YFP components of the PKA-based sensor (*left panel*) and distribution of the R-CFP subunit in rat neonatal cardiac myocytes (*right panel*). The localization of the probe in parallel striations was shown to depend on anchoring of R-CFP to endogenous AKAPs (Zaccolo and Pozzan 2002). The distribution of the C-YFP subunit (not shown) overlays the distribution of the R-CFP subunit. **(c)** Kinetics of FRET changes recorded in rat neonatal cardiac myocytes overexpressing the R-CFP/C-YFP sensor (*left panel*) or the R_{R230K}-CFP/C-YFP sensor (*right panel*). R, PKA regulatory subunit; C, catalytic PKA subunit; DD, dimerization/docking domain; IS, inhibitory sequence; *domain* A, cAMP binding domain A; *domain* B, cAMP binding domain B; Rol, rolipram; IBMX, 3-isobutyl-1-methylxanthine; NE, norepinephrine

for FRET to occur. When cAMP levels increase and the two subunits dissociate, CFP and YFP disengage, and FRET is no longer possible. When FRET occurs and CFP is excited at its proper wavelength (430 nm), part of its excited-state energy is transferred to YFP, which can emit at its own wavelength (545 nm). When

FRET is abolished upon cAMP binding, only CFP emission (480 nm) can be detected upon excitation of CFP. FRET changes can be measured as changes in the emission spectrum of the probe (emission YFP/emission CFP) upon illumination at a wavelength that excites selectively the donor CFP (430 nm). Being entirely genetically encoded, the R-CFP/C-YFP sensor can be easily introduced into cells by transfection or infection (Warrier et al. 2005), therefore extending the application of this methodology to most cell types.

The R-CFP/C-YFP sensor shows an EC_{50} for cAMP of about 0.3 μM (Table 1), and its catalytic activity is comparable to wild-type PKA (Mongillo et al. 2004).

One specific advantage of the PKA-based sensors for cAMP is that they report fluctuations of the second messenger specifically in the compartments in which PKA resides. Thus, being the R component of the probe the isoform type II, the overexpressed R-CFP subunit binds, via its DD domain, to those AKAPs that are present in the cell under study, thereby localizing the sensor in that specific compartment. Overexpression of the R-CFP/C-YFP sensor in cardiac myocytes provides a clear example of such localization (Fig. 1b).

Using the R-CFP/C-YFP sensor, it was possible to visualize, for the first time, microdomains of cAMP in cardiac cells in correspondence with signaling units organized by AKAPs (Zaccolo and Pozzan 2002). In addition, using the R-CFP/C-YFP sensor, insight into the role of PDEs in shaping the intracellular gradients of cAMP in cardiac myocytes has been gained, leading to the conclusion that different PDEs are selectively engaged in degrading cAMP pools generated by the activation of specific G-protein coupled receptors (Mongillo et al. 2004, 2006).

In specific experimental set-ups, the affinity of the PKA-based sensor may result in being too high, and changes in cAMP concentration may not be detected

Table 1 Design and sensitivity of different probes for real-time detection of cAMP. Approximate EC_{50} for each sensor is indicated.

Sensor	Design	EC_{50}	References
FlCRhR	Tetrameric PKA	90 nM	Adams et al. (1991)
R-CFP/C-YFP	Tetrameric PKA	0.3 μM	Mongillo et al. (2004)
R_{R230K}-CFP/C-YFP	Mutant tetrameric PKA	31.3 μM	Mongillo et al. (2004)
Wild-type CNGA2	CNG channel	36 μM	Rich et al. (2001)
Δ61-90C460W/E583M	Mutant CNG channel	15 μM	Rich et al. (2001)
H30	Mutant Epac 1	12.5 μM[a]	Terrin et al. (2006)
*mp*H30	Mutant Epac 1	20 μM[a]	Terrin et al. (2006)
*nls*H30	Mutant Epac 1	17.5 μM[a]	Terrin et al. (2006)
Epac 1-camps	cAMP-binding domain from Epac 1	2.4 μM	Nikolaev et al. (2004)
Epac 2-camps	cAMP-binding domain B from Epac 2	0.9 μM	Nikolaev et al. (2004)
PKA-camps	cAMP-binding domain B from PKA	1.9 μM	Nikolaev et al. (2004)
HCN 2-camps	cAMP-binding domain from HCN 2	5.9 μM	Nikolaev et al. (2006)

[a]Values determined in living cells. All other values are determined in vitro

accurately because of probe saturation. To overcome this limitation, the R230K mutation was introduced in the R subunit, thus generating a probe (R_{R230K}-CFP/C-YFP) with a two-order of magnitude lower affinity for cAMP (Table 1). Figure 1c compares the cAMP signal detected by either the R-CFP/C-YFP or the R_{R230K}-CFP/C-YFP sensor in cardiac myocytes in which the concentration of cAMP was raised by different stimuli. The R-CFP/C-YFP sensor cannot discriminate the cAMP response to norepinephrine (NE) from the amount of cAMP released upon selective PDE4 inhibition with rolipram (Rol) or non-selective inhibition of all PDEs with 3-Isobutyl-1-methylxanthine (IBMX). This is clearly the result of probe saturation as the R_{R230K}-CFP/C-YFP sensor can easily report the peak response to these different stimuli.

In spite of the specific advantages described above, the PKA-based sensors for cAMP have some drawbacks. First, because of the multimeric nature of the sensor, equimolar concentration of R and C subunits is required in order to form a functional tetramer, and this is not easy to control in transfected cells. Second, the cAMP-dependent dissociation of R and C subunits occurs through a complex cooperative mechanism (Taylor et al. 2005), and, therefore, the kinetics of FRET change reported by the sensor may result slower as compared to the actual kinetics of cAMP changes. In addition, the C-YFP subunit is catalytically active. This may affect the level of cAMP inside the cell by, for example, hyperactivation of PKA-sensitive PDEs. In order to overcome these limitations, a new generation of unimolecular and catalytically inactive cAMP sensors has been designed.

5.2 *Epac-Based Sensors*

The first unimolecular FRET probes for cAMP exploited Epac as the sensor for the cyclic nucleotide (DiPilato et al. 2004; Ponsioen et al. 2004). One of these indicators was generated by fusing the amino terminus of the Epac 1 protein to CFP and the C-terminus to YFP (Ponsioen et al. 2004). Such sensors localize to the cytosol and to membranes, in particular to the nuclear envelope and to perinuclear compartments (Ponsioen et al. 2004). To generate a cytosolic variant, the DEP domain (amino acids 1–148) was deleted. Indeed, this chimera locates almost exclusively in the cytosol. Additional mutations (T781A, F782A) were introduced to generate the sensor CFP–Epac(δDEP-CD)–YFP (also termed H30), which is catalytically inactive (Ponsioen et al. 2004) (Fig. 2a,b).

The lower sensitivity to cAMP of these Epac-based sensors (20 μM, Table 1) as compared to the PKA-based probes limits their application to systems characterized by large changes in cAMP concentration.

Further modifications to the Epac-based indicators have been introduced to target the sensor to specific subcellular compartments. In particular, a short polypeptide called *mp* and corresponding to the N-terminal targeting signal from the Lyn kinase (Resh 1999) was fused to the N-terminal end of H30. The *mp* sequence is post-translationally myristoylated and palmitoylated, therefore targeting the sensor to the

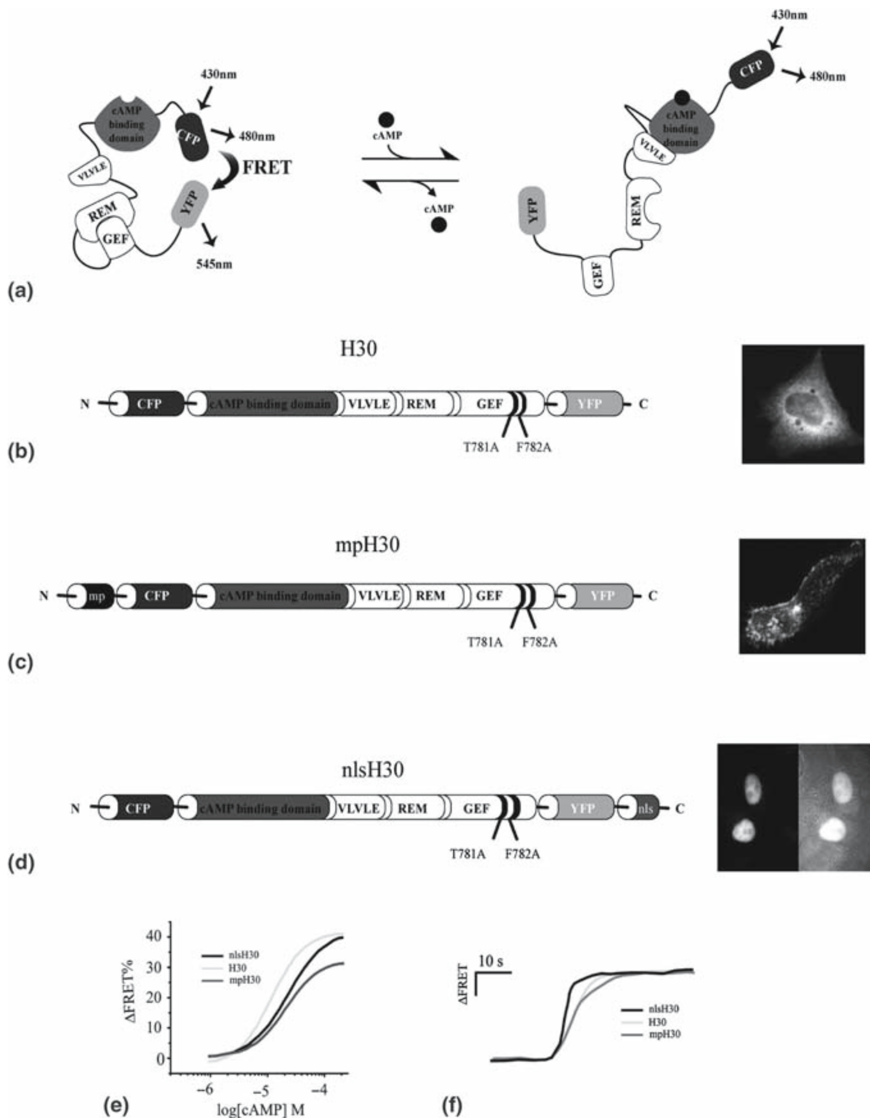

Fig. 2 Epac 1-based sensor. **(a)** Schematic representation of the mechanism of action of the Epac 1-based sensor H30. **(b)** Schematic structure of H30 (*left panel*) and cytosolic distribution of the probe (*right panel*). **(c)** Schematic structure of *mp*H30 (*left panel*) and localization of the probe at the plasma membrane (*right panel*). **(d)** Schematic structure of *nls*H30 (*left panel*) and localization of the probe in the nucleus (*right panel*) shown as image of the fluorescence emission (*left*) and its overlay with the transmitted light image of the same cells (*right*). **(e)** Dose–response curves showing FRET changes at different cAMP concentration for the three Epac 1-based sensors. For these measurements, HeLa cells expressing each cAMP sensor were injected with known concentrations of cAMP via a patch pipette and FRET changes recorded (Terrin et al. 2006). **(f)** Kinetics of FRET changes recorded with the three different sensors upon addition, via a patch pipette, of 100 μM cAMP. Each curve represents the average of five independent measurements

plasma membrane (Fig. 2c). Another variant of H30 was generated by fusing a nuclear localization sequence (*nls*) to the C-terminus of the sensor, which effectively targets the cAMP indicator to the nuclear compartment (Fig. 2d). These differently targeted probes have proved to be very useful tools to study compartmentalized cAMP signaling. The combined use of the differently targeted H30 probes allowed the dissection of the cAMP response in the different subcellular compartments in the model cell line HEK293 (Terrin et al. 2006). This study shows that prostaglandin 1 (PGE1) stimulation of HEK293 cells generates multiple contiguous intracellular domains with different cAMP concentration. In particular, PGE1 stimulation generates a larger pool of cAMP in the sub-plasma membrane compartment as compared to the bulk cytosol. In addition, this study shows that in a subcellular compartment, such as the nucleus, located deep inside the cell and far away from the site of synthesis of the second messenger, the concentration of cAMP can be higher as compared to the surrounding cytosol. This is an important observation because it confirms that mechanisms are in place that allow a subset of PKA that are anchored at a distance from the plasma membrane to be selectively activated without the concomitant activation of other PKA subsets that may be located closer to the site of cAMP synthesis. What is the mechanism responsible for such compartmentalization of cAMP? Pharmacological inhibition of PDEs associated with genetic manipulation of these enzymes by using small RNA interfering and overexpression of dominant negative constructs of selected PDEs demonstrate that compartmentalized PDE4B and PDE4D are responsible for selectively modulating the concentration of cAMP in individual subcellular compartments in HEK293 cells (Terrin et al. 2006).

The obvious advantage of using the same sensor targeted to different subcellular compartments is that, in principle, the readings of the different probes are directly comparable. However, careful characterization of the individual sensors is necessary as even minor changes in the sequence of a probe may affect its performance. For example, in the case of the H30, *mp*H30 and *nls*H30 indicators, although the introduction of targeting sequences did not significantly affect the sensitivity of each probe variant to cAMP (Fig. 2e and Table 1), the kinetics of FRET changes resulted in being significantly faster for the *nls*H30 sensor as compared to the H30 and *mp*H30 variants (Fig. 2f), therefore hindering reliable conclusions on the different kinetics of cAMP changes in the individual compartments.

5.3 Sensors Based on Single cAMP-Binding Domains

Crystallographic studies of cyclic nucleotide-binding domains from Epac and PKA revealed that cAMP binding induces conformational changes even in the absence of other parts of the protein (Diller et al. 2001; Rehmann et al. 2003). Based on these observations, a family of FRET sensor based on a single cAMP-binding domain from Epac 1, Epac 2, the PKA R subunit (Nikolaev et al. 2004) or a HCN channel

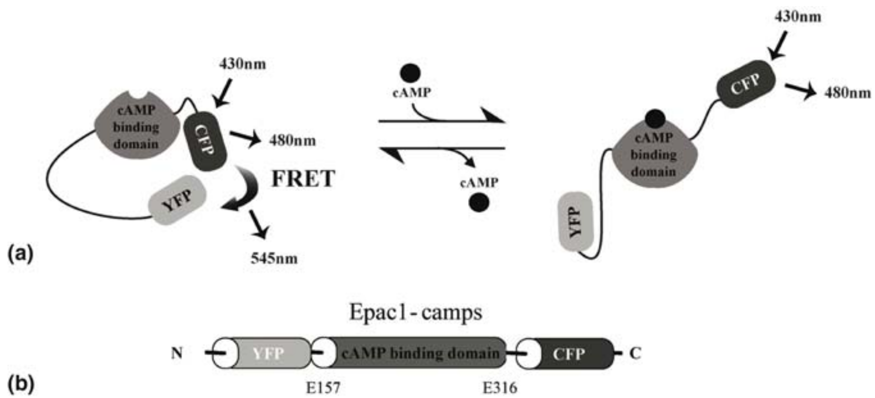

Fig. 3 Sensor based on a single cAMP-binding domain. **(a)** Schematic representation of the mechanism of action of Epac-camps and **(b)** schematic structure of Epac 1-camps

(Nikolaev et al. 2006) was generated. Such sensors have the advantage of an extremely simplified design with a single cAMP-binding domain sandwiched between YFP and CFP (Fig. 3), reducing in this way the chance to generate artifacts resulting from catalytic activity or potential interaction of the sensor with endogenous proteins. In addition, the sensitivity of such single-domain sensors proved to be reasonably high (0.9–5.9 μM) (Nikolaev et al. 2004), allowing the measurement of cAMP within the physiological range of concentrations. In addition, they show rapid cAMP-dependent FRET changes both in vitro and in intact cells, therefore allowing the achievement of high temporal resolution (Nikolaev et al. 2004).

6 Conclusions

Real-time measurement of cAMP fluctuations in the cell is of paramount importance to understand the inner works of the cAMP-dependent signaling pathway. Development of new techniques, relying both on CNG channels and on FRET-based sensors, is leading to the acquisition of detailed information on how cAMP signal propagation occurs in the complexity of the intact cell and is contributing to defining the complex molecular mechanisms that regulate signal transmission in space and time. Being genetically encoded, such sensors can be expressed in transgenic animals, thus opening the possibility to study cAMP signaling in intact, living organisms.

Acknowledgements This work was supported by grants from Telethon Italy (TCP00089 and GGP05113), the Italian Cystic Fibrosis Research Foundation, the Fondazione Compagnia di San Paolo, the HFSPO (RGP0001/2005-C), the Fondation Leducq (O6 CVD 02) and the EC (LSHB-CT-2006-037189).

References

Adams SR, Harootunian AT, Buechler YJ, Taylor SS, Tsien RY (1991) Fluorescence ratio imaging of cyclic AMP in single cells. Nature 349:694–697

Bacskai BJ, Hochner B, Mahaut-Smith M, Adams SR, Kaang BK, Kandel ER, Tsien RY (1993) Spatially resolved dynamics of cAMP and protein kinase A subunits in Aplysia sensory neurons. Science 260:222–266

Bender AT, Beavo JA (2006) Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol Rev 58:488–520

Broillet MC (2000) A single intracellular cysteine residue is responsible for the activation of the olfactory cyclic nucleotide-gated channel by NO. J Biol Chem 275:15135–15141

Brunton LL, Hayes JS, Mayer SE (1981) Functional compartmentation of cyclic AMP and protein kinase in heart. Adv Cyclic Nucleotide Res 14:391–397

Davare MA, Avdonin V, Hall DD, Peden EM, Burette A, Weinberg RJ, Horne MC, Hoshi T, Hell JW (2001) A beta2 adrenergic receptor signaling complex assembled with the Ca2+ channel Cav1.2. Science 293:98–101

de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396:474–477

de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL (2003) Epac is a Rap1 guanine-nucleotide-exchange-factor directly activated by cAMP. Nature 396:474–477

Dhallan RS, Yau KW, Schrader KA, Reed RR (1990) Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. Nature 347:184–187

Diller TC, Madhusudan, Xuong NH, Taylor SS (2001) Molecular basis for regulatory subunit diversity in cAMP-dependent protein kinase: crystal structure of the type II beta regulatory subunit. Structure 9:73–82

DiPilato LM, Cheng X, Zhang J (2004) Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signalling within discrete subcellular compartments. Proc Natl Acad Sci 101:16513–16518

Finn JT, Grunwald ME, Yau KW (1996) Cyclic nucleotide-gated ion channels: an extended family with diverse functions. Annu Rev Physiol 58:395–426

Förster T (1948) Intermolecular energy migration and fluorescence. Ann Phys 2:55–75

Francis SH, Corbin JD (1999) Cyclic nucleotide-dependent protein kinases: intracellular receptors for cAMP and cGMP action. Crit Rev Clin Lab Sci 36:275–328

Goaillard JM, Vincent PV, Fischmeister R (2001) Simultaneous measurements of intracellular cAMP and L-type Ca2+ current in single frog ventricular myocytes. J Physiol 530:79–91

Golla R, Seethala R (2002) A homogeneous enzyme fragment complementation cyclic AMP screen for GPCR agonists. J Biomol Screen 7:515–525

Jurevicius J, Fischmeister R (1996) cAMP compartmentation is responsible for a local activation of cardiac Ca2+ channels by beta-adrenergic agonists. Proc Natl Acad Sci USA 93:295–299

Kasai H, Petersen OH (1994) Spatial dynamics of second messengers: IP3 and cAMP as long-range and associative messengers. Trends Neurosci 17:95–101

Lissandron V, Terrin A, Collini M, D'Alfonso L, Chirico G, Pantano S, Zaccolo M (2005) Improvement of a FRET-based indicator for cAMP by linker design and stabilization of donor-acceptor interaction. J Mol Biol 354:546–555

Liu M, Chen TY, Ahamed B, Li J, Yau KW (1994) Calcium-calmodulin modulation of the olfactory cyclic nucleotide-gated cation channel. Science 266:1348–1354

Mongillo M, McSorley T, Evellin S, Sood A, Lissandron V, Terrin A, Huston E, Hannawacker A, Lohse MJ, Pozzan T, Houslay MD, Zaccolo M (2004) Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. Circ Res 95:67–75

Mongillo M, Tocchetti CG, Terrin A, Lissandron V, Cheung YF, Dostmann WR, Pozzan T, Kass DA, Paolocci N, Houslay MD, Zaccolo M (2006) Compartmentalized phosphodiesterase-2 activity blunts beta-adrenergic cardiac inotropy via an NO/cGMP-dependent pathway. Circ Res 98:226–234

Nikolaev VO, Bunemann M, Hein L, Hannawacker A, Lohse MJ (2004) Novel single chain cAMP sensors for receptor-induced signal propagation. J Biol Chem 279:37215–37218

Nikolaev VO, Gambaryan S, Engelhardt S, Walter U, Lohse MJ (2005) Real-time monitoring of the PDE2 activity of live cells: hormone-stimulated cAMP hydrolysis is faster than hormone-stimulated cAMP synthesis. J Biol Chem 280:1716–1719

Nikolaev VO, Bunemann M, Schmitteckert E, Lohse MJ, Engelhardt S (2006) Cyclic AMP imaging in adult cardiac myocytes reveals far-reaching beta1-adrenergic but locally confined beta2-adrenergic receptor-mediated signaling. Circ Res 99:1084–1091

Ponsioen B, Zhao J, Riedl J, Zwartkruis FJ, van der Krogt G, Zaccolo M, Moolenaar WH, Bos JL, Jalink K (2004) Detecting cAMP-induced activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. EMBO Rep 5:1–5

Rehmann H, Prakash B, Wolf E, Rueppel A, De Rooij J, Bos JL, Wittinghofer A (2003) Structure and regulation of the cAMP-binding domains of Epac2. Nat Struct Biol 10:26–32

Resh MD (1999) Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochim Biophys Acta 1451:1–16

Rich TC, Fagan KA, Nakata H, Schaack J, Cooper DM, Karpen JW (2000) Cyclic nucleotide-gated channels colocalize with adenylyl cyclase in regions of restricted cAMP diffusion. J Gen Physiol 116:147–161

Rich TC, Fagan KA, Tse TE, Schaack J, Cooper DM, Karpen JW (2001a) A uniform extracellular stimulus triggers distinct cAMP signals in different compartments of a simple cell. Proc Natl Acad Sci USA 98:13049–13054

Rich TC, Tse TE, Rohan JG, Schaack J, Karpen JW (2001b) In vivo assessment of local phosphodiesterase activity using tailored cyclic nucleotide-gated channels as cAMP sensors. J Gen Physiol 118:63–78

Steinberg SF, Brunton LL (2001) Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. Annu Rev Pharmacol Toxicol 41:751–773

Steiner AL, Kipnis DM, Utiger R, Parker C (1969) Radioimmunoassay for the measurement of adenosine 3′,5′-cyclic phosphate. Proc Natl Acad Sci USA 64:367–373

Tasken K, Aandahl EM (2004) Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol Rev 84:137–167

Taylor SS, Kim C, Vigil D, Haste NM, Yang J, Wu J, Anand GS (2005) Dynamics of signaling by PKA. Biochim Biophys Acta 1754:25–37

Terrin A, Di Benedetto G, Pertegato V, Cheung YF, Baillie G, Elvassore N, Prinz A, Herberg FW, Houslay MD, Zaccolo M (2006) PGE1 stimulation of HEK293 cells generates multiple contiguous domains with different [cAMP]: role of compartmentalized phosphodiesterases. J Cell Biol

Warrier S, Belevych AE, Ruse M, Eckert RL, Zaccolo M, Pozzan T, Harvey RD (2005) Beta-adrenergic- and muscarinic receptor-induced changes in cAMP activity in adult cardiac myocytes detected with FRET-based biosensor. Am J Physiol Cell Physiol 289:C455–C461

Williams C (2004) cAMP detection methods in HTS: selecting the best from the rest. Nat Rev Drug Discov 3:125–135

Wong W, Scott JD (2004) AKAP signalling complexes: focal points in space and time. Nat Rev Mol Cell Biol 5:959–970

Zaccolo M, Pozzan T (2002) Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. Science 295:1711–1715

Zaccolo M, De Giorgi F, Cho CY, Feng L, Knapp T, Negulescu PA, Taylor SS, Tsien RY, Pozzan T (2000) A genetically encoded, fluorescent indicator for cyclic AMP in living cells. Nat Cell Biol 2:25–29

Zaccolo M, Magalhaes P, Pozzan T (2002) Compartmentalisation of cAMP and Ca(2+) signals. Curr Opin Cell Biol 14:160–166

Molecular Architecture of Signal Complexes Regulating Immune Cell Function

K.M. Torgersen, E.M. Aandahl, and K. Taskén(✉)

Contents

1 Scaffolding of Proximal TCR Signaling . . . 328
1.1 Transmembrane Adaptor Proteins . . . 329
1.2 Slp-76 and Vav: Important Integrators of Complex Cellular Responses . . . 336
1.3 Integration of TCR Signaling by PI3K Signaling . . . 339
2 Modulation of Proximal Signaling Events by cAMP . . . 340
2.1 cAMP Modulates Immune Functions . . . 340
2.2 A cAMP–PKA–Csk Inhibitory Pathway in Lipid Rafts Regulates T-Cell Immune Function . . . 341
2.3 cAMP Levels Are Increased in Lipid Rafts upon TCR Stimulation . . . 343
2.4 PDE4 Is Recruited to Lipid Rafts upon TCR and CD28 Co-Stimulation . . . 344
2.5 Control of cAMP Levels Is Implicated in Normal and Diseased T-Cell Function . . . 344
3 Protein Assembly Linking TCR to NF-κB Activation . . . 345
4 Other Receptor Systems Controlling Immune Responses . . . 348
4.1 Scaffolding of SLAM Receptor Signaling . . . 349
4.2 Scaffolding of Toll-Like Receptor Signaling . . . 350
5 Targeting of Anchored Signal Molecules as a Basis for Therapy . . . 351
References . . . 353

Abstract Signals transmitted via multichain immunoreceptors control the development, differentiation and activation of hematopoetic cells. The cytoplasmic parts of these receptors contain immunoreceptor tyrosine-based activation motifs (ITAMs) that upon phosphorylation by members of the Src tyrosine kinase family orchestrate a complex set of signaling events involving tyrosine phosphorylation, generation of second messengers like DAG, IP3 and Ca^{2+}, activation of effector

K. Taskén
The Biotechnology Centre of Oslo, Gaustadalléen 21, PO Box 1125 Blindern, 0317 Oslo, Norway
kjetil.tasken@biotek.uio.no

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,

© Springer-Verlag Berlin Heidelberg 2008

molecules like Ras and MAPKs and the translocation and activation of transcription factors like NFAT, AP1 and NF-kB. Spatial and temporal organization of these signaling events is essential both to connect the receptors to downstream cascades as well as to control the functional outcome of the immune activation. Throughout this process control and fine-tuning of the different signals are necessary both for effective immune function and in order to avoid inappropriate or exaggerated immune activation and autoimmunity. This control includes modulating mechanisms that set the threshold for activation and reset the activation status after an immune response has been launched. One immunomodulating pathway is the cAMP-protein kinase A-Csk pathway scaffolded by a supramolecular complex residing in lipid rafts with the A kinase-anchoring protein (AKAP) ezrin, the Csk-binding protein PAG and a linker between the two, EBP50. Failure of correct scaffolding and loss of spatiotemporal control can potentially have severe consequences, leading to immune failure or autoimmunity. The clinical relevance of supramolecular complexes specifically organized by scaffolding proteins in regulating immune activity and the specter of genetic diseases linked to different signaling components suggest that protein–protein contact surfaces can be potential targets for drug intervention. It is also of interest to note that different pathogens have evolved strategies to specifically modulate signal integration, thereby rewiring the signal in a way beneficial for their survival. In addition to demonstrating the importance of different signal processes, these adaptations are elegant illustrations of the potential for drug targeting of protein assembly. This chapter reviews some of the important scaffolding events downstream of immunoreceptors with focus on signaling transduction through the T-cell receptor (TCR).

1 Scaffolding of Proximal TCR Signaling

The T-cell receptor is composed of a ligand-binding TCRαβ heterodimer and signal-transducing dimers of CD3εγ, CD3εδ and ζζ that upon interaction with its MHC-peptide ligand initiate an activation process that probably involves both receptor clustering and conformational change of the cytoplasmic part of the CD3 component (Call and Wucherpfennig 2005; Minguet et al. 2007). These signaling components contain ITAMs with the consensus sequence YxxL/I/V x(6–8) YxxL/I/V that are essential for TCR-mediated activation (Reth 1989). The tyrosine residues within this motif are phosphorylated by the protein tyrosine kinases Lck and Fyn and play a critical role in the recruitment of Src homology 2 (SH2) domain-containing proteins to the TCR (Chan and Shaw 1996; Kane et al. 2000). In this respect, the receptor itself acts as an adaptor where specific assembly of signaling components can be regulated. Phosphorylated ITAMs recruit the tandem SH2-containing tyrosine kinase Zap-70 that is further regulated by a complex interplay between the tyrosine kinases Lck and Abl (Chu et al. 1998; Zipfel et al. 2004). In addition to phosphorylation in the activation loop of the kinase, several regulatory events have been coupled to tyrosine phosphorylation sites in interdomain B of Zap-70, between the second SH2 domain and the kinase domain (Brdicka

et al. 2005; Deindl et al. 2007; Di et al. 1999; Pelosi et al. 1999). This includes the recruitment of several adaptor proteins, such as c-Cbl, Vav and Crk, that can direct the activation process, suggesting that protein assembly at the level of Zap-70 is functionally important both in developing and mature cells. In support of this notion, Zap-70-deficient mice fail to develop mature T cells, and humans harboring mutations in the Zap-70 gene develop a severe form of immune deficiency due to the lack of circulating CD8+ cells and functional CD4+ cells (Negishi et al. 1995; Wiest et al. 1997). An interesting observation that further illustrates the importance of intact Zap-70 recruitment is seen in SKG mice that carry a single nucleotide substitution in the second SH2 domain of Zap-70 (W163C). These mice develop an autoimmune disease resembling human rheumatoid arthritis, probably due to a switch in the selection process that leads to survival of T cells with a high degree of self-reactivity (Sakaguchi et al. 2003, 2006b). Importantly, the disease does not manifest itself until T cells in the periphery are activated by pathogens, suggesting that only then is the higher self-reactivity able to overcome the reduced signaling ability. Hence, it seems that a partial defect in Zap-70 is enough to change the signaling threshold essential for correct thymic selection. This suggests that fine-tuning of signals is functionally very important in balancing immune regulation. Other immunoreceptors that utilize ITAM phosphorylation and recruitment of Syk family tyrosine kinases include the B-cell receptor (BCR), Fc receptors for IgG (FcγRIII and FcεRI) and the collagen receptor on platelets, indicating that the ITAM-mediated recruitment of tandem SH2-containing kinases is a general paradigm for transmitting signals in response to immunoreceptor-ligand interaction (Underhill and Goodridge 2007). Following the recruitment and activation of Zap-70, the TCR represents an activated immune receptor that is ready to initiate downstream signals. The next step in immune cell signaling is to connect the activated receptor complex to relevant downstream pathways to elicit adequate immune responses. This involves the specific recruitment of different signaling components to the inner leaflet of the plasma membrane through a highly sophisticated network of protein interactions.

1.1 Transmembrane Adaptor Proteins

Transmembrane adaptor proteins (TRAPs) are a group of integral membrane proteins that recruit and assemble signaling molecules proximal to the antigen receptors (Horejsi et al. 2004). These molecules are characterized by short extracellular domains followed by a transmembrane domain and long cytoplasmatic tails containing mainly tyrosine-based interaction motifs. The phosphorylation status of these motifs changes after immunoreceptor ligation and Zap-70 activation, thereby regulating their ability to bind SH2-domain-containing effector proteins. So far, seven different members have been identified that can be further divided into two groups based on their targeting to lipid rafts in the plasma membrane. Lipid raft-associated TRAPs include LAT (linker for activation of T cell), PAG (protein associated with glycosphingolipid-enriched microdomains, also called Csk-binding protein Cbp), NTAL (non-T-cell activation linker) and LIME (Lck-interacting membrane protein),

all of which have a juxtamembrane CxxC motif that when palmitoylated targets TRAPs to membrane microdomains. Although this posttranslational modification seems to be essential for LAT function (further discussed below), the significance of dual acylation for targeting of the other TRAPs remains unclear. The non-lipid raft-associated TRAPs encompass LAX (linker for activation of X cells), SIT (SHP2-interacting TRAP) and TRIM (TCR-interacting molecule).

Together TRAPs provide an arsenal of targeting sites that can connect immunoreceptor signaling to downstream effectors. Several members contain multiple motifs for recruitment of Grb2 or GADS through YxN motifs. This suggests that the different TRAPs in general are associated with the fine-tuning and signal assembly related to specific receptor systems and that there is some level of redundancy between members. Gene targeting studies of different TRAPs support this notion to some extent, but they also suggest that defects in signal organization due to lack of specific TRAPs can have severe long-term effects, including autoimmunity. It is important to point out that TRAPs contribute in a unique way in the fine-tuning of different signals, including negative regulation of immune receptor activation. Interestingly, recent results suggest that there also can be interplay by two functionally opposing TRAPS in the fine-tuning of TCR signaling. This added complexity together with the different membrane distribution and binding abilities provides the immune cell with a set of focal points for spatiotemporal signal regulation. A detailed description of all TRAPS and their functions can be found elsewhere (Horejsi et al. 2004; Simeoni et al. 2005). Here we will focus on two functionally opposing scaffolding complexes involving TRAPs.

1.1.1 LAT Scaffolding

LAT has an essential role in TCR signaling and provides a hallmark example of the complex signal integration needed for T-cell activation (Fig. 1) (Weber et al. 1998; Zhang et al. 1998a). Gene targeting of *Lat* in mice leads to a complete arrest early in T-cell development (DN3 stage) due to defect signaling downstream of the pre-TCR (Zhang et al. 1999b). Cell line defects in LAT display normal ζ-chain phosphorylation and recruitment of Zap-70, but show defects in PLC-γ activation, calcium flux and activation of Ras and Erk (Finco et al. 1998; Zhang et al. 1999a). The signaling defects in these cell lines can be rescued by wild-type LAT, but not by a mutant with a disrupted CxxC motif, suggesting that lipid raft association is essential for normal function (Zhang et al. 1998b). The cytoplasmic part of LAT contains a total of nine tyrosine-based interaction motifs where the four distal sites (Y^{132}LVV, Y^{171}VNV, Y^{191}VNV and Y^{226}ENL) seem to be the primary targets for phosphorylation by Zap-70 or Syk and functionally most important. In agreement with this, mice with knock-in mutation in all four of these distal tyrosines (LATY-4F) have similar phenotype as the LAT−/− mice (Sommers et al. 2001). Tyrosine 132 binds specifically the SH2 domain of PLCγ, whereas the three distal tyrosines bind the SH2 domains of Grb2 or the Grb2-related adaptor protein GADS (Zhang et al. 2000). Binding of Grb2 leads to recruitment of the Ras guanine

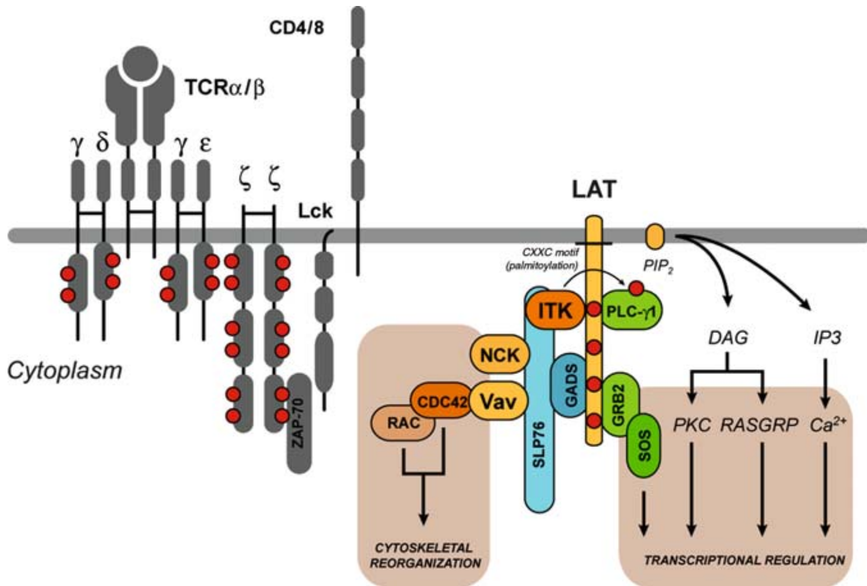

Fig. 1 Integrated scaffolding by LAT and Slp-76 couples TCR to downstream mediators. Phosphorylation of LAT by Zap-70 after TCR activation leads to recruitment of Grb2-SOS and GADS-Slp76 to the three C-terminal YxN motifs. Grb2-SOS is involved in activating the Ras-Erk pathway, while recruitment of GADS brings Slp-76 to the membrane, where it is phosphorylated by Zap-70 at critical tyrosine residues. This creates binding sites for the SH2 domains of Vav, Nck and the Tec kinase ITK. Vav and Nck regulate cytoskeletal reorganization, while ITK phosphorylates and activates PLC-γ. The SH2 domain of PLC-γ binds directly to phosphorylated Y^{132}LVV in LAT, and after activation PLC-γ will hydrolyze PIP_2 to generate IP3 and DAG. IP3 induces release of cytoplasmic Ca^{2+} and activation of NFAT through calcineurin. DAG activates PKC isoforms and RasGRP to regulate the activity of NF-κB and AP1. Together, this complex scaffolding connects the activated TCR complex to the transcriptional machinery regulation of the immune response

exchange factor SOS, thereby contributing to the activation of the Ras-MAPK pathway. The SH2 domain of PLCγ is specifically recruited to Y132 and places the enzyme in close proximity of its substrate PtdIns(4,5)P2, leading to an increase in cytoplasmic calcium by IP3 and activation of both PKC isozymes and RasGRP by DAG. Results suggest that the recruitment of RasGRP by DAG is the preferred pathway for activation of Erk downstream of the TCR (Dower et al. 2000; Layer et al. 2003; Priatel et al. 2002). The activation of PLCγ is dependent on the GADS-mediated recruitment of the multifunctional adaptor Slp-76 to LAT, since both LAT-deficient and Slp-76-deficient cells are unable to activate PLCγ (Finco et al. 1998; Yablonski et al. 1998). Recruitment of GADS to LAT brings Slp-76 to the membrane, where it is phosphorylated by Zap-70 at critical tyrosine residues in the N-terminal region (Singer et al. 2004). This creates binding sites for the SH2 domains of Vav, Nck and the Tec-kinase ITK. PLCγ is then phosphorylated by the

recruited ITK and activated. Like LAT, Slp-76 can bind PLCγ, directly creating an interconnected signaling module of LAT, GADS, Slp-76, ITK and PLCγ that controls the generation of the second messengers IP3 and DAG downstream of immunoreceptors (Koretzky et al. 2006). Additional regulation is introduced since both PLCγ and ITK contain PH domains for binding to phosphatidylinositols in the membrane (Schwartzberg et al. 2005). The Slp-76-Vav-Nck interactions are important in regulating processes leading to reorganization of the actin cytoskeleton and generation of the immunological synapse (further discussed below).

The importance of the LAT-PLCγ interaction in immune homeostasis is dramatically illustrated in knock-in mice where the PLCγ binding site is mutated to phenylalanine (Aguado et al. 2002; Sommers et al. 2002). T cells from these mice display similar defects in signaling, as seen in cell lines with the same mutation characterized by reduced phosphorylation of PLCγ and an inability to induce intra-cellular calcium release after TCR stimulation. This results in a severe defect in T-cell development and supports the notion that the LAT-PLCγ interaction is necessary for pre-TCR signaling. Surprisingly, these mice develop a polyclonal lymphoproliferative disease and autoimmunity involving constitutively active CD4+ cells in the periphery, a high amount of T-helper-2 (TH2) cytokines secreted and induced B-cell maturation. Interestingly, the activation of Erk was relatively normal in these mice, suggesting additional mechanisms for signal regulation of the Ras-Erk pathway. Another (and very likely) explanation for the drastic lymphoproliferative disease is the defect in the development of CD4+CD25+Foxp3+ regulatory T cells (Treg) seen in these mice (Koonpaew et al. 2006). Considering the essential role of Treg in immune tolerance (Sakaguchi et al. 2006a), the absence of these cells would clearly be problematic and contribute to the phenotype. A clear defect in immune development is also seen in knock-in mice where the three distal tyrosines in LAT are mutated (Nunez-Cruz et al. 2003). These LAT-Y3F mice also have an early developmental block, but retain a significant amount of γδT cells with an activated phenotype that produce large amounts of TH2 cytokines. Jointly, these models suggest that both binding of PLCγ and Grb2/GADS complexes to LAT contribute to the balancing of signals needed for correct development, while at the same time selecting T cells with an appropriate antigen repertoire. Signal integration at the level of LAT can also involve proteins complexes with inhibitory functions. The adaptor protein Gab2 is recruited to LAT via its association with the SH3 domain of Grb2 or GADS. Gab2 has a negative effect on TCR activation, probably by recruiting the protein tyrosine phosphatase (PTP) SHP-2 to LAT (Yamasaki et al. 2003). A recent report also describes the clustering of a Grb2-Dok-2-SHIP regulatory complex to LAT, which when disrupted by the use of siRNA increases proximal signaling and IL-2 production (Dong et al. 2006). These results fit well with results from Dok-1/Dok-2 double deficient mice describing enhanced TCR-induced cytokine production and proliferation (Yasuda et al. 2007). Targeting of the 5′-phosphoinositol-phosphatase SHIP1 to LAT in the membrane will localize this phosphatase in the proximity of its lipid substrate and thereby enable modulation of the recruitment and activation of PH-domain-containing signaling proteins. Together, these examples add to the complexity of signal integration at the level of

LAT and may indicate that the multiple Grb2-binding sites seen in some of the TRAPs will recruit functionally opposing protein complexes. Dissecting the spatiotemporal control of such LAT scaffolding will be necessary to fully understand the multitude of proximal signal integration downstream of ITAM-containing immunoreceptors. Furthermore, this information will also be relevant to the analysis of the complex phenotypes seen in different LAT mouse models.

Reversible palmitoylation through the opposing activity of acyltransferases and thioesterases is important in the functional regulation of several proteins (Resh 2006). In immune cell signaling, lipid modification, including palmitoylation, is central for correct membrane targeting of key signaling molecules, such as Src kinases and Ras. Although the significance of LAT targeting to lipid rafts is still controversial, it seems clear that the dual palmitoylation of LAT contributes to the correct tethering in the plasma membrane (Zhang et al. 1998b). In agreement with this, the dynamic regulation of LAT palmitoylation has recently been suggested to be an important mechanism in inducing non-responsiveness in T cells and anergy (Hundt et al. 2006). Considering that T-cell anergy is not caused by a permanent failure in TCR-induced signaling pathways, but represents a temporal and reversible defect in activation due to specific uncoupling of downstream signal pathways, a dynamic modulation of LAT function would provide an attractive model for anergy regulation.

Together this rather extensive analysis of the molecular mechanism associated with LAT-mediated scaffolding in T cells has revealed that site-specific recruitment and specific protein assembly are critical in fine-tuning proximal TCR signals.

1.1.2 NTAL and PAG: Transmembrane Adaptors with Regulatory Functions

In the absence of an appropriate activating stimulus, the TCR-signaling machinery needs to be kept in an inactive state. Regulation of signals controlling the initiation of lymphocyte activation is central in preventing the cell from launching an attack on normal cells. Equally important is the release from inhibition when activation is necessary. This also includes mechanisms that are able to terminate an immune response after successful clearance of the pathogen. It is now evident that dynamic scaffolding of inhibitory complexes at the plasma membrane is functionally important for immune homeostasis. Adaptor molecules that negatively regulate T-cell activation include NTAL, PAG, SIT and LAX, in addition to cytoplasmatic adaptors such as Cbl (casitas B-lineage lymphoma) and SAP (SLAM-associated protein) (Horejsi et al. 2004; Latour and Veillette 2004; Mueller 2004). The scaffolding mediated by the two TRAPs NTAL and PAG plays different roles in immune control. NTAL is expressed in B cells, mast cells and NK cells, but not in naïve T cells. Five of the eight tyrosine phosphorylation motifs in the cytoplasmatic region are YxN motifs that become tyrosine phosphorylated after BCR or Fc-receptor stimulation and associate with Grb2, Gab1 and Cbl (Brdicka et al. 2002; Janssen et al. 2003). Due to its lack of expression in naïve T cells and its similarity with LAT, NTAL was originally thought to be a LAT-like linker downstream of the B

cell receptor. However, deletion of the gene encoding NTAL (LAB in mouse) did not significantly affect B-cell development or BCR-mediated signaling (Wang et al. 2005; Zhu et al. 2004). Although a recent report suggests a regulatory role in the internalization of BCR (Mutch et al. 2007), the significance of NTAL in B cell function remains unclear. Interestingly, NTAL can partly rescue the phenotype of LAT-deficient cells and the development of peripheral T cells in LAT −/− mice. However, NTAL does not bind PLC-γ or recruit Slp proteins, and transgenic mice expressing NTAL on a LAT-deficient background develop a severe lymphoproliferative syndrome almost identical to the LATY136F mice (Janssen et al. 2004; Koonpaew et al. 2004). So far, these results suggest that the Grb2-binding sites in NTAL can compensate for the loss of LAT except for the critical recruitment and activation of PLCγ. An important development in the understanding of NTAL in immune cells was reported recently (Zhu et al. 2006). It turns out that the expression of NTAL is upregulated during T-cell activation and that aged mice (>6 months) deficient in NTAL develop an autoimmune syndrome characterized by enlarged spleens and production of autoantibodies. T cells from these mice are hyperactive, and TCR stimulation results in increased phosphorylation of LAT, PLCγ, Erk and Akt. In comparison, T cells from NTAL transgenic mice display reduced activation of the same molecules, further supporting a negative effect on T-cell activation. Interestingly, the data suggest that there is a functional connection between NTAL and LAT, since the targeting of LAT to lipid rafts is somehow related to NTAL expression. In the absence of NTAL, more LAT was distributed to rafts leading to increased LAT phosphorylation and calcium mobilization, whereas overexpression of NTAL had the opposite effect. Together these data support a model where NTAL deficiency leads to enhanced TCR signaling in the periphery and gradual accumulation of autoreactive T cells that result in the development of an autoimmune syndrome. Thus, upregulation of NTAL after TCR activation provides a negative feedback loop important for T-cell homeostasis.

The cloning and initial characterization of PAG led to some interesting models of proximal control of TCR signaling. Cbp/PAG is ubiquitously expressed and was originally observed as an 80-kDa phosphoprotein present in lipid rafts and as a component of a Fyn-associated complex in T cells (Brdicka et al. 2000; Kawabuchi et al. 2000; Marie-Cardine et al. 1999). Like other TRAPs, it is a type-III transmembrane protein with a short extracellular domain, a transmembrane region followed by a palmitoylation motif and a cytoplasmatic part containing multiple sites for tyrosine phosphorylation. These sites serve as substrates for the Src kinase Fyn as Cbp/PAG phosphorylation in Fyn −/− cells is almost absent (Yasuda et al. 2002). Analyzed in vitro, PAG phosphorylation creates binding sites for the SH2 domains of several signaling molecules, but in vivo only the C-terminal Src kinase (Csk) and Fyn itself are reproducibly found to interact with PAG (Brdicka et al. 2000; Kawabuchi et al. 2000). Csk plays an important role in negative regulation of Src kinases by phosphorylation of a C-terminal inhibitory tyrosine residue that induces an intramolecular interaction between this site and the SH2 domain (Bergman et al. 1992; Chow et al. 1993; Okada et al. 1991). Since Src kinases are membrane targeted, this attenuation requires the membrane recruitment of the

cytosolic Csk. In agreement with this, constitutive targeting of Csk to the plasma membrane leads to almost complete block of TCR signaling (Cloutier et al. 1995). The inhibitory action by Csk is counteracted by the tyrosine phosphatase CD45 through dephosphorylation of the C-terminal site (for review, see Thomas and Brown, 1999; Veillette et al. 2002). Site-directed mutagenesis of Cbp/PAG identified Tyr317 (Tyr314 in mouse) in an YSSV motif as the site for Csk binding (Brdicka et al. 2000; Kawabuchi et al. 2000; Thomas and Brown 1999). In a naïve T cell, PAG is kept in a hyperphosphorylated state, probably due to its association with Fyn, and thereby constitutively targets Csk to lipid rafts. Interestingly, the binding to Cbp/PAG significantly increases Csk activity that can be further modulated by PKA-mediated phosphorylation of Csk (Vang et al. 2003). This could provide a system for tonic inhibition of Src kinases that is localized in proximity to the Cbp/PAG-associated Csk. Upon TCR stimulation, PAG is rapidly dephosphorylated by a mechanism that appears to involve CD45 activity, and Csk is subsequently removed from lipid rafts (Brdicka et al. 2000; Davidson et al. 2003; Torgersen et al. 2001). This dissociation of Csk results in less negative control of Src kinases, allows for phosphorylation of specific substrates including ITAM sequences and is a necessary permissive event to allow T-cell activation to proceed.

The physiological significance of Csk targeting to Cbp/PAG has been seriously questioned by two independent reports describing the absence of any clear phenotype in Cbp/PAG deficient mice (Dobenecker et al. 2005; Xu et al. 2005). Specifically, embryogenesis, T-cell development and T-cell functions appeared normal in the absence of Cbp/PAG. Taken together with the severe phenotype of Csk-deficient mice (Imamoto and Soriano 1993; Nada et al. 1993), this information clearly suggests that Cbp/PAG is not the only anchoring molecule for Csk and that some redundancy must exist. In fact, several Csk-binding adaptor proteins have been described including Dok-related adaptors, paxillin, LIME and SIT, arguing for coordinated action of several scaffolding proteins in the regulation of Csk (Lemay et al. 2000; Pfrepper et al. 2001; Sabe et al. 1994). Together this opens for a model where Csk targeting by distinct adaptors couple Csk modulation onto specific pathways on an individual basis and provide control modules for the Src kinases involved.

The non-covalent association of Fyn to Cbp/PAG has not been fully characterized. Dephosphorylation of Cbp/PAG after TCR stimulation of normal human T cells was originally reported not to affect the binding of Fyn to Cbp/PAG, suggesting a phosphotyrosine-independent association. However, recent results from mice demonstrate a similar release of Fyn from Cbp/PAG after TCR crosslinking as with Csk (Davidson et al. 2007). In fact, it is suggested that the release of Fyn precedes the dephosphorylation of Tyr314 and release of Csk. The reason for this discrepancy is unknown, but could potentially be influenced by Fyn SH3 domain binding to PAG (see below). This study also includes the analyses of Cbp/PAG transgenic mice arguing for a distinct role of Cbp/PAG-associated Fyn in the regulation of T-cell anergy, implying that Fyn can regulate other processes than Csk recruitment when associated with Cbp/PAG. Whether Cbp/PAG truly provides

independent regulation of Fyn or Csk remains to be clarified, but studies like this might provide insight into the functional role of scaffolding to Cbp/PAG.

Structurally Cbp/PAG also has certain unique features like two proline-rich regions and a PDZ-domain binding motif in its C-terminus. We recently identified the first proline-rich region as a ligand for the SH3 domain of Fyn, suggesting that Fyn interacts with PAG through both the SH3 and SH2 domains (Solheim et al. 2008). This provides an example of coordinated, dual domain docking of Fyn as seen in certain other cases (Arold et al. 2001; Nakamoto et al. 1996). Furthermore, it opens for the possibility that Fyn associated with PAG is insensitive to Csk-mediated phosphorylation at the regulatory Y528 site, since the SH2 domain already is engaged and unable to form a closed conformation. The possible transient release of Fyn after T-cell activation described above suggests that the SH2 domain is most important in binding. However, it appears that the engagement of the SH3 domain is more significant in regulation of Fyn kinase activity. This would be in agreement with the general model of Src regulation where engagement of the SH3 domain causes a structural change that releases Fyn from an inhibitory intramolecular interaction between the SH3 domain and the SH2-kinase linker region (for review, see Hubbard and Till 2000; Sicheri and Kuriyan 1997).

The C-terminal VTRL sequence of PAG interacts with the PDZ-domain of the cytoplasmic adaptor EBP-50 [ezrin, radixin, moesin (ERM)-binding protein 50], which in turn can bind to ERM proteins and connect this protein complex to the actin cytoskeleton (Brdickova et al. 2001; Itoh et al. 2002). This opens the possibility that PAG is involved in the rearrangement of the cellular framework influencing formation of the immunological synapse. In this context it is interesting to note that reduction of PAG expression by siRNA seems to influence Src kinase activity related to cell adhesion and to spread in a similar way as in Csk-deficient cells (Shima et al. 2003).

1.2 Slp-76 and Vav: Important Integrators of Complex Cellular Responses

An integrating role of Slp-76 in the specific assembly of signaling molecules downstream of the TCR is demonstrated in mice deficient in Slp-76 (Clements et al. 1998; Pivniouk et al. 1998). These mice have a block in T-cell development at the same stage as LAT-deficient mice, where signaling through the pre-TCR is necessary for thymic selection. However, due to the expression of Slp-76 in most hematopoietic cells, Slp-76 deficient mice have a complex phenotype with signaling defects downstream of different receptors on mast cells, platelets and neutrophils described in addition to T-cell defects. Importantly, Slp-76 seems to have a pivotal role in mediating signals downstream of integrin receptors on these cells. Furthermore, Slp-76-deficient mice have a vascular phenotype probably due to the failure to separate the

blood and lymphatic vascular networks during development (Abtahian et al. 2003). This chimeric vessel system results in a bleeding phenotype where blood enters the lymphatic system. The molecular mechanism behind this severe phenotype remains to be identified. A detailed description of the different phenotypes in Slp-76-deficient mice can be found elsewhere (Koretzky et al. 2006). Thus, there is an essential interplay between LAT and Slp-76 that sets the stage for immune activation downstream of the TCR. Classical structure-function studies in Slp-76-deficient Jurkat cells have clarified the functional significance of the domain-specific interactions in this scaffolding process (reviewed in Koretzky et al. 2006). Slp-76 consists of an N-terminal domain with three tyrosine phosphorylation motifs, a central proline-rich region and a C-terminal SH2 domain. The central region provides binding sites for PLCγ and the SH3 domains of GADS. Interestingly, GADS binds constitutively to Slp-76 through a RxxK motif (class-III binding motif), and this interaction is essential for translocation and membrane targeting of Slp-76 to LAT after TCR activation (Liu et al. 2003). Specific disruption of the GADS-Slp-76 interaction by mutation or peptide competition has a negative effect on TCR signaling. The N-terminal part of Slp-76 contains functionally important tyrosine motifs that mediate the interaction with the SH2 domains of Vav (Y^{112} and Y^{128}) (Raab et al. 1997; Tuosto et al. 1996; Wu et al. 1996), NCK (Y^{128}) (Bubeck et al. 1998; Wunderlich et al. 1999) and ITK(Y^{145}) (Su et al. 1999). Both in vivo and in vitro data support an essential role of these N-terminal sites in Slp-76-mediated scaffolding (Fang et al. 1996). For complete T-cell activation, all three sites need to be phosphorylated and to recruit their specific partners, suggesting that the molecules attaching to these sites play important roles in signal integration during T-cell activation. Tyrosine 145 is phosphorylated by Lck and Zap-70 after recruitment to LAT and creates a binding site for Itk. This Tec kinase can then phosphorylate and activate PLCγ associated either with Slp-76 itself (Yablonski et al. 2001) or LAT. In addition, Vav seems to play a part in this complex by regulating the function of Itk. Combined scaffolding by LAT and Slp-76 thereby creates multiple interactions relevant for the fine-tuning of signals leading to the generation of Ca^{2+} and DAG (Fig. 2). This cooperative binding system might have significance in increasing specificity and structural stability at a critical point in the T-cell activation process. Together, these events set the stage for the initial responses and further direct the activation process through the generation of the immunological synapse and relevant immune responses.

As indicated above, Vav is an important effector in the scaffolding of immune receptor signaling and has been demonstrated to mediate a critical function during T-cell development and activation (Tybulewicz 2005). Vav proteins are guanine-exchange factors (GEFs) for the Rho/Rac family of small GTPases and exist in three functionally distinct forms (Vav 1–3) that have identical domain structure (reviewed in Turner and Billadeau 2002). The N-terminal part contains a calponin-homology (CH) domain that is followed by an acidic stretch containing tyrosines involved in autoinhibition of its GEF activity. This is followed by the DBL-homology domain responsible for the interaction with the GTPases and GEF activity. Next follows a PH domain, a zinc-finger domain contributing to the GEF

activity, a proline-rich region that binds the SH3 domain of Grb2 and two SH3 domains separated by a SH2 domain. Structural studies using NMR revealed that an intramolecular regulation mechanism exists where the acidic region around tyrosine 174 associates with the DH domain and inhibits GEF activity. Upon phosphorylation of tyrosine 174, this closed structure opens, giving the DH domain access to binding. The complexity of its domain structure suggests that Vav can act through a multitude of mechanisms. The GEF activity of Vav1 is important for T-cell development as Vav1-deficient mice have a partial block in thymic development at the CD4-/CD8-double-negative stage that is dependent on its GEF activity towards Rac-family members of small GTPases (Gomez et al. 2001; Turner et al. 1997). Interestingly, T-cell development in Vav2- and Vav3-deficient mice is normal, supporting a specific role of each member (Doody et al. 2000; Fujikawa et al. 2003). TCR signaling in Vav1-deficient T cells has partial defects in MAPK activation, intracellular Ca^{2+} release and NF-κB activation, while T cells deficient in all three members have a complete block in these signaling pathways (Costello et al. 1999; Fujikawa et al. 2003). Hence, it seems that there is some degree of functional redundancy between Vav isoforms in addition to their individual functions.

The ability of Vav to activate GTPases and the subsequent accumulation of F-actin is essential in the formation of a defined and organized contact zone between the immune cell and the antigen presenting cell (APC) called the immunological synapse (Tybulewicz 2005). While the complex integration of signals described above is a prerequisite for the formation of the synapse, this defined junction is the structural basis for the sustained signaling needed for a functional immune response (Billadeau et al. 2007). In general, T-cell activation through an immunological synapse can be divided into several steps that include polarization, adhesion to target, engagement of the TCR, generation of the immunological synapse and finally contact termination (Dustin 2005). After the initial contact between the T cell and the APC, a characteristic pattern forms with a central area of adhesion molecules surrounded by a ring of TCR-MHC complexes. The intriguing part is that over a period of minutes this pattern is inverted, resulting in a central cluster of TCR-MHC surrounded by a ring of adhesion molecules. This represents the mature synapse and is defined by so-called central and peripheral supramolecular activation clusters (c-SMAC and p-SMAC). Interestingly, activation of Rac by Vav can also mediate signals leading to the dephosphorylation and inactivation of ERM proteins and detachment, resulting in a less rigid cell structure that will be more dynamic and facilitate formation of T cell-APC interfaces (Faure et al. 2004). Furthermore, after detachment ERM proteins will redistribute away from the active contact zone and become part of a distal pole complex. The functional significance of this is uncertain, but considering the ability of ERM proteins to bind different proteins, it is tempting to speculate that it promotes specific scaffolding of a functionally important complex. Together this dynamic modulation of the cellular framework allows the redistribution of cellular components and formation of cellular polarization that is essential for immune cell functions.

1.3 Integration of TCR Signaling by PI3K Signaling

As indicated above, phosphatidylinositols contribute to the proximal signal integration downstream of the T-cell receptor. Two different signaling pathways originate from PtdIns(4,5)P_2. Hydrolysis of PtdIns(4,5)P_2 by activated PLCγ tethered to the LAT/Slp-76 complex yields Ins(1,4,5)P_3 and DAG, which both act as signaling molecules. Binding of Ins(1,4,5)P_3 to specific intracellular receptors leads to the release of Ca^{2+} from intracellular stores and subsequent opening of calcium channels in the plasma membrane. Calcium contributes to the activation of several proteins, including calcineurin that dephosphorylates NFAT transcription factors enabling nuclear translocation and transcriptional activity. DAG activates Ras-GRP (which is also Ca^{2+} dependent) and isoforms of PKC that contributes to the activation of the Ras-Erk-Ap1 and NF-κB pathways, respectively. The complex scaffolding involved in the regulation of NF-κB will be discussed below. In T cells, PtdIns(4,5)P_2 can also act as a substrate for class IA of phosphoinositide 3-kinases (PI3Ks) that are activated upon T-cell stimulation (Okkenhaug and Vanhaesebroeck, 2003). Class IA PI3Ks are heterodimers of a p85 regulatory subunit isoform and a p110 catalytic isoform. PI3Ks phosphorylate PtdIns(4,5)P_2 at the 3′ position of the inositol ring, generating PtdIns(3,4,5)P_3 at the plasma membrane. Interestingly, PtdIns(3,4,5)P_3 is generated very early after TCR triggering, indicating a direct link between PI3K activation and early signaling events (Costello et al. 2002; Harriague and Bismuth 2002; Ward et al. 1993). Exactly how PI3Ks are linked to the antigen receptor is still not resolved, but it probably involves the coordinated recruitment and binding of the SH2 domain of the p85 regulatory subunit of PI3K to canonical YxxM motifs located in different adaptor proteins (Bruyns et al. 1998; Ward and Cantrell 2001). The CD28 receptor also contains this motif, and the binding of PI3K is thought to be part of the essential co-stimulation signal that leads to increased IL-2 production, proliferation and prevention of anergy (Sharpe and Freeman 2002). However, although mice carrying a mutation in the CD28 YxxM motif failed to bind PI3K, it did not affect the ability of CD28 to transmit the co-stimulatory signal, suggesting that PI3K bound to CD28 is involved in a different signaling pathway (Burr et al. 2001; Harada et al. 2001; Okkenhaug et al. 2001). In agreement with this, results indicate that activation of PI3K through CD28 is important in promoting cell survival through PKB and upregulation of BCL-X1 (Boise et al. 1995; Dahl et al. 2000; Jones et al. 2000) in addition to the β-arrestin-mediated recruitment of phosphodiesterases (PDEs) regulating the levels of cAMP (Abrahamsen et al. 2004).

The rapid accumulaton of PtdIns(3,4,5)P_3 in the membrane induces immediate translocation of proteins that contain PH domains with preference for this phosphoinositide (Rameh and Cantley 1999). These include the protein kinases PKB/Akt, Tec family members like Itk and the 3′-phosphoinositide-dependent kinase-1 (Pdk1) in addition to different adaptors like Vav and Gab2. As described above, Itk and Vav are involved in the assembly of the multimeric LAT/Slp-76 scaffolding complex, supporting the notion that PI3K signaling is integrated with the early

tyrosine phosphorylation events. The membrane recruitment of the PH-domain containing Ser/Thr kinase PDK-1 as a result of PtdIns(3,4,5)P_3 generation is a key regulatory step in the activation of downstream cascades. As described later, PDK-1-mediated phosphorylation of PKCθ probably is central in the TCR-mediated activation of NF-κB through the CARMA1 scaffolding complex. In addition, PDK-1 plays an important role in regulating T-cell activation and survival through phosphorylation of Akt/PKB, which plays an important role in transmitting signals. Once recruited, Akt/PKB undergoes a conformational change and is phosphorylated by PDK-1 on two key residues. Activated Akt/PKB then regulates a number of substrates containing the recognition sequence RXRXXS/T. A full description of all Akt/PKB substrates is beyond the scope of this review and can be found elsewhere (Vanhaesebroeck and Alessi 2000). However, among the functionally important targets of Akt/PKB in T cells are GSK3 and members of the FOXO (forkhead box class O) family of forkhead box transcription factors, particulary FOXO3a (Brunet et al. 1999). In a resting cell, FOXO3a is transcriptionally active in the nucleus where it contributes to the suppression of NF-κB activation. Upon T-cell activation, FOXO3a is phosphorylated by Akt/PKB at three sites that induce binding to 14-3-3 proteins and sequestration in the nucleus (Brownawell et al. 2001; Brunet et al. 2001; Plas and Thompson 2003). This functional inactivation of FOXO3a opens for NF-κB activation and transcription of genes involved in the immune response. The functional significance of this was illustrated in mice deficient in FOXO3a that develop a spontaneous, multisystemic inflammatory syndrome due to increased NF-κB activation and hyperactivation of CD4+ cells (Lin et al. 2004). Combined with the complex scaffolding in the regulation of NF-κB described below, it is evident that cells need multiple-level control of this key mediator.

2 Modulation of Proximal Signaling Events by cAMP

The cyclic AMP (cAMP)-protein kinase A (PKA) pathway is strongly involved in the regulation and modulation of immune responses and is the most potent and acute inhibitor of activation of lymphocytes. The mechanisms of cAMP-mediated immunomodulation are discussed in more detail below.

2.1 cAMP Modulates Immune Functions

cAMP, generated by G-protein-mediated activation of adenylyl cyclase (AC), is a versatile second messenger controlling a variety of cellular processes and is known to inhibit TCR-induced T-cell proliferation (Kammer 1988). cAMP activates PKA (reviewed in Tasken and Aandahl 2004), Epac (exchange

protein directly activated by cAMP; de Rooij et al. 1998) and cAMP-regulated ion channels (Kaupp and Seifert 2002). Effects mediated by cAMP in T cells are, however, most likely elicited by PKA, being the dominant effector in T cells. The mechanism for inactivation of cAMP involves degradation by cAMP-specific phosphodiesterases (PDEs) (Conti and Jin, 1999; Houslay and Adams 2003). Compartmentalization of receptors, ACs and PKA by A-kinase-anchoring proteins (AKAPs) (Michel and Scott 2002) as well as generation of local pools of cAMP within the cell by the action of PDEs (Zaccolo and Pozzan 2002) generates a high degree of specificity in PKA-mediated signaling despite the broad substrate specificity of PKA. AKAPs contribute specificity by targeting PKA towards specific substrates as well as versatility by assembling multi-protein signal complexes allowing for signal termination by phosphoprotein phosphatases and crosstalk between different signaling pathways (Michel and Scott 2002; Tasken and Aandahl 2004). Integrating PDEs into these anchoring complexes adds a further temporal aspect to the spatial regulation of cAMP signals (reviewed in Baillie et al. 2005; Smith and Scott 2002).

In the immune system, prostaglandin E_2 (PGE_2) and other ligands elevating cAMP by binding to G-protein-coupled receptors inhibit TCR-induced T-cell activation and thereby exert important immunoregulatory functions (Kammer 1988). Based on studies with selective agonists, activation of PKA type I ($RI\alpha_2C_2$) has been shown to be necessary and sufficient for mediating these effects of cAMP (Skalhegg et al. 1992). Similarly, PKA type I negatively regulates activation of B cells through the B-cell antigen receptor (Levy et al. 1996) and NK cell cytotoxicity elicited through specific NK cell receptors (Torgersen et al. 1997). Although PKA can modulate TCR signaling at multiple levels (reviewed in Torgersen et al. 2002), the observed inhibitory effects of cAMP on TCR-induced ζ-chain phosphorylation point towards an important role for Csk, which is the most up-stream PKA target reported so far.

2.2 A cAMP–PKA–Csk Inhibitory Pathway in Lipid Rafts Regulates T-Cell Immune Function

Proteins involved in proximal TCR signaling events are localized in lipid rafts, representing small regions of detergent-resistant lipid domains of the membrane (Montixi et al. 1998; Xavier et al. 1998). Both the cAMP-generating machinery (adenylyl cyclase) and the effectors (PKA type I and Csk) are localized in lipid rafts. Analyses of lipid raft purifications from normal resting T cells for the presence of different subunits of PKA revealed that both the catalytic subunit and the regulatory subunit RIα (but not RII subunits) are constitutively associated with the lipid rafts (Vang et al. 2001). This suggests that the observed co-localization of PKA type I and TCR in capped T cells (Skalhegg et al. 1994) occurs in lipid rafts

and that there are mechanisms for specific targeting of PKA type I to these areas involving interaction with an AKAP in lipid rafts, which we recently identified as ezrin (Ruppelt et al. 2007).

Csk is constitutively localized to lipid rafts in resting T cells, but is transiently displaced to the cytosol during T-cell activation (Torgersen et al. 2001) in order to allow the activation cascade to proceed. The phosphatase responsible for the dephosphorylation of Cbp/PAG and the release of Csk was recently identified as CD45 (Davidson et al. 2003). Fyn-mediated phosphorylation of Cbp/PAG (Brdicka et al. 2000; Kawabuchi et al. 2000) leads to re-recruitment of Csk and re-establishment of the inhibitory pathway.

Csk regulates Lck activity by phosphorylation of a C-terminal inhibitory tyrosine residue (Lck-Y505). So far, two different mechanisms are reported to regulate Csk activity. PKA type I, through phosphorylation of Ser-364, increases Csk kinase activity two- to four-fold, leading to reduced Lck activity and TCR ζ-chain phosphorylation, which inhibits T-cell activation (Fig. 2). The other mechanism involves the adaptor molecule Cbp/PAG. Csk is recruited to lipid rafts and the site of action by binding to Y314/Y317-phosphorylated (rat/human) Cbp/PAG through its SH2 domain (Brdicka et al. 2000; Kawabuchi et al. 2000), and the interaction between Csk-SH2 and Cbp/PAG increases Csk activity

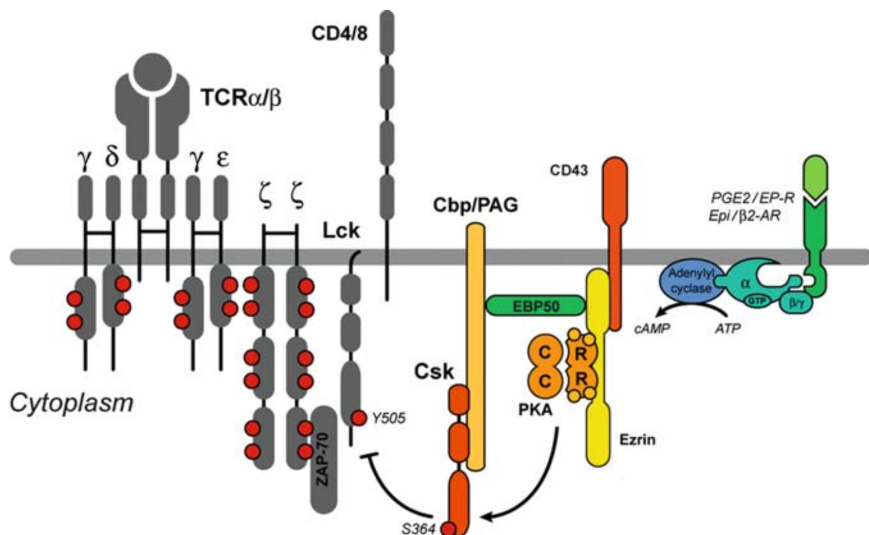

Fig. 2 Modulation of proximal TCR signaling by cAMP and PKA. Generation of local pools of cAMP through G protein-coupled receptors and adenylyl cyclase activity is able to attenuate proximal TCR signaling by activating a PKA-Csk-Lck inhibitory pathway. Ezrin acts as an AKAP and targets PKA type I to lipid rafts where it can phosphorylate and activate Csk. PKA-mediated phosphorylation and targeting to the transmembrane adaptor Cbp/PAG both contribute to increased Csk activity and thereby increased tonic inhibition of Src kinases such as Lck. Engagement of the T-cell receptor leads to dephosphorylation of Cbp/PAG, dissociation of Csk from lipid rafts and increased Lck activity

(Takeuchi et al. 2000). Addition of either recombinant Cbp/PAG or phosphopeptides corresponding to the Csk-SH2 binding site in Cbp/PAG significantly increased Csk kinase activity towards a Src substrate in vitro. Lastly, the PKA phosphorylation of Csk and its interaction with Cbp/PAG act together in turning on Csk activity in a time- and space-regulated fashion (Vang et al. 2003), providing a powerful mechanism for terminating activation through receptors eliciting Src kinase signaling (Fig. 2).

Immunofluorescense and immunoprecipitation studies of ezrin in normal T cells revealed colocalization with PKA RIα and unveiled a complex including Csk and Cbp/PAG interacting as well as the ezrin-Cbp/PAG linker EBP50 (Ruppelt et al. 2007). Together this demonstrates formation of a lipid raft-associated complex where ezrin brings PKA type I in the proximity of its downstream substrate Csk by forming a supramolecular signaling complex consisting of PKA type I, ezrin, EBP50, Cbp/PAG and Csk. Ezrin is bound via CD43, CD44 or ICAMs to the plasma membrane (Heiska et al. 1998; Tsukita et al. 1994; Yonemura et al. 1998) and via its C-terminus to the actin cytoskeleton (Algrain et al. 1993), and EBP50 links ezrin to Cbp/PAG (Itoh et al. 2002). Thus, ezrin positions PKA type I in proximity of its substrate Csk, which is bound to Cbp/PAG (Fig. 2), leading to PKA phosphorylation of the pool of Csk anchored to Cbp/PAG (Vang et al. 2001, 2003). This, together with the observations that downstream effector functions modulated by the PKA-Csk pathway are released from cAMP inhibition by knockdown of ezrin, argues that the PKA regulation of Csk is discretely coordinated and spatiotemporally restricted to this supramolecular complex (Ruppelt et al. 2007).

2.3 cAMP Levels Are Increased in Lipid Rafts upon TCR Stimulation

It has previously been demonstrated that stimulation of the TCR results in elevated cAMP levels in the cell (Ledbetter et al. 1986). However, since increased cAMP concentrations inhibit T-cell function and proliferation (Aandahl et al. 2002; Skalhegg et al. 1992), it is important that TCR-mediated cAMP production is tightly regulated. The significance of activation-induced cAMP production has been poorly understood to date, as has the location in the cell where cAMP is generated. However, we were recently able to show that upon engagement of the TCR in primary T cells, cAMP was rapidly produced in lipid rafts, resulting in raft-associated PKA activation (Abrahamsen et al. 2003). The G proteins G_i, G_s and AC have been reported to segregate into lipid rafts (Oh and Schnitzer 2001), and our data indicate that recruitment to lipid rafts of the stimulatory G protein G_s and dissociation of the inhibitory G protein G_i play an important role for the cAMP production that occurs upon TCR activation. A local increase in cAMP is therefore generated in T-cell lipid rafts upon activation. In contrast, T cells activated by TCR and CD28 cross-ligation revealed

decreased cAMP levels compared to control cells, and an increase in cAMP levels was only observed in the presence of the non-selective PDE inhibitor IBMX. Furthermore, phosphorylation of PKA substrates in lipid rafts was less abundant when cells were only activated through the TCR compared to concomitant TCR and CD28 stimulation. We therefore hypothesized that TCR-induced cAMP production must be accompanied by cAMP degradation by PDEs to allow full T-cell activation to proceed.

2.4 PDE4 Is Recruited to Lipid Rafts upon TCR and CD28 Co-Stimulation

We observed PDE4 activity in lipid rafts upon T-cell activation. In particular, TCR and CD28 co-stimulation resulted in profound raft-associated increase in PDE4 activity (Abrahamsen et al. 2004). This specific increase in PDE4 activity in lipid raft fractions upon TCR and CD28 engagement indicates that temporal changes in PDE4 activity can play a key role in tuning intracellular activation-induced gradients of cAMP in T-cell lipid rafts and thereby increase signal propagation upon co-stimulation. The mechanisms by which PDE4 isoforms are recruited to specific locations upon T-cell activation are now being unraveled (Abrahamsen et al. 2004; Arp et al. 2003). We have demonstrated that PDE4A4, 4B2 and 4D1/2 are recruited to lipid rafts upon TCR and CD28 co-stimulation in human peripheral T cells (Abrahamsen et al. 2004). Members of the PDE4 family have previously been described to associate with the scaffolding protein β-arrestin, and β-arrestin has been shown to be responsible for bringing PDE4 to the plasma membrane of HEK293 cells and to the activated G-protein-coupled receptor (GPCR) where cAMP production is taking place (Perry et al. 2002). Intriguingly, we found that both TCR and CD28 co-stimulation and CD28 stimulation alone caused a clear recruitment of β-arrestin to T-cell lipid raft fractions concurrently with PDE4. In addition, immunoprecipitation from both unstimulated and co-stimulated cells revealed that β-arrestin and PDE4 pre-exist in a complex prior to stimulation, indicating that they are recruited to rafts together (Abrahamsen et al. 2004).

2.5 Control of cAMP Levels Is Implicated in Normal and Diseased T-Cell Function

PKA is activated upon TCR-induced cAMP production in lipid rafts and inhibits proximal T-cell signaling. However, overexpression of PDE4 isoforms or β-arrestin has been demonstrated to increase T-cell activation, revealing regulatory roles for both proteins in T-cell signaling (Abrahamsen et al. 2004). Other regulatory roles for β-arrestin in T cells have also been described. For example, β-arrestin plays a positive regulatory role in chemotaxis (Fong et al. 2002) and in migration

into the airways during asthma (Walker et al. 2003). The activities of both PKA and PDE4 therefore seem to be important for regulation of TCR-induced signaling and T-cell function. We propose a novel role for TCR and CD28 co-stimulation in down-modulation of TCR-induced cAMP-mediated inhibitory signals through the recruitment of β-arrestin and PDE4 to lipid rafts and thus allowing a full T-cell response to occur. Interestingly, the cAMP inhibitory pathway has also been shown to be implicated in several immune diseases. T cells from HIV-infected patients have elevated levels of cAMP and hyperactivation of PKA. Targeting of the cAMP-PKA type-I pathway by selective antagonists reverses T-cell dysfunction in HIV T cells ex vivo (Aandahl et al. 1998, 1999), and targeting cyclooxygenase 2 to reduce PGE_2 production lowers cAMP and increases T-cell function in vivo (Johansson et al. 2004; Kvale et al. 2006). A similar mechanism contributes to the T-cell dysfunction in a subset of patients with common variable immunodeficiency (Aukrust et al. 1999) and to the severe T-cell anergy in a murine immunodeficiency model termed MAIDS (mouse AIDS) (Rahmouni et al. 2004, 2001).

3 Protein Assembly Linking TCR to NF-κB Activation

Transcription of immune regulatory genes involves the coordinated action of several transcription factors, including NFAT, NF-κB, AP1 and members of the forkhead box family (Coffer and Burgering 2004; Kuo and Leiden 1999). It is therefore relevant to examine how receptor proximal events are linked to these mediators and how specificity in the regulation of the different transcription factors is maintained. There is accumulating evidence demonstrating that fine-tuning of signals at this level in the signaling cascade is as complex as the fine-tuning of signals proximal to the immune receptors and that scaffolding proteins again are essential for signal integration to occur. This is well illustrated by the molecular mechanisms connecting the T-cell receptor to NF-κB activation (Fig. 3) (Bonizzi and Karin 2004). Engagement of the TCR results in the nuclear translocation and activation of NF-κB, which regulates various aspects of lymphocyte development, homeostasis, survival and functions. Dysregulation of NF-κB is associated with cancer, chronic inflammation and autoimmunity, and it is hence of great interest to identify the molecular mechanisms that both connect proximal immune-receptor signaling to NF-κB and that regulate the activity of this transcription factor (Karin and Greten 2005). NF-κB represents a group of structurally related and evolutionary conserved proteins that contains Rel homology DNA-binding domains (RHD) and forms various homo- or heterodimers (Ghosh and Karin 2002). In resting cells, NF-κB is sequestrered in the cytosol by a family of inhibitory proteins called IκB that binds to NF-κB through their ankyrin repeats and masks a nuclear localization signal in the RHD of NF-κB. Stimulation of T cells by CD3-CD28 crosslinking initiates signaling cascades leading to activation of IκB kinase (IKK) and phosphorylation of regulatory serine residues in IκB. This targets IκB for ubiquitination and degradation by the proteasome

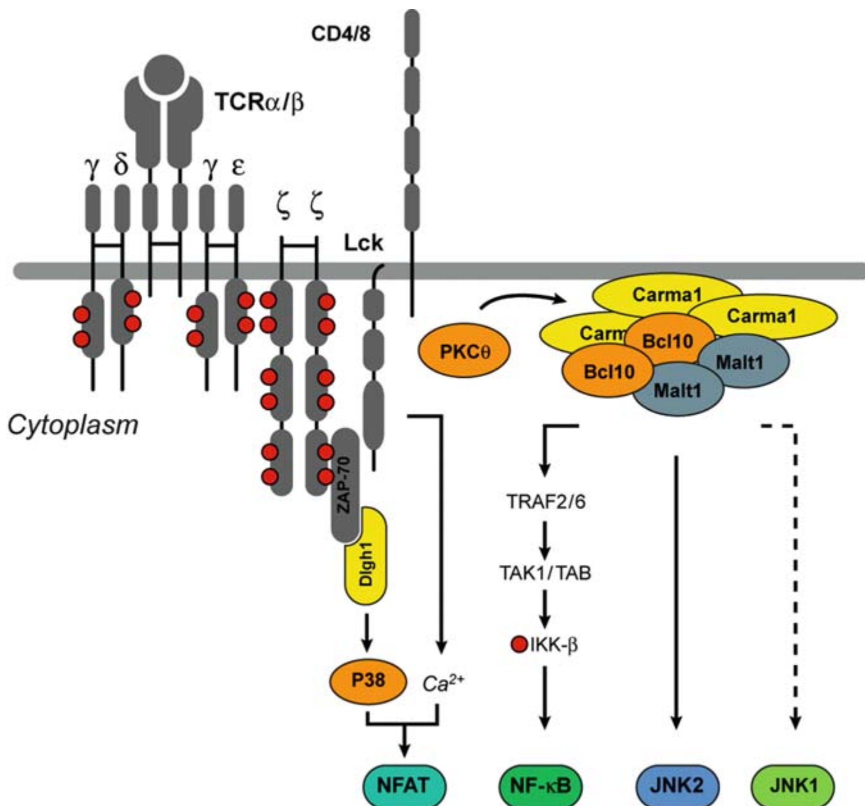

Fig. 3 Coordination of TCR signaling by MAGUK-family proteins. Activation of PKC isoforms downstream of TCR leads to phosphorylation and activation of the MAGUK protein CARMA1. Phosphorylated CARMA1 will interact with Bcl-10/MALT1 complexes to form oligomerized scaffolds that in turn activate TRAF2/6. TRAF2 and TRAF6 will induce ubiquitinylation and assembly of proteins that lead to the phosphorylation of IKKβ and activation of the IKK complex. This results in the phosphorylation and ubiquitinylation of IκB subunits and release of NF-κB. Results also suggest that signaling through CARMA1 can control the specific activation of JNK2. Binding of Dlgh1 to Zap-70 is supposed to induce an alternative pathway for activation of p38 and control of NFAT activity

complex, leading to release of NF-κB and unmasking of the nuclear localization signal. NF-κB will then translocate to the nucleus and engage its cognate κB enhancer elements. Recent studies have described an important mechanism in connecting T-cell receptor signaling to NF-κB activation. In this model, IKK complex activation occurs through PKCθ-mediated regulation of a supramolecular scaffold complex formed by the adaptor proteins CARMA1 (CARD-domain- and MAGUK-domain-containing protein 1), BCL10 (B-cell lymphoma 10) and MALT1 (mucosa-associated-lymphoid-tissue lymphoma translocation gene 1) (Rawlings et al. 2006; Schulze-Luehrmann and Ghosh 2006; Thome 2004). CARMA1 is a lymphocyte member of the membrane-associated guanylate kinase (MAGUK) protein family

and contains N-terminal CARD domain, followed by a coiled-coil domain and a region containing PDZ, SH3 and GUK domains. Lymphocytes from CARMA1-deficient mice fail to proliferate after antigen receptor stimulation and cannot activate IKK or JNK (Egawa et al. 2003; Hara et al. 2003). Furthermore, a portion of CARMA1 seems to constitutively associate with lipid rafts where it is able to recruit and bind BCL-10, TAK1 [transforming growth factor-β (TGFβ)-activated kinase 1] and IKKγ upon receptor stimulation and initiate activation of the IKK complex (Gaide et al. 2002). Between the coiled-coil (CC) and the PDZ of CARMA1 is an important flexible linker region with several serine residues that are targets for phosphorylation by atypical PKC isoforms (PKCβ or PKCθ) (Matsumoto et al. 2005; Sommer et al. 2005). This leads to an open active conformation, oligomerization of CARMA1 through its CC domain and binding of BCL-10 through CARD-CARD interactions, suggesting that CARMA1 acts upstream of BCL-10 in this process. Each BCL-10 molecule can then act as a binding site for MALT1 and under stimulating conditions form cytoplasmic structures called POLKADOTS, which are dynamic protein structures formed by multiple BCL-10 protein–protein interactions (Rossman et al. 2006; Schaefer et al. 2004). BCL-10 is part of the larger family of proteins that contain caspase recruitment domains (CARD) and has been described to interact with numerous proteins including CARMA1 and MALT 1. Chromosomal translocations resulting in BCL-10 overexpression lead to aggressive B-cell-derived MALT lymphomas characterized by constitutive NF-κB activation (Willis et al. 1999). In contrast, B or T cells from BCL-10 knockout mice are unable to activate NF-κB after antigen-receptor stimulation despite having intact calcium flux and relatively normal phosphotyrosine signals (Ruland et al. 2001). The specific CARD-domain interaction with CARMA1 seems to be functionally important since disruption of this interaction abrogates the ability of CARMA-1 to activate IKK and NF-κB (Gaide et al. 2002; Pomerantz et al. 2002). A downstream target of BCL-10 is MALT1, which is a protein characterized by an N-terminal death domain, two Ig-like domains and a caspase-like domain (Lucas et al. 2001; Uren et al. 2000). MALT1 knockout mice have defects in responses to TCR activation, including proliferation, IL-2 production and IKK activation (Ruefli-Brasse et al. 2003; Ruland et al. 2003). Like BCL-10, it is targeted by chromosomal translocation in some MALT lymphomas, leading to overexpression and constitutive NF-κB activation. Similarly, overexpression and oligomerization of MALT1 are sufficient to activate NF-κB, and combined overexpression of both MALT and BCL-10 has a synergistic effect on NF-κB activation (Lucas et al. 2001; Uren et al. 2000). In summary, data suggest that BCL-10 bound to CARMA1 acts as a scaffold in the assembly and oligomerization of MALT1, thereby creating a signalosome controlling NF-κB activation. The next step involves the positioning of TRAF 2 and 6 (tumor necrosis factor–receptor-associated factor) in complex with trimerized MALT1 and activation of their ubiquitin ligase activity (Baud et al. 1999; Park et al. 1999; Sun et al. 2004). TRAF2 and TRAF6 will induce ubiquitinylation and assembly of proteins that lead to the phosphorylation of IKKβ and activation of the IKK complex. Finally, this results in the phosphorylation and ubiquitinylation of IκB subunits and release of NF-κB. The events downstream of MALT1 are conserved among different NF-κB-activating

pathways and represent a convergence point in the NF-κB cascade. In conclusion, TCR activation of atypical PKCs leads to the recruitment and oligomerization of CARMA1, recruitment of BCL-10 and MALT1 and nucleation of an oligomeric protein complex that directs the activation of NF-κB. Interestingly, results suggest that CARMA1 has an additional role in generating specificity in the regulation of c-Jun N-terminal kinases (JNKs) downstream of TCR activation (Blonska et al. 2007). T cells express JNK1 and JNK2 isoforms, which both are activated after TCR stimulation, and CARMA1 deficiency seems to affect this activation. In fact, there appears to be a selective defect in JNK2 activation in a CARMA1-deficient cell line or T cells from CARMA1-deficient mice, suggesting that CARMA1 scaffolding through BCL-10 is able to specifically regulate the assembly of a JNK2-MKK7-TAK1 protein complex. Activation of JNK2 by this protein assembly seems to control the accumulation of c-Jun after TCR stimulation (Blonska et al. 2007).

The significance of MAGUK proteins in the scaffolding and connection of signaling downstream of TCR has been further substantiated by the possible role of Dlgh1 in the direct activation of the MAPK p38 by Zap-70. Like CARMA1, Dlgh1 is an adaptor with several modular domains. It contains a proline-rich N-terminal followed by three PDZ domains, a SH3 domain and a GUK domain. T-cell stimulation leads to the recruitment of Dlgh1 to the immunological synapse where it forms a complex with Lck, Zap-70 and p38 MAPK (Hanada et al. 1997; Round et al. 2007; Xavier et al. 2004). This facilitates Zap-70-mediated phosphorylation of Tyr323 on p38 (Salvador et al. 2005), which induces a conformational change, dimerization and autophosphorylation on Thr180 and Tyr182. This alternative pathway seems to correlate with phosphorylation of NFAT at Ser 54 and increased transactivation. Overexpression of Dlgh1 in T-cell hybridoma cells resulted in a specific increase in NFAT reporter activity, while knockdown of Dlgh1 in T cells from TCR transgenic mice gave reduced p38 and NFAT activation (Round et al. 2005). It is important to note that Dlgh1 seems to skew TCR proximal events towards NFAT activation independent of the classical Ca^{2+}-dependent activation of calcineurin, adding to the complexity in the regulation of this transcription factor. Together the recent studies of CARMA1 and Dlgh1 place MAGUK proteins at key positions in orchestrating signal specificity downstream of immune receptors and channeling toward distinct targets (Rebeaud et al. 2007). In this regard, it will be interesting to see if MAGUK proteins play a role in the unique transcriptional profile seen in CD4+/CD25+/Foxp3+ regulatory T cells and thereby can influence signaling underlying immune homeostasis.

4 Other Receptor Systems Controlling Immune Responses

Signaling through immunoreceptors alone is not sufficient to control all aspects of T-cell biology. Other receptors, such as cytokine receptors, chemokine receptors, integrins and tumor-necrosis factor (TNF)-related receptors, can all influence

T-cell functions in different ways. Some involve the recruitment of functionally unique adaptors that connect these receptors to intracellular signaling pathways.

4.1 Scaffolding of SLAM Receptor Signaling

A small group of receptors implicated in immune cell regulation are members of the signaling lymphocytic activation molecule (SLAM) family, which belongs to the CD2 subfamily of Ig receptors. These include SLAM (CD150), 2B4, CD84, NTBA (NK-,T- and B-cell antigen), Ly9 (CD229) and CD2-like receptor activating cytotoxic cells or CRACC (CD319) (Ma et al. 2007; Veillette 2006). They have Ig-like domains in their extracellular regions and are expressed in different subsets of lymphocytes. Except for 2B4, which binds CD48, the ligands for SLAM receptors are not well characterized, but almost all members of the family appear to be involved in homotypic interaction with other SLAM expressing cells. SLAM/CD150 is expressed on thymocytes, T cells, B cells, macrophages and dendritic cells and interestingly serves as the lymphoid specific receptor for morbilliviruses (including measles) (Tatsuo et al. 2000; Veillette 2006). T cells from SLAM-deficient mice have a defect in the production of IL-4 and IL-13, but not INF-γ, suggesting that SLAM is involved in skewing the immune response towards a Th2 response (Davidson et al. 2004; Wang et al. 2004). Furthermore, SLAM is thought to be involved in the communication between T cells and antigen-presenting cells (APCs) in vivo, and SLAM expression is upregulated on APCs after inflammatory stimuli (Bleharski et al. 2001; Kruse et al. 2001). However, further work is required to fully understand the role of SLAM interactions in immune development and regulation.

A common denominator of the SLAM family of receptors is their ability to interact with a small family of adaptors called SLAM-associated proteins or SAPs (Ma et al. 2007; Veillette 2006). The three members of this family are SAP, Ewing's sarcoma-associated transcript-2 (EAT-2) and the rodent member EAT-2-related transducer (ERT). They have a very simple structure consisting of an SH2 domain and a short C-terminal tail. The SH2 domain interacts with the consensus TxYxxV/I present in the cytoplasmic part of some, but not all forms of SLAM receptors. In contrast to classical SH2 domain interactions, SAP can bind to this motif independently of tyrosine phosphorylation, suggesting that SAP constitutively interacts with SLAM receptors (Latour et al. 2001). This association to SAP makes the receptor able to signal through tyrosine phosphorylation by recruiting the Src kinase Fyn through a unique type of interaction (Chan et al. 2003; Latour et al. 2003). An arginine-based motif (RFFRKVKN) localized between the sixth β-sheet and the second α-helix of the SAP SH2-domain specifically interacts with the SH3 domain of Fyn. This means that this interaction occurs on the external surface away from the phosphotyrosine pocket interacting with the tyrosine-based motif of the SLAM receptors. The association with Fyn is induced upon receptor engagement and leads to activation of Fyn kinase activity and subsequent phosphorylation of

additional tyrosine motifs present in the C-terminal part of the receptors. In the case of SLAM, this leads to the recruitment of the inositol phosphatase SHIP and assembly of a complex involving Dok1, Dok2 and RasGAP (Latour et al. 2001). Hence, the dual-binding capabilities of the SAP SH2 domain form a unique scaffolding module that alone connects the SLAM receptors to downstream signaling.

The identification of SAP (*SH2D1A)* as the aberrant gene that causes X-linked lymphoproliferative (XLP) syndrome clearly points to a central role of SAP in immune homeostasis (Coffey et al. 1998; Nichols et al. 1998; Sayos et al. 1998). XLP is a complex disorder characterized by a defect in immune regulation usually manifested by an uncontrolled immune response to primary infection of Epstein Barr virus (EBV). Other common clinical features are lymphoproliferative disorders and dysgammaglobulinemia. Importantly, the phenotype of SAP-deficient mice recapitulates several important aspects of the immune defect seen in XLP, including defects in Th2 cytokine production, hyper-responsive CD8+ population after virus infection, defects in B-cell function and absence of NKT cells (Ma et al. 2007). The lack of NKT cells in SAP-deficient mice is striking, since the development of T and NK cells appears normal. Interestingly, Fyn is also implicated in the development of NKT cells, suggesting that the SAP/Fyn signaling unit transmits signals necessary for this process. Furthermore, the Th2-cell defect seen in SLAM-deficient mice is reminiscent of the phenotype observed in SAP and Fyn-deficient mice, suggesting that SLAM primarily signals through SAP-Fyn. Together these data support a central role of the SLAM-SAP-Fyn signaling in the control of immune effector functions during infections.

4.2 Scaffolding of Toll-Like Receptor Signaling

As part of the defence against microbial infection, the immune system has evolved mechanisms for detection of abundantly expressed unique molecules critical for pathogen replication and survival. The recognition of these pathogen-associated molecular patterns (PAMPS) is mediated by Toll-like receptors (TLRs) that either alone or through cooperation form a repertoire that can recognize a broad array of PAMPs (West et al. 2006). Acting like sensors, TLRs are able to initiate signaling cascades that lead to activation of the NF-κB, AP-1 and IRF families of transcription factors and subsequent transcription of distinct target genes required for an effective immune response. Essential in TLR signaling is a structural motif called Toll/IL-1 receptor (TIR) domain present in all TLRs and the IL-1 receptor family (Dunne and O’Neill 2003). This domain forms homotypic interaction and couples these receptors to a unique set of TIR-domain-containing scaffolding proteins. These adaptors include myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor (TIRAP), TIR-domain-containing adaptor inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) (West et al. 2006). So far, results suggest that MyD88 is the key mediator of signals downstream of most TLRs. In addition to its TIR-domain, MyD88 has an N-terminal death domain that can interact and activate

members of the interleukin 1 receptor-associated kinase (IRAK) family. This leads to the recruitment and activation of the ubiquitin ligase TRAF-6 and activiation of the kinase TAK1 through poly-ubiquitinylation. Tak 1 activates the classical NF-κB activation resulting in activation of the IKK complex, IκB degradation and NF-κB translocation and activation (Chen 2005; Dunne and O'Neill 2003).

The central role of MyD88 is well illustrated by results from MyD88-deficient mice (Adachi et al. 1998; Kawai et al. 1999). Cells from these mice do not respond to a broad spectrum of PAMPs known to activate TLRs 2, 5, 7/8, 9 and 11, suggesting that MyD88 is essential to connect these receptors to intracellular pathways. TLR4-induced signals are not affected to the same degree, as cells from these mice still can signal through MAPK and NF-κB after LPS stimulation. However, the response profile is reduced and delayed, suggesting that other molecules are involved in this pathway. In fact, this MyD88-independent signaling led to the identification of other TIR-domain containing adaptors, including TRIF, which seems to independently control signals through TLR4 (Akira and Takeda 2004; West et al. 2006). TLR4 signaling through TRIF is thought to activate IRF3 and IFN gene transcription by engaging the downstream kinase TBK1. Thus, the current model suggests that after LPS binding and dimerization, TLR4 assembles two distinct scaffolding units to the receptor complex that regulates signals through MyD88 and TRIF. Furthermore, recent results suggest that this process is coordinated by two of the other TIR adaptor proteins, TRAM and TIRAP. TRAM contains an N-terminal myristoylation site that targets it to the plasma membrane where it seems to colocalize with TLR4 (Rowe et al. 2006). This places TRAM in a position to recruit and target TRIF to the receptor and initiate signaling through TBK1. Similarly, TIRAP plays an important role in TLR signaling and can facilitate MyD88 delivery to activated TLR4. Interestingly, TIRAP is targeted to membrane structures by binding to phosphatidylinositol 4,5,-bisphosphate (PIP2), suggesting that signal initiation by TLR4 is linked to lipid metabolism (Kagan and Medzhitov 2006). Together these data suggest that spatial regulation and membrane tethering of adaptors is an important process in the sorting of signals downstream of TLRs.

5 Targeting of Anchored Signal Molecules as a Basis for Therapy

Typically drug-targeting strategies have focused on cell-surface receptors and enzymes. In contrast, targeting of interacting surfaces of anchoring, docking and adaptor proteins that localize signal molecules have not been subject to similar interest up to now, but offer an expansion of strategies for perturbation of signal pathways and offer great opportunities for specificity because of the opportunity of targeting particular subpopulations of signal molecules. However, interruption of protein–protein interactions involved in signal localization also poses challenges as interacting surfaces may be large and with a less suitable topology and charge

distribution for the binding of small molecules. A complicating factor for specific targeting disruption is the cooperative binding involved in protein scaffolds that together create specificity and stability to the signaling module. This suggests that targeting of single interactions may affect the whole complex and cause multiple signal alterations.

Peptide-array technology, intracellular delivery of optimized peptides and in vivo use of stabilized peptidomimetics represent powerful tools to address the possibility of drug targeting of protein–protein interactions and anchored complexes with huge potential for specifically addressing cell-type-specific and signal-pathway-specific effects. Peptidomimetics are typically protected against exopeptidases as they are capped or have internal modifications to remove or protect the peptide bonds of a corresponding peptide. This technology can be used to develop molecules to perturb anchoring and provide accurate proof of principle and target validation and would typically precede a decision to screen for small molecular compounds that perturb the same anchored complexes.

As an example, the inhibitory effect of protein kinase A on T-cell function is dependent on anchoring of the enzyme by an A-kinase-anchoring protein (AKAP) called ezrin that anchors PKA in the vicinity of the T-cell receptor as discussed above (Ruppelt et al. 2007). Isozyme-specific PKA anchoring disruptors have been developed by designing small peptides that competitively bind to the anchoring site and displace distince PKA isozymes. Such peptide disruptors may represent future means for targeting cell-specific and isoenzyme-specific anchored signaling processes mediated by PKA (Carlson et al. 2006; Gold et al. 2006). This strategy is suitable to test and validate anchored PKA as a target and could have therapeutically relevant applications in regulation of, for example, immune function.

Development of assays suitable to find drug candidates, primarily small synthetic molecular entities, interfering with protein–protein interactions could be cell-based assays, such as bioluminescence resonance energy transfer assay, amplified luminescence proximity homogenous assay (AlphaScreen) or fluorescence polarization, all of which offer means to characterize molecular interactions such as protein–protein interactions, and subsequently this could be used to detect disruption by small molecular compounds in a high-throughput screen. In an example using AlphaScreen, an assay was established using biotinylated PKA RIα/RIIα and GST-fused truncated AKAP95/149 immobilized to these beads via streptavidin and anti-GST, respectively. When the beads were brought in proximity by an R-AKAP complex, they produced an AlphaScreen signal. Peptide antagonists that competed with the AKAP for binding to the D/D domain in the R subunit of PKA reduced the AlphaScreen signal in a concentration-dependent manner, and kinetic properties of such peptide antagonists could be characterized, leading up to future chemical biology screens (Stokka et al. 2006).

In summary, we anticipate that we will see an increasing number of drug-targeting strategies aimed at perturbing hyperactive signaling pathways in disease that make use of the opportunities to interfere with anchoring to disrupt the discrete spatio-temporal regulation provided by scaffolding proteins.

Acknowledgments Our work is supported by grants from the Functional Genomics Programme, the Research Council of Norway, the Norwegian Cancer Society, Novo Nordic Foundation and the European Union (grant no. 037189, thera-cAMP).

References

Aandahl EM, Aukrust P, Skalhegg BS, Muller F, Froland SS, Hansson V, Tasken K (1998) Protein kinase A type I antagonist restores immune responses of T cells from HIV-infected patients. FASEB J 12:855–862

Aandahl EM, Aukrust P, Muller F, Hansson V, Tasken K, Froland SS (1999) Additive effects of IL-2 and protein kinase A type I antagonist on function of T cells from HIV-infected patients on HAART. AIDS 13:F109–F114

Aandahl EM, Moretto WJ, Haslett PA, Vang T, Bryn T, Tasken K, Nixon DF (2002) Inhibition of antigen-specific T cell proliferation and cytokine production by protein kinase A type I. J Immunol 169:802–808

Abrahamsen H, Vang T, Tasken K (2003) Protein kinase A intersects SRC signaling in membrane microdomains. J Biol Chem 278:17170–17177

Abrahamsen H, Baillie G, Ngai J, Vang T, Nika K, Ruppelt A, Mustelin T, Zaccolo M, Houslay M, Tasken K (2004) TCR- and CD28-mediated recruitment of phosphodiesterase 4 to lipid rafts potentiates TCR signaling. J Immunol 173:4847–4858

Abtahian F, Guerriero A, Sebzda E, Lu MM, Zhou R, Mocsai A, Myers EE, Huang B, Jackson DG, Ferrari VA, Tybulewicz V, Lowell CA, Lepore JJ, Koretzky GA, Kahn ML (2003) Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. Science 299:247–251

Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 9:143–150

Aguado E, Richelme S, Nunez-Cruz S, Miazek A, Mura AM, Richelme M, Guo XJ, Sainty D, He HT, Malissen B, Malissen M (2002) Induction of T helper type 2 immunity by a point mutation in the LAT adaptor. Science 296:2036–2040

Akira S, Takeda K (2004) Toll-like receptor signaling. Nat Rev Immunol 4:499–511

Algrain M, Turunen O, Vaheri A, Louvard D, Arpin M (1993) Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. J Cell Biol 120:129–139

Arold ST, Ulmer TS, Mulhern TD, Werner JM, Ladbury JE, Campbell ID, Noble ME (2001) The role of the Src homology 3-Src homology 2 interface in the regulation of Src kinases. J Biol Chem 276:17199–17205

Arp J, Kirchhof MG, Baroja ML, Nazarian SH, Chau TA, Strathdee CA, Ball EH, Madrenas J (2003) Regulation of T-cell activation by phosphodiesterase 4B2 requires its dynamic redistribution during immunological synapse formation. Mol Cell Biol 23:8042–8057

Aukrust P, Aandahl EM, Skalhegg BS, Nordoy I, Hansson V, Tasken K, Froland SS, Muller F (1999) Increased activation of protein kinase A type I contributes to the T cell deficiency in common variable immunodeficiency. J Immunol 162:1178–1185

Baillie GS, Scott JD, Houslay MD (2005) Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract. FEBS Lett 579:3264–3270

Baud V, Liu ZG, Bennett B, Suzuki N, Xia Y, Karin M (1999) Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. Genes Dev 13:1297–1308

Bergman M, Mustelin T, Oetken C, Partanen J, Flint NA, Amrein KE, Autero M, Burn P, Alitalo K (1992) The human p50csk tyrosine kinase phosphorylates p56lck at Tyr-505 and down regulates its catalytic activity. EMBO J 11:2919–2924

Billadeau DD, Nolz JC, Gomez TS (2007) Regulation of T-cell activation by the cytoskeleton. Nat Rev Immunol 7:131–143

Bleharski JR, Niazi KR, Sieling PA, Cheng G, Modlin RL (2001) Signaling lymphocytic activation molecule is expressed on CD40 ligand-activated dendritic cells and directly augments production of inflammatory cytokines. J Immunol 167:3174–3181

Blonska M, Pappu BP, Matsumoto R, Li H, Su B, Wang D, Lin X (2007) The CARMA1-Bcl10 signaling complex selectively regulates JNK2 kinase in the T cell receptor-signaling pathway. Immunity 26:55–66

Boise LH, Minn AJ, Noel PJ, June CH, Accavitti MA, Lindsten T, Thompson CB (1995) CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. Immunity 3:87–98

Bonizzi G, Karin M (2004) The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol 25:280–288

Brdicka T, Pavlistova D, Leo A, Bruyns E, Korinek V, Angelisova P, Scherer J, Shevchenko A, Hilgert I, Cerny J, Drbal K, Kuramitsu Y, Kornacker B, Horejsi V, Schraven B (2000) Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed transmembrane adaptor protein, binds the protein tyrosine kinase csk and is involved in regulation of T cell activation. J Exp Med 191:1591–1604

Brdicka T, Imrich M, Angelisova P, Brdickova N, Horvath O, Spicka J, Hilgert I, Luskova P, Draber P, Novak P, Engels N, Wienands J, Simeoni L, Osterreicher J, Aguado E, Malissen M, Schraven B, Horejsi V (2002) Non-T cell activation linker (NTAL): a transmembrane adaptor protein involved in immunoreceptor signaling. J Exp Med 196:1617–1626

Brdicka T, Kadlecek TA, Roose JP, Pastuszak AW, Weiss A (2005) Intramolecular regulatory switch in ZAP-70: analogy with receptor tyrosine kinases. Mol Cell Biol 25:4924–4933

Brdickova N, Brdicka T, Andera L, Spicka J, Angelisova P, Milgram SL, Horejsi V (2001) Interaction between two adapter proteins, PAG and EBP50: a possible link between membrane rafts and actin cytoskeleton. FEBS Lett 507:133–136

Brownawell AM, Kops GJ, Macara IG, Burgering BM (2001) Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. Mol Cell Biol 21:3534–3546

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96:857–868

Brunet A, Park J, Tran H, Hu LS, Hemmings BA, Greenberg ME (2001) Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). Mol Cell Biol 21:952–965

Bruyns E, Marie-Cardine A, Kirchgessner H, Sagolla K, Shevchenko A, Mann M, Autschbach F, Bensussan A, Meuer S, Schraven B (1998) T cell receptor (TCR) interacting molecule (TRIM), a novel disulfide-linked dimer associated with the TCR-CD3-zeta complex, recruits intracellular signaling proteins to the plasma membrane. J Exp Med 188:561–575

Bubeck WJ, Pappu R, Bu JY, Mayer B, Chernoff J, Straus D, Chan AC (1998) Regulation of PAK activation and the T cell cytoskeleton by the linker protein SLP-76. Immunity 9:607–616

Burr JS, Savage ND, Messah GE, Kimzey SL, Shaw AS, Arch RH, Green JM (2001) Cutting edge: distinct motifs within CD28 regulate T cell proliferation and induction of Bcl-XL. J Immunol 166:5331–5335

Call ME, Wucherpfennig KW (2005) The T cell receptor: critical role of the membrane environment in receptor assembly and function. Annu Rev Immunol 23:101–125

Carlson CR, Lygren B, Berge T, Hoshi N, Wong W, Tasken K, Scott JD (2006) Delineation of type I protein kinase A-selective signaling events using an RI anchoring disruptor. J Biol Chem 281:21535–21545

Chen ZJ (2005) Ubiquitin signaling in the NF-kappaB pathway. Nat Cell Biol 7:758–765

Chan AC, Shaw AS (1996) Regulation of antigen receptor signal transduction by protein tyrosine kinases. Curr Opin Immunol 8:394–401

Chan B, Lanyi A, Song HK, Griesbach J, Simarro-Grande M, Poy F, Howie D, Sumegi J, Terhorst C, Eck MJ (2003) SAP couples Fyn to SLAM immune receptors. Nat Cell Biol 5:155–160
Chow LM, Fournel M, Davidson D, Veillette A (1993) Negative regulation of T-cell receptor signaling by tyrosine protein kinase p50csk. Nature 365:156–160
Chu DH, Morita CT, Weiss A (1998) The Syk family of protein tyrosine kinases in T-cell activation and development. Immunol Rev 165:167–180
Clements JL, Yang B, Ross-Barta SE, Eliason SL, Hrstka RF, Williamson RA, Koretzky GA (1998) Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. Science 281:416–419
Cloutier JF, Chow LM, Veillette A (1995) Requirement of the SH3 and SH2 domains for the inhibitory function of tyrosine protein kinase p50csk in T lymphocytes. Mol Cell Biol 15:5937–5944
Coffer PJ, Burgering BM (2004) Forkhead-box transcription factors and their role in the immune system. Nat Rev Immunol 4:889–899
Coffey AJ, Brooksbank RA, Brandau O, Oohashi T, Howell GR, Bye JM, Cahn AP, Durham J, Heath P, Wray P, Pavitt R, Wilkinson J, Leversha M, Huckle E, Shaw-Smith CJ, Dunham A, Rhodes S, Schuster V, Porta G, Yin L, Serafini P, Sylla B, Zollo M, Franco B, Bolino A, Seri M, Lanyi A, Davis JR, Webster D, Harris A, Lenoir G, de St BG, Jones A, Behloradsky BH, Achatz H, Murken J, Fassler R, Sumegi J, Romeo G, Vaudin M, Ross MT, Meindl A, Bentley DR (1998) Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. Nat Genet 20:129–135
Conti M, Jin SL (1999) The molecular biology of cyclic nucleotide phosphodiesterases. Prog Nucleic Acid Res Mol Biol 63:1–38
Costello PS, Walters AE, Mee PJ, Turner M, Reynolds LF, Prisco A, Sarner N, Zamoyska R, Tybulewicz VL (1999) The Rho-family GTP exchange factor Vav is a critical transducer of T cell receptor signals to the calcium, ERK, and NF-kappaB pathways. Proc Natl Acad Sci USA 96:3035–3040
Costello PS, Gallagher M, Cantrell DA (2002) Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse. Nat Immunol 3:1082–1089
Dahl AM, Klein C, Andres PG, London CA, Lodge MP, Mulligan RC, Abbas AK (2000) Expression of bcl-X(L) restores cell survival, but not proliferation off effector differentiation, in CD28-deficient T lymphocytes. J Exp Med 191:2031–2038
Davidson D, Bakinowski M, Thomas ML, Horejsi V, Veillette A (2003) Phosphorylation-dependent regulation of T-cell activation by PAG/Cbp, a lipid raft-associated transmembrane adaptor. Mol Cell Biol 23:2017–2028
Davidson D, Shi X, Zhang S, Wang H, Nemer M, Ono N, Ohno S, Yanagi Y, Veillette A (2004) Genetic evidence linking SAP, the X-linked lymphoproliferative gene product, to Src-related kinase FynT in T(H)2 cytokine regulation. Immunity 21:707–717
Davidson D, Schraven B, Veillette A (2007) PAG-associated FynT regulates calcium signaling and promotes anergy in T lymphocytes. Mol Cell Biol 27:1960–1973
Deindl S, Kadlecek TA, Brdicka T, Cao X, Weiss A, Kuriyan J (2007) Structural basis for the inhibition of tyrosine kinase activity of ZAP-70. Cell 129:735–746
de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396:474–477
Di B, V, Mege D, Germain V, Pelosi M, Dufour E, Michel F, Magistrelli G, Isacchi A, Acuto O (1999) Tyrosine 319, a newly identified phosphorylation site of ZAP-70, plays a critical role in T cell antigen receptor signaling. J Biol Chem 274:6285–6294
Dobenecker MW, Schmedt C, Okada M, Tarakhovsky A (2005) The ubiquitously expressed Csk adaptor protein Cbp is dispensable for embryogenesis and T-cell development and function. Mol Cell Biol 25:10533–10542
Dong S, Corre B, Foulon E, Dufour E, Veillette A, Acuto O, Michel F (2006) T cell receptor for antigen induces linker for activation of T cell-dependent activation of a negative signaling complex involving Dok-2, SHIP-1, and Grb-2. J Exp Med 203:2509–2518

Doody GM, Billadeau DD, Clayton E, Hutchings A, Berland R, McAdam S, Leibson PJ, Turner M (2000) Vav-2 controls NFAT-dependent transcription in B- but not T-lymphocytes. EMBO J 19:6173–6184

Dower NA, Stang SL, Bottorff DA, Ebinu JO, Dickie P, Ostergaard HL, Stone JC (2000) RasGRP is essential for mouse thymocyte differentiation and TCR signaling. Nat Immunol 1:317–321

Dunne A, O'Neill LA (2003) The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. Sci STKE 2003:re3

Dustin ML (2005) A dynamic view of the immunological synapse. Semin Immunol 17:400–410

Egawa T, Albrecht B, Favier B, Sunshine MJ, Mirchandani K, O'Brien W, Thome M, Littman DR (2003) Requirement for CARMA1 in antigen receptor-induced NF-kappa B activation and lymphocyte proliferation. Curr Biol 13:1252–1258

Fang N, Motto DG, Ross SE, Koretzky GA (1996) Tyrosines 113, 128, and 145 of SLP-76 are required for optimal augmentation of NFAT promoter activity. J Immunol 157:3769–3773

Faure S, Salazar-Fontana LI, Semichon M, Tybulewicz VL, Bismuth G, Trautmann A, Germain RN, Delon J (2004) ERM proteins regulate cytoskeleton relaxation promoting T cell-APC conjugation. Nat Immunol 5:272–279

Finco TS, Kadlecek T, Zhang W, Samelson LE, Weiss A (1998) LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. Immunity 9:617–626

Fong AM, Premont RT, Richardson RM, Yu YR, Lefkowitz RJ, Patel DD (2002) Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice. Proc Natl Acad Sci USA 99:7478–7483

Fujikawa K, Miletic AV, Alt FW, Faccio R, Brown T, Hoog J, Fredericks J, Nishi S, Mildiner S, Moores SL, Brugge J, Rosen FS, Swat W (2003) Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cells. J Exp Med 198:1595–1608

Gaide O, Favier B, Legler DF, Bonnet D, Brissoni B, Valitutti S, Bron C, Tschopp J, Thome M (2002) CARMA1 is a critical lipid raft-associated regulator of TCR-induced NF-kappa B activation. Nat Immunol 3:836–843

Ghosh S, Karin M (2002) Missing pieces in the NF-kappaB puzzle. Cell 109 (Suppl):S81–S96

Gold MG, Lygren B, Dokurno P, Hoshi N, McConnachie G, Tasken K, Carlson CR, Scott JD, Barford D (2006) Molecular basis of AKAP specificity for PKA regulatory subunits. Mol Cell 24:383–395

Gomez M, Kioussis D, Cantrell DA (2001) The GTPase Rac-1 controls cell fate in the thymus by diverting thymocytes from positive to negative selection. Immunity 15:703–713

Hanada T, Lin L, Chandy KG, Oh SS, Chishti AH (1997) Human homologue of the Drosophila discs large tumor suppressor binds to p56lck tyrosine kinase and Shaker type Kv1.3 potassium channel in T lymphocytes. J Biol Chem 272:26899–26904

Hara H, Wada T, Bakal C, Kozieradzki I, Suzuki S, Suzuki N, Nghiem M, Griffiths EK, Krawczyk C, Bauer B, D'Acquisto F, Ghosh S, Yeh WC, Baier G, Rottapel R, Penninger JM (2003) The MAGUK family protein CARD11 is essential for lymphocyte activation. Immunity 18:763–775

Harada Y, Tokushima M, Matsumoto Y, Ogawa S, Otsuka M, Hayashi K, Weiss BD, June CH, Abe R (2001) Critical requirement for the membrane-proximal cytosolic tyrosine residue for CD28-mediated costimulation in vivo. J Immunol 166:3797–3803

Harriague J, Bismuth G (2002) Imaging antigen-induced PI3K activation in T cells. Nat Immunol 3:1090–1096

Heiska L, Alfthan K, Gronholm M, Vilja P, Vaheri A, Carpen O (1998) Association of ezrin with intercellular adhesion molecule-1 and -2 (ICAM-1 and ICAM-2). Regulation by phosphatidylinositol 4, 5-bisphosphate. J Biol Chem 273:21893–21900

Horejsi V, Zhang W, Schraven B (2004) Transmembrane adaptor proteins: organizers of immunoreceptor signaling. Nat Rev Immunol 4:603–616

Houslay MD, Adams DR (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signaling cross-talk, desensitization and compartmentalization. Biochem J 370:1–18

Hubbard SR, Till JH (2000) Protein tyrosine kinase structure and function. Annu Rev Biochem 69:373–398

Hundt M, Tabata H, Jeon MS, Hayashi K, Tanaka Y, Krishna R, De GL, Liu YC, Fukata M, Altman A (2006) Impaired activation and localization of LAT in anergic T cells as a consequence of a selective palmitoylation defect. Immunity 24:513–522

Imamoto A, Soriano P (1993) Disruption of the csk gene, encoding a negative regulator of Src family tyrosine kinases, leads to neural tube defects and embryonic lethality in mice. Cell 73:1117–1124

Itoh K, Sakakibara M, Yamasaki S, Takeuchi A, Arase H, Miyazaki M, Nakajima N, Okada M, Saito T (2002) Cutting edge: negative regulation of immune synapse formation by anchoring lipid raft to cytoskeleton through Cbp-EBP50-ERM assembly. J Immunol 168:541–544

Janssen E, Zhu M, Zhang W, Koonpaew S, Zhang W (2003) LAB: a new membrane-associated adaptor molecule in B cell activation. Nat Immunol 4:117–123

Janssen E, Zhu M, Craven B, Zhang W (2004) Linker for activation of B cells: a functional equivalent of a mutant linker for activation of T cells deficient in phospholipase C-gamma1 binding. J Immunol 172:6810–6819

Johansson CC, Bryn T, Yndestad A, Eiken HG, Bjerkeli V, Froland SS, Aukrust P, Tasken K (2004) Cytokine networks are pre-activated in T cells from HIV-infected patients on HAART and are under the control of cAMP. AIDS 18:171–179

Jones RG, Parsons M, Bonnard M, Chan VS, Yeh WC, Woodgett JR, Ohashi PS (2000) Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo. J Exp Med 191:1721–1734

Kagan JC, Medzhitov R (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell 125:943–955

Kammer GM (1988) The adenylate cyclase-cAMP-protein kinase A pathway and regulation of the immune response. Immunol Today 9:222–229

Kane LP, Lin J, Weiss A (2000) Signal transduction by the TCR for antigen. Curr Opin Immunol 12:242–249

Karin M, Greten FR (2005) NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 5:749–759

Kaupp UB, Seifert R (2002) Cyclic nucleotide-gated ion channels. Physiol Rev 82:769–824

Kawabuchi M, Satomi Y, Takao T, Shimonishi Y, Nada S, Nagai K, Tarakhovsky A, Okada M (2000) Transmembrane phosphoprotein Cbp regulates the activities of Src-family tyrosine kinases. Nature 404:999–1003

Kawai T, Adachi O, Ogawa T, Takeda K, Akira S (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 11:115–122

Koonpaew S, Janssen E, Zhu M, Zhang W (2004) The importance of three membrane-distal tyrosines in the adaptor protein NTAL/LAB. J Biol Chem 279:11229–11235

Koonpaew S, Shen S, Flowers L, Zhang W (2006) LAT-mediated signaling in CD4+CD25+ regulatory T cell development. J Exp Med 203:119–129

Koretzky GA, Abtahian F, Silverman MA (2006) SLP76 and SLP65: complex regulation of signaling in lymphocytes and beyond. Nat Rev Immunol 6:67–78

Kruse M, Meinl E, Henning G, Kuhnt C, Berchtold S, Berger T, Schuler G, Steinkasserer A (2001) Signaling lymphocytic activation molecule is expressed on mature CD83+ dendritic cells and is up-regulated by IL-1 beta. J Immunol 167:1989–1995

Kuo CT, Leiden JM (1999) Transcriptional regulation of T lymphocyte development and function. Annu Rev Immunol 17:149–187

Kvale D, Ormaasen V, Kran AM, Johansson CC, Aukrust P, Aandahl EM, Froland SS, Tasken K (2006) Immune modulatory effects of cyclooxygenase type 2 inhibitors in HIV patients on combination antiretroviral treatment. AIDS 20:813–820

Latour S, Veillette A (2004) The SAP family of adaptors in immune regulation. Semin Immunol 16:409–419

Latour S, Gish G, Helgason CD, Humphries RK, Pawson T, Veillette A (2001) Regulation of SLAM-mediated signal transduction by SAP, the X-linked lymphoproliferative gene product. Nat Immunol 2:681–690

Latour S, Roncagalli R, Chen R, Bakinowski M, Shi X, Schwartzberg PL, Davidson D, Veillette A (2003) Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signaling in immune regulation. Nat Cell Biol 5:149–154

Layer K, Lin G, Nencioni A, Hu W, Schmucker A, Antov AN, Li X, Takamatsu S, Chevassut T, Dower NA, Stang SL, Beier D, Buhlmann J, Bronson RT, Elkon KB, Stone JC, Van PL, Lim B (2003) Autoimmunity as the consequence of a spontaneous mutation in Rasgrp1. Immunity 19:243–255

Ledbetter JA, Parsons M, Martin PJ, Hansen JA, Rabinovitch PS, June CH (1986) Antibody binding to CD5 (Tp67) and Tp44 T cell surface molecules: effects on cyclic nucleotides, cytoplasmic free calcium, and cAMP-mediated suppression. J Immunol 137:3299–3305

Lemay S, Davidson D, Latour S, Veillette A (2000) Dok-3, a novel adapter molecule involved in the negative regulation of immunoreceptor signaling. Mol Cell Biol 20:2743–2754

Levy FO, Rasmussen AM, Tasken K, Skalhegg BS, Huitfeldt HS, Funderud S, Smeland EB, Hansson V (1996) Cyclic AMP-dependent protein kinase (cAK) in human B cells: co-localization of type I cAK (RI alpha 2 C2) with the antigen receptor during anti-immunoglobulin-induced B cell activation. Eur J Immunol 26:1290–1296

Lin L, Hron JD, Peng SL (2004) Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. Immunity 21:203–213

Liu Q, Berry D, Nash P, Pawson T, McGlade CJ, Li SS (2003) Structural basis for specific binding of the Gads SH3 domain to an RxxK motif-containing SLP-76 peptide: a novel mode of peptide recognition. Mol Cell 11:471–481

Lucas PC, Yonezumi M, Inohara N, lister-Lucas LM, Abazeed ME, Chen FF, Yamaoka S, Seto M, Nunez G (2001) Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-kappa B signaling pathway. J Biol Chem 276:19012–19019

Ma CS, Nichols KE, Tangye SG (2007) Regulation of cellular and humoral immune responses by the SLAM and SAP families of molecules. Annu Rev Immunol 25:337–379

Marie-Cardine A, Kirchgessner H, Schraven B (1999) Molecular alterations of the Fyn-complex occur as late events of human T cell activation. Eur J Immunol 29:1175–1187

Matsumoto R, Wang D, Blonska M, Li H, Kobayashi M, Pappu B, Chen Y, Wang D, Lin X (2005) Phosphorylation of CARMA1 plays a critical role in T Cell receptor-mediated NF-kappaB activation. Immunity 23:575–585

Michel JJ, Scott JD (2002) AKAP mediated signal transduction. Annu Rev Pharmacol Toxicol 42:235–257

Minguet S, Swamy M, Alarcon B, Luescher IF, Schamel WW (2007) Full activation of the T cell receptor requires both clustering and conformational changes at CD3. Immunity 26:43–54

Montixi C, Langlet C, Bernard AM, Thimonier J, Dubois C, Wurbel MA, Chauvin JP, Pierres M, He HT (1998) Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. EMBO J 17:5334–5348

Mueller DL (2004) E3 ubiquitin ligases as T cell anergy factors. Nat Immunol 5:883–890

Mutch CM, Sanyal R, Unruh TL, Grigoriou L, Zhu M, Zhang W, Deans JP (2007) Activation-induced endocytosis of the raft-associated transmembrane adaptor protein LAB/NTAL in B lymphocytes: evidence for a role in internalization of the B cell receptor. Int Immunol 19:19–30

Nada S, Yagi T, Takeda H, Tokunaga T, Nakagawa H, Ikawa Y, Okada M, Aizawa S (1993) Constitutive activation of Src family kinases in mouse embryos that lack Csk. Cell 73:1125–1135

Nakamoto T, Sakai R, Ozawa K, Yazaki Y, Hirai H (1996) Direct binding of C-terminal region of p130Cas to SH2 and SH3 domains of Src kinase. J Biol Chem 271:8959–8965

Negishi I, Motoyama N, Nakayama K, Nakayama K, Senju S, Hatakeyama S, Zhang Q, Chan AC, Loh DY (1995) Essential role for ZAP-70 in both positive and negative selection of thymocytes. Nature 376:435–438

Nichols KE, Harkin DP, Levitz S, Krainer M, Kolquist KA, Genovese C, Bernard A, Ferguson M, Zuo L, Snyder E, Buckler AJ, Wise C, Ashley J, Lovett M, Valentine MB, Look AT, Gerald W, Housman DE, Haber DA (1998) Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. Proc Natl Acad Sci U S A 95:13765–13770

Nunez-Cruz S, Aguado E, Richelme S, Chetaille B, Mura AM, Richelme M, Pouyet L, Jouvin-Marche E, Xerri L, Malissen B, Malissen M (2003) LAT regulates gammadelta T cell homeostasis and differentiation. Nat Immunol 4:999–1008
Oh P, Schnitzer JE (2001) Segregation of heterotrimeric G proteins in cell surface microdomains. G(q) binds caveolin to concentrate in caveolae, whereas G(i) and G(s) target lipid rafts by default. Mol Biol Cell 12:685–698
Okada M, Nada S, Yamanashi Y, Yamamoto T, Nakagawa H (1991) CSK: a protein-tyrosine kinase involved in regulation of src family kinases. J Biol Chem 266:24249–24252
Okkenhaug K, Vanhaesebroeck B (2003) PI3K in lymphocyte development, differentiation and activation. Nat Rev Immunol 3:317–330
Okkenhaug K, Wu L, Garza KM, La RJ, Khoo W, Odermatt B, Mak TW, Ohashi PS, Rottapel R (2001) A point mutation in CD28 distinguishes proliferative signals from survival signals. Nat Immunol 2:325–332
Park YC, Burkitt V, Villa AR, Tong L, Wu H (1999) Structural basis for self-association and receptor recognition of human TRAF2. Nature 398:533–538
Pelosi M, Di B, V, Mounier V, Mege D, Pascussi JM, Dufour E, Blondel A, Acuto O (1999) Tyrosine 319 in the interdomain B of ZAP-70 is a binding site for the Src homology 2 domain of Lck. J Biol Chem 274:14229–14237
Perry SJ, Baillie GS, Kohout TA, McPhee I, Magiera MM, Ang KL, Miller WE, McLean AJ, Conti M, Houslay MD, Lefkowitz RJ (2002) Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. Science 298:834–836
Pfrepper KI, Marie-Cardine A, Simeoni L, Kuramitsu Y, Leo A, Spicka J, Hilgert I, Scherer J, Schraven B (2001) Structural and functional dissection of the cytoplasmic domain of the transmembrane adaptor protein SIT (SHP2-interacting transmembrane adaptor protein). Eur J Immunol 31:1825–1836
Pivniouk V, Tsitsikov E, Swinton P, Rathbun G, Alt FW, Geha RS (1998) Impaired viability and profound block in thymocyte development in mice lacking the adaptor protein SLP-76. Cell 94:229–238
Plas DR, Thompson CB (2003) Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. J Biol Chem 278:12361–12366
Pomerantz JL, Denny EM, Baltimore D (2002) CARD11 mediates factor-specific activation of NF-kappaB by the T cell receptor complex. EMBO J 21:5184–5194
Priatel JJ, Teh SJ, Dower NA, Stone JC, Teh HS (2002) RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. Immunity 17:617–627
Raab M, da Silva AJ, Findell PR, Rudd CE (1997) Regulation of Vav-SLP-76 binding by ZAP-70 and its relevance to TCR zeta/CD3 induction of interleukin-2. Immunity 6:155–164
Rahmouni S, Aandahl EM, Trebak M, Boniver J, Tasken K, Moutschen M (2001) Increased cAMP levels and protein kinase (PKA) type I activation in CD4+ T cells and B cells contribute to retrovirus-induced immunodeficiency of mice (MAIDS): a useful in vivo model for drug testing. FASEB J 15:1466–1468
Rahmouni S, Aandahl EM, Nayjib B, Zeddou M, Giannini S, Verlaet M, Greimers R, Boniver J, Tasken K, Moutschen M (2004) Cyclo-oxygenase type 2-dependent prostaglandin E2 secretion is involved in retrovirus-induced T-cell dysfunction in mice. Biochem J 384:469–476
Rameh LE, Cantley LC (1999) The role of phosphoinositide 3-kinase lipid products in cell function. J Biol Chem 274:8347–8350
Rawlings DJ, Sommer K, Moreno-Garcia ME (2006) The CARMA1 signalosome links the signaling machinery of adaptive and innate immunity in lymphocytes. Nat Rev Immunol 6:799–812
Rebeaud F, Hailfinger S, Thome M (2007) Dlgh1 and Carma1 MAGUK proteins contribute to signal specificity downstream of TCR activation. Trends Immunol 28:196–200
Resh MD (2006) Palmitoylation of ligands, receptors, and intracellular signaling molecules. Sci STKE 2006:re14

Reth M (1989) Antigen receptor tail clue. Nature 338:383–384
Rossman JS, Stoicheva NG, Langel FD, Patterson GH, Lippincott-Schwartz J, Schaefer BC (2006) POLKADOTS are foci of functional interactions in T-Cell receptor-mediated signaling to NF-kappaB. Mol Biol Cell 17:2166–2176
Round JL, Tomassian T, Zhang M, Patel V, Schoenberger SP, Miceli MC (2005) Dlgh1 coordinates actin polymerization, synaptic T cell receptor and lipid raft aggregation, and effector function in T cells. J Exp Med 201:419–430
Round JL, Humphries LA, Tomassian T, Mittelstadt P, Zhang M, Miceli MC (2007) Scaffold protein Dlgh1 coordinates alternative p38 kinase activation, directing T cell receptor signals toward NFAT but not NF-kappaB transcription factors. Nat Immunol 8:154–161
Rowe DC, McGettrick AF, Latz E, Monks BG, Gay NJ, Yamamoto M, Akira S, O'Neill LA, Fitzgerald KA, Golenbock DT (2006) The myristoylation of TRIF-related adaptor molecule is essential for Toll-like receptor 4 signal transduction. Proc Natl Acad Sci USA 103:6299–6304
Ruefli-Brasse AA, French DM, Dixit VM (2003) Regulation of NF-kappaB-dependent lymphocyte activation and development by paracaspase. Science 302:1581–1584
Ruland J, Duncan GS, Elia A, del BB, I, Nguyen L, Plyte S, Millar DG, Bouchard D, Wakeham A, Ohashi PS, Mak TW (2001) Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. Cell 104:33–42
Ruland J, Duncan GS, Wakeham A, Mak TW (2003) Differential requirement for Malt1 in T and B cell antigen receptor signaling. Immunity 19:749–758
Ruppelt A, Mosenden R, Grönholm M, Aandahl EM, Tobin D, Carlson CR, Abrahamsen H, Herberg FW, Carpén O, Tasken K (2007) Inhibition of T cell activation by cAMP requires lipid raft targeting of protein kinase A type I by the A-kinase anchoring protein Ezrin. J Immunol 179:5159–5168
Sabe H, Hata A, Okada M, Nakagawa H, Hanafusa H (1994) Analysis of the binding of the Src homology 2 domain of Csk to tyrosine-phosphorylated proteins in the suppression and mitotic activation of c-Src. Proc Natl Acad Sci USA 91:3984–3988
Sakaguchi N, Takahashi T, Hata H, Nomura T, Tagami T, Yamazaki S, Sakihama T, Matsutani T, Negishi I, Nakatsuru S, Sakaguchi S (2003) Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. Nature 426:454–460
Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S, Fehervari Z, Shimizu J, Takahashi T, Nomura T (2006a) Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol Rev 212:8–27
Sakaguchi S, Sakaguchi N, Yoshitomi H, Hata H, Takahashi T, Nomura T (2006b) Spontaneous development of autoimmune arthritis due to genetic anomaly of T cell signal transduction: Part 1. Semin Immunol 18:199–206
Salvador JM, Mittelstadt PR, Guszczynski T, Copeland TD, Yamaguchi H, Appella E, Fornace AJ Jr, Ashwell JD (2005) Alternative p38 activation pathway mediated by T cell receptor-proximal tyrosine kinases. Nat Immunol 6:390–395
Sayos J, Wu C, Morra M, Wang N, Zhang X, Allen D, van SS, Notarangelo L, Geha R, Roncarolo MG, Oettgen H, De Vries JE, Aversa G, Terhorst C (1998) The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. Nature 395:462–469
Schaefer BC, Kappler JW, Kupfer A, Marrack P (2004) Complex and dynamic redistribution of NF-kappaB signaling intermediates in response to T cell receptor stimulation. Proc Natl Acad Sci USA 101:1004–1009
Schulze-Luehrmann J, Ghosh S (2006) Antigen-receptor signaling to nuclear factor kappa B. Immunity 25:701–715
Schwartzberg PL, Finkelstein LD, Readinger JA (2005) TEC-family kinases: regulators of T-helper-cell differentiation. Nat Rev Immunol 5:284–295
Sharpe AH, Freeman GJ (2002) The B7-CD28 superfamily. Nat Rev Immunol 2:116–126
Shima T, Nada S, Okada M (2003) Transmembrane phosphoprotein Cbp senses cell adhesion signaling mediated by Src family kinase in lipid rafts. Proc Natl Acad Sci USA 100: 14897–14902

Sicheri F, Kuriyan J (1997) Structures of Src-family tyrosine kinases. Curr Opin Struct Biol 7:777–785
Simeoni L, Smida M, Posevitz V, Schraven B, Lindquist JA (2005) Right time, right place: the organization of membrane proximal signaling. Semin Immunol 17:35–49
Singer AL, Bunnell SC, Obstfeld AE, Jordan MS, Wu JN, Myung PS, Samelson LE, Koretzky GA (2004) Roles of the proline-rich domain in SLP-76 subcellular localization and T cell function. J Biol Chem 279:15481–15490
Skalhegg BS, Landmark BF, Doskeland SO, Hansson V, Lea T, Jahnsen T (1992) Cyclic AMP-dependent protein kinase type I mediates the inhibitory effects of 3′,5′-cyclic adenosine monophosphate on cell replication in human T lymphocytes. J Biol Chem 267:15707–15714
Skalhegg BS, Tasken K, Hansson V, Huitfeldt HS, Jahnsen T, Lea T (1994) Location of cAMP-dependent protein kinase type I with the TCR-CD3 complex. Science 263:84–87
Smith FD, Scott JD (2002) Signaling complexes: junctions on the intracellular information super highway. Curr Biol 12:R32–R40
Solheim SA, Torgersen KM, Taskén K, Berge T (2008) Regulation of FynT function by dual-domain docking on Cbp/PAG. J Biol Chem 283:2773–2783.
Sommer K, Guo B, Pomerantz JL, Bandaranayake AD, Moreno-Garcia ME, Ovechkina YL, Rawlings DJ (2005) Phosphorylation of the CARMA1 linker controls NF-kappaB activation. Immunity 23:561–574
Sommers CL, Menon RK, Grinberg A, Zhang W, Samelson LE, Love PE (2001) Knock-in mutation of the distal four tyrosines of linker for activation of T cells blocks murine T cell development. J Exp Med 194:135–142
Sommers CL, Park CS, Lee J, Feng C, Fuller CL, Grinberg A, Hildebrand JA, Lacana E, Menon RK, Shores EW, Samelson LE, Love PE (2002) A LAT mutation that inhibits T cell development yet induces lymphoproliferation. Science 296:2040–2043
Stokka AJ, Gesellchen F, Carlson CR, Scott JD, Herberg FW, Tasken K (2006) Characterization of A-kinase-anchoring disruptors using a solution-based assay. Biochem J 400:493–499
Su YW, Zhang Y, Schweikert J, Koretzky GA, Reth M, Wienands J (1999) Interaction of SLP adaptors with the SH2 domain of Tec family kinases. Eur J Immunol 29:3702–3711
Sun L, Deng L, Ea CK, Xia ZP, Chen ZJ (2004) The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. Mol Cell 14:289–301
Takeuchi S, Takayama Y, Ogawa A, Tamura K, Okada M (2000) Transmembrane phosphoprotein Cbp positively regulates the activity of the carboxyl-terminal Src kinase, Csk. J Biol Chem 275:29183–29186
Tasken K, Aandahl EM (2004) Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol Rev 84:137–167
Tatsuo H, Ono N, Tanaka K, Yanagi Y (2000) SLAM (CDw150) is a cellular receptor for measles virus. Nature 406:893–897
Thomas ML, Brown EJ (1999) Positive and negative regulation of Src-family membrane kinases by CD45. Immunol Today 20:406–411
Thome M (2004) CARMA1, BCL-10 and MALT1 in lymphocyte development and activation. Nat Rev Immunol 4:348–359
Torgersen KM, Vaage JT, Levy FO, Hansson V, Rolstad B, Tasken K (1997) Selective activation of cAMP-dependent protein kinase type I inhibits rat natural killer cell cytotoxicity. J Biol Chem 272:5495–5500
Torgersen KM, Vang T, Abrahamsen H, Yaqub S, Horejsi V, Schraven B, Rolstad B, Mustelin T, Tasken K (2001) Release from tonic inhibition of T cell activation through transient displacement of C-terminal Src kinase (Csk) from lipid rafts. J Biol Chem 276:29313–29318
Torgersen KM, Vang T, Abrahamsen H, Yaqub S, Tasken K (2002) Molecular mechanisms for protein kinase A-mediated modulation of immune function. Cell Signal 14:1–9
Tsukita S, Oishi K, Sato N, Sagara J, Kawai A, Tsukita S (1994) ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J Cell Biol 126:391–401

Tuosto L, Michel F, Acuto O (1996) p95vav Associates with tyrosine-phosphorylated SLP-76 in antigen-stimulated T cells. J Exp Med 184:1161–1166
Turner M, Billadeau DD (2002) VAV proteins as signal integrators for multi-subunit immune-recognition receptors. Nat Rev Immunol 2:476–486
Turner M, Mee PJ, Walters AE, Quinn ME, Mellor AL, Zamoyska R, Tybulewicz VL (1997) A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. Immunity 7:451–460
Tybulewicz VL (2005) Vav-family proteins in T-cell signaling. Curr Opin Immunol 17:267–274
Underhill DM, Goodridge HS (2007) The many faces of ITAMs. Trends Immunol 28:66–73
Uren AG, O'Rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV, Dixit VM (2000) Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol Cell 6:961–967
Vang T, Torgersen KM, Sundvold V, Saxena M, Levy FO, Skalhegg BS, Hansson V, Mustelin T, Tasken K (2001) Activation of the COOH-terminal Src kinase (Csk) by cAMP-dependent protein kinase inhibits signaling through the T cell receptor. J Exp Med 193:497–507
Vang T, Abrahamsen H, Myklebust S, Horejsi V, Tasken K (2003) Combined spatial and enzymatic regulation of Csk by cAMP and protein kinase a inhibits T cell receptor signaling. J Biol Chem 278:17597–17600
Vanhaesebroeck B, Alessi DR (2000) The PI3K-PDK1 connection: more than just a road to PKB. Biochem J 346 (Pt 3):561–576
Veillette A (2006) Immune regulation by SLAM family receptors and SAP-related adaptors. Nat Rev Immunol 6:56–66
Veillette A, Latour S, Davidson D (2002) Negative regulation of immunoreceptor signaling. Annu Rev Immunol 20:669–707
Walker JK, Fong AM, Lawson BL, Savov JD, Patel DD, Schwartz DA, Lefkowitz RJ (2003) Beta-arrestin-2 regulates the development of allergic asthma. J Clin Invest 112:566–574
Wang N, Satoskar A, Faubion W, Howie D, Okamoto S, Feske S, Gullo C, Clarke K, Sosa MR, Sharpe AH, Terhorst C (2004) The cell surface receptor SLAM controls T cell and macrophage functions. J Exp Med 199:1255–1264
Wang Y, Horvath O, Hamm-Baarke A, Richelme M, Gregoire C, Guinamard R, Horejsi V, Angelisova P, Spicka J, Schraven B, Malissen B, Malissen M (2005) Single and combined deletions of the NTAL/LAB and LAT adaptors minimally affect B-cell development and function. Mol Cell Biol 25:4455–4465
Ward SG, Cantrell DA (2001) Phosphoinositide 3-kinases in T lymphocyte activation. Curr Opin Immunol 13:332–338
Ward SG, Westwick J, Hall ND, Sansom DM (1993) Ligation of CD28 receptor by B7 induces formation of D-3 phosphoinositides in T lymphocytes independently of T cell receptor/CD3 activation. Eur J Immunol 23:2572–2577
Weber JR, Orstavik S, Torgersen KM, Danbolt NC, Berg SF, Ryan JC, Tasken K, Imboden JB, Vaage JT (1998) Molecular cloning of the cDNA encoding pp36, a tyrosine-phosphorylated adaptor protein selectively expressed by T cells and natural killer cells. J Exp Med 187:1157–1161
West AP, Koblansky AA, Ghosh S (2006) Recognition and signaling by toll-like receptors. Annu Rev Cell Dev Biol 22:409–437
Wiest DL, Ashe JM, Howcroft TK, Lee HM, Kemper DM, Negishi I, Singer DS, Singer A, Abe R (1997) A spontaneously arising mutation in the DLAARN motif of murine ZAP-70 abrogates kinase activity and arrests thymocyte development. Immunity 6:663–671
Willis TG, Jadayel DM, Du MQ, Peng H, Perry AR, bdul-Rauf M, Price H, Karran L, Majekodunmi O, Wlodarska I, Pan L, Crook T, Hamoudi R, Isaacson PG, Dyer MJ (1999) Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types. Cell 96:35–45
Wu J, Motto DG, Koretzky GA, Weiss A (1996) Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. Immunity 4:593–602

Wunderlich L, Farago A, Downward J, Buday L (1999) Association of Nck with tyrosine-phosphorylated SLP-76 in activated T lymphocytes. Eur J Immunol 29:1068–1075
Xavier R, Brennan T, Li Q, McCormack C, Seed B (1998) Membrane compartmentation is required for efficient T cell activation. Immunity 8:723–732
Xavier R, Rabizadeh S, Ishiguro K, Andre N, Ortiz JB, Wachtel H, Morris DG, Lopez-Ilasaca M, Shaw AC, Swat W, Seed B (2004) Discs large (Dlg1) complexes in lymphocyte activation. J Cell Biol 166:173–178
Xu S, Huo J, Tan JE, Lam KP (2005) Cbp deficiency alters Csk localization in lipid rafts but does not affect T-cell development. Mol Cell Biol 25:8486–8495
Yablonski D, Kuhne MR, Kadlecek T, Weiss A (1998). Uncoupling of nonreceptor tyrosine kinases from PLC-gamma1 in an SLP-76-deficient T cell. Science 281:413–416
Yablonski D, Kadlecek T, Weiss A (2001) Identification of a phospholipase C-gamma1 (PLC-gamma1) SH3 domain-binding site in SLP-76 required for T-cell receptor-mediated activation of PLC-gamma1 and NFAT. Mol Cell Biol 21:4208–4218
Yamasaki S, Nishida K, Sakuma M, Berry D, McGlade CJ, Hirano T, Saito T (2003) Gads/Grb2-mediated association with LAT is critical for the inhibitory function of Gab2 in T cells. Mol Cell Biol 23:2515–2529
Yasuda K, Nagafuku M, Shima T, Okada M, Yagi T, Yamada T, Minaki Y, Kato A, Tani-Ichi S, Hamaoka T, Kosugi A (2002) Cutting edge: Fyn is essential for tyrosine phosphorylation of Csk-binding protein/phosphoprotein associated with glycolipid-enriched microdomains in lipid rafts in resting T cells. J Immunol 169:2813–2817
Yasuda T, Bundo K, Hino A, Honda K, Inoue A, Shirakata M, Osawa M, Tamura T, Nariuchi H, Oda H, Yamamoto T, Yamanashi Y (2007) Dok-1 and Dok-2 are negative regulators of T cell receptor signaling. Int Immunol 19:487–495
Yonemura S, Hirao M, Doi Y, Takahashi N, Kondo T, Tsukita S, Tsukita S (1998) Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. J Cell Biol 140:885–895
Zaccolo M, Pozzan T (2002) Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. Science 295:1711–1715
Zhang W, Sloan-Lancaster J, Kitchen J, Trible RP, Samelson LE (1998a) LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell 92:83–92
Zhang W, Trible RP, Samelson LE (1998b) LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. Immunity 9:239–246
Zhang W, Irvin BJ, Trible RP, Abraham RT, Samelson LE (1999a) Functional analysis of LAT in TCR-mediated signaling pathways using a LAT-deficient Jurkat cell line. Int Immunol 11:943–950
Zhang W, Sommers CL, Burshtyn DN, Stebbins CC, DeJarnette JB, Trible RP, Grinberg A, Tsay HC, Jacobs HM, Kessler CM, Long EO, Love PE, Samelson LE (1999b) Essential role of LAT in T cell development. Immunity 10:323–332
Zhang W, Trible RP, Zhu M, Liu SK, McGlade CJ, Samelson LE (2000) Association of Grb2, Gads, and phospholipase C-gamma 1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T cell angigen receptor-mediated signaling. J Biol Chem 275:23355–23361
Zhu M, Liu Y, Koonpaew S, Granillo O, Zhang W (2004) Positive and negative regulation of FcepsilonRI-mediated signaling by the adaptor protein LAB/NTAL. J Exp Med 200:991–1000
Zhu M, Koonpaew S, Liu Y, Shen S, Denning T, Dzhagalov I, Rhee I, Zhang W (2006) Negative regulation of T cell activation and autoimmunity by the transmembrane adaptor protein LAB. Immunity 25:757–768
Zipfel PA, Zhang W, Quiroz M, Pendergast AM (2004) Requirement for Abl kinases in T cell receptor signaling. Curr Biol 14:1222–1231

Scaffolding Proteins at the Postsynaptic Density: Shank as the Architectural Framework

H.-J. Kreienkamp

Contents

1 Scaffold Proteins of the Postsynaptic Density 366
2 Domain Structure of the Shank Proteins 367
3 Physiological Role of Shank Proteins 368
4 Molecular Interactions of Shank Scaffolds: Self Association 368
5 Molecular Interactions of Shank Scaffolds: Anchoring of Postsynaptic Membrane Proteins. 370
6 Molecular Interactions of Shank Scaffolds: Linkages to the Actin-Based Cytoskeleton 372
7 Molecular Interactions of Shank Scaffolds: Signaling Proteins. 373
8 Dynamic Regulation of PSD Scaffold Proteins at the Synapse 375
References. 376

Abstract Shank proteins are multidomain scaffold proteins of the postsynaptic density, connecting neurotransmitter receptors and other membrane proteins with signaling proteins and the actin cytoskeleton. By virtue of their protein interactions, Shank proteins assemble signaling platforms for G-protein-mediated signaling and the control of calcium homeostasis in dendritic spines. In addition, they participate in morphological changes, leading to maturation of dendritic spines and synapse formation. The importance of the Shank scaffolding function is demonstrated by genetically determined forms of mental retardation, which may be caused by haploinsufficiency for the *SHANK3* gene. Consistent with its central function within the postsynaptic density, the availability of Shank is tightly controlled by local synthesis and degradation, as well as actin-dependent dynamic rearrangements within the dendritic spine.

H.-J. Kreienkamp
Institut für Humangenetik, Universitätsklinikum Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg Germany
kreienkamp@uke.uni-hamburg.de

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.* Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

1 Scaffold Proteins of the Postsynaptic Density

In the postsynaptic density (PSD) of excitatory (usually glutamatergic) synapses of the central nervous system, cell surface transmitter receptors are physically connected with components of their signal transduction machinery and the actin cytoskeleton. The PSD is usually located at the tip of dendritic protrusions of about 1–2 μm length, termed dendritic spines, separated from the presynaptic transmitter-containing terminal by the synaptic cleft (Fig. 1).

One of the hallmarks of the PSD is its size; hundred(s) of different proteins contribute to a large insoluble complex that accumulates a molecular weight of about 1.5 GDa (Chen et al. 2005). Thus, there is a strong need for scaffolds to keep this assembly together through various protein interaction motifs. Some of the scaffold proteins of the PSD, such as A-kinase anchoring proteins (AKAPs) or S-Scam/MAGI-proteins, are important in other large protein complexes; others, such as the SAP/PSD-95 family members, SAPAP/GKAPs, Homer and Shank proteins, are rather specific to the PSD of excitatory synapses. Here I will focus on the role of SH3- and ankyrin-containing proteins (Shank1-3; Naisbitt et al. 1999, also known as somatostatin receptor interacting protein, SSTRIP; Zitzer et al. 1999a; proline-rich synapse-associated protein, ProSAP;

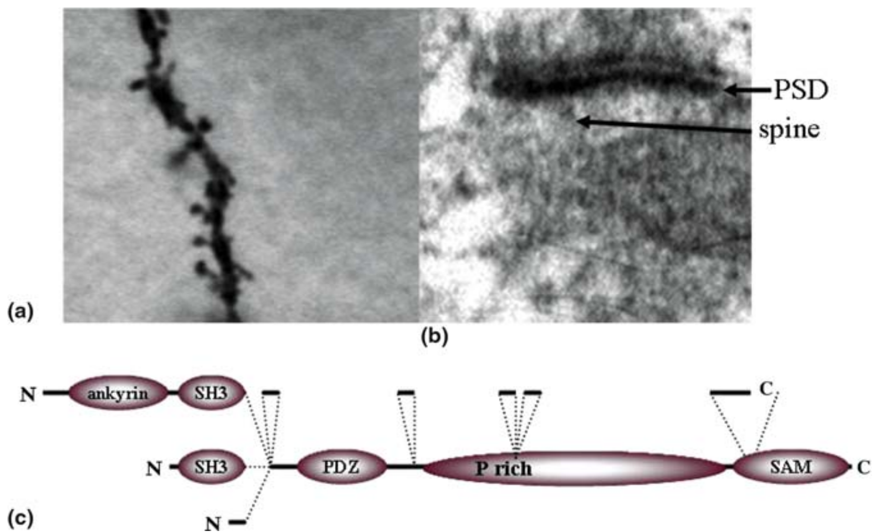

Fig. 1 **(a)** Section of a dendrite from the mouse cerebral cortex, stained by the Golgi technique. Numerous dendritic spines are clearly visible. **(b)** Electron micrograph of a section from the mouse hippocampus. The PSD is visible as a *black* (i.e., electron dense) *structure* at the top of the spine. **(c)** Domain structure and possible splice variants of Shank proteins. Note that not all alternatively spliced inserts have been described for all three Shank genes

Boeckers et al. 1999a; synamon, Yao et al. 1999; cortactin binding protein, CortBP1; Du et al. 1998) in the maturation, function and dynamics of postsynaptic specializations.

2 Domain Structure of the Shank Proteins

Three genes coding for Shank isoforms have been discovered in various mammalian species; all three forms, labeled Shank1-3, are expressed strongly in the central nervous system. In addition, individual isoforms are expressed at lower levels in peripheral tissues (Lim et al. 1999). All three Shank forms are characterized by a common series of protein interaction motifs or domains, i.e., a set of ankyrin repeats, followed by SH3- and PDZ-domains, a rather long proline-rich region and a C-terminal SAM domain (Fig. 1). The calculated molecular weights range from 130 to 230 kDa; in Western blot experiments, these forms migrate at apparent molecular weights ranging from 180 kDa to more than 250 kDa. This difference is probably due to a large proportion of unfolded protein regions within the 1,000-aa-long proline-rich region.

Western blot analysis also indicates the presence of multiple molecular forms of Shank proteins in which individual parts of the domain structure may be missing due to alternative splicing and alternative promoter usage (Lim et al. 1999; Boeckers et al. 1999a; see Fig. 1). Thus, the major form of Shank2 expressed in the brain does not contain the ankyrin and SH3 domains; the term “Shank” therefore does not really apply to this protein, and the alternative ProSAP makes indeed more sense. This form occurs due to a transcriptional start site within the long intron separating the genomic segments coding for SH3 and PDZ domains. A “full-length” form of Shank2 containing SH3 and ankyrin regions is expressed mostly in epithelial cells and has been labeled Shank2E (McWilliams et al. 2004).

In neurons, Shank proteins have been localized almost exclusively to the postsynaptic specialization of excitatory synapses. Biochemical preparations indicate a very strong enrichment in the PSD, and Shank immunoreactivity is in fact one of the most reliable markers for the PSD (Boeckers 1999a; Naisbitt et al. 1999; Zitzer et al. 1999a). Quantitative analyses have led to an estimate of about 100 Shank molecules per mature PSD complex (Chen et al. 2005; Sugiyama et al. 2005). A detailed analysis by immunogold electron microscopy indicated that the majority of Shank is located deep within the PSD, at a position remote from the synaptic plasma membrane. In contrast, glutamate receptors and their directly associated scaffold proteins from the SAP/PSD-95 family are located at the surface of the PSD, in direct contact with the plasma membrane (Valtschanoff and Weinberg 2001). Shank proteins are therefore not considered to provide a direct scaffolding function for transmitter receptors, but rather work indirectly by connecting different types of scaffold/receptor complexes, leading to the concept of a “master” or “higher order” scaffold. This view is strongly supported by the different protein interactions in which Shank proteins are involved, as detailed below.

3 Physiological Role of Shank Proteins

The physiological relevance of Shank proteins is most likely directly related to its role as a scaffold protein in the PSD. Initial overexpression experiments by Sala et al. (2001) demonstrated that increased levels of Shank lead to earlier maturation of the postsynapse and an increased size of dendritic spines, both through the enhanced recruitment of associated proteins (most notably Homer) and structures (Homer/IP3-receptor-associated ER membranes) to the postsynaptic site. Shank over-expression is sufficient to induce the generation of spines in otherwise spineless neurons, such as the cerebellar granule cells (Roussignol et al. 2005). Mice deficient in the Shank1 gene have been generated and are viable (Hung et al. 2005), but a detailed analysis of these mice remains to be published.

So far, it is unclear whether individual Shank family members fulfill unique physiological functions. The structural similarity between Shank isoforms led to the observation that many (but not all) interaction partners of Shank proteins at the synapse, such as GKAP/SAPAP or Homer, are equally recognized by all three family members (Boeckers et al. 1999b; Naisbitt et al. 1999). The expression patterns of the three genes in different brain regions, as determined by in situ hybridization experiments, are only partially overlapping. Thus, the thalamus is characterized by high levels of Shank3 transcripts, whereas Shank1 and Shank2 are only weakly expressed. Within the cerebellum, expression of Shank3 is restricted to granule cells, while Shank1 and Shank2 are found only in the Purkinje cell layer. Principal neurons of cortex and hippocampus are likely to coexpress all three Shank genes (Boeckers et al. 2004; Lim et al. 1999).

The relatively strong effect of mutations in the human *SHANK3* gene argues for a specific functional role of this protein, as similar disease-associated mutations in the other *SHANK* genes have not been detected so far. The *SHANK3* gene is located on the distal arm of chromosome 22q13.3 and may be lost due to chromosomal translocations (Bonaglia et al. 2001) or deletions (in the 22q13.3 microdeletion syndrome, a disorder that affects about 1 in 200,000 people). In addition, point mutations leading to frame shifts or alterations in the protein sequence have been described recently (Durand et al. 2007). Affected patients suffer from severe mental retardation, delayed speech and autism. The current view is that the haploinsufficiency of the *SHANK3* gene leads to a lack of functional Shank proteins, which may be critical for the maturation of dendritic spines and formation of synapses.

4 Molecular Interactions of Shank Scaffolds: Self Association

One of the most prominent interaction partners of Shank proteins is the Shank proteins themselves. The C-terminal *sterile alpha* motif (the SAM domain), which is required for the correct synaptic targeting of Shank2 and Shank3 (but not

Shank1; Boeckers et al. 2005; Sala et al. 2001), has a strong ability to self-associate. Three-dimensional structures of the recombinant Shank3 SAM domain led to the identification of an extended surface area that is available for polymerization.

The SAM domains first assemble into helical filamentous structures, which then may pack alongside each other, leading to the formation of a two-dimensional array (Baron et al. 2006). Interdomain contacts generate binding sites for Zn^{2+} that further stabilize the sheet. Site-directed mutagenesis and expression of mutant constructs in cultured neurons indicated that intact Zn^{2+}-binding sites and the ability to polymerize are required for correct postsynaptic targeting of the Shank3 protein. Baron et al. (2006) argued that the arraying capacity of the Shank SAM domains may provide a driving force in PSD assembly and that the Zn^{2+}-dependent addition or removal of Shank monomers might contribute to structural plasticity of the postsynaptic scaffold (see also Gundelfinger et al. 2006). Intriguingly, Jan et al. (2002) have reported that Zn^{2+} can trigger the reassembly of a denatured PSD preparation in vitro, further supporting the functional relevance of the SAM domain/Zn^{2+} complex.

Further self-association is provided by the PDZ domain, which, besides binding various membrane receptors and signaling proteins (see below), also has the capacity to form dimers, as observed in the high-resolution structure obtained from the Shank1 PDZ domain in complex with the PDZ ligand motif derived from GKAP/SAPAP (Im et al. 2003). Taken together with the remarkable capacities of the SAM domain, this should already be enough self-association to provide for a complex network of Shank protein molecules. Nevertheless, one more mode of binding between two Shank molecules has been detected in the N-terminal part of the proteins. Direct binding between SH3- and ankyrin-repeat domains also contributes to the ability of Shank to form large molecular aggregates, independent of the C-terminal domains. When expressed recombinantly in COS cells (i.e., in the absence of other synaptic interaction partners of Shank), SH3-Ank concatemerization leads to filamentous aggregates of Shank1 that are deposited in aggresomes, possibly followed by proteasomal degradation (Romorini et al. 2004). Association with GKAP and PSD-95, either from endogenous supplies in neurons or coexpressed in COS cells, prevents aggregation/degradation, leading to formation of Shank/GKAP/PSD-95 clusters instead. Besides pointing to an intricate network of intra- and intermolecular interactions regulating the assembly of Shank-containing clusters, this may also tell us two things: (1) Shank is instable on its own, particularly when not attached to the GKAP/PSD-95 complex; (2) the fact that overexpressed Shank in neurons is not instable suggests that there are enough interaction partners (enough GKAP/PSD-95 in particular) to support it; in turn, the availability of Shank (rather than GKAP/PSD-95) should be the limiting factor for postsynaptic complex formation. This would provide a straightforward explanation for the striking effects of haploinsufficiency for the *SHANK3* gene. The lack of one functional copy of the gene would thus lead to severe neurological deficits because individual neurons cannot provide enough Shank protein for synapse development and maintenance. In addition, it makes sense that the amount of Shank protein in dendrites is tightly controlled, most likely by local synthesis derived from dendritically transported mRNAs, and

activity-dependent degradation through the ubiquitin proteasome system (Boeckers et al. 2004; Ehlers, 2003; see below).

5 Molecular Interactions of Shank Scaffolds: Anchoring of Postsynaptic Membrane Proteins

Shank proteins interact directly as well as indirectly with postsynaptic membrane proteins and membrane proteins in epithelial tissues, such as kidney and lung; direct interactions are mediated by the PDZ domain, which is recognized by type I PDZ ligand motifs (sequence: …Xxx-Ser/Thr-Xxx-Ile/Leu-COO^-) at the C-termini of various transmembrane proteins, including G-protein coupled receptors (GPCRs), cell adhesion molecules, ion channels and transport proteins (Kim et al. 2004; Kreienkamp et al. 2000; Lee et al. 2007; Olson et al. 2005; Tobaben et al. 2000; Shi et al. 2004; Schuetz et al. 2004; Uemura et al. 2004; Zhang et al. 2005; Zitzer et al. 1999a,b). Postsynaptic ionotropic glutamate receptors may also be attached directly to the PDZ domain of Shank (in the case of the GluR-δ2 subunit; Uemura et al. 2004). However, the majority of AMPA- and NMDA-type receptors does not bind to Shank directly; instead, these are anchored via PSD-95 proteins, also through PDZ-type interactions. In case of AMPA receptors this is achieved through the accessory protein stargazin (e.g., Chen et al. 2000), whereas NMDA receptors interact directly with PSD-95 through the C-terminal tails of their NMDAR2-subunits (Kornau et al. 1995). PSD-95 contains a domain similar to guanylate kinases, which does not harbor any enzymatic activity but binds to guanylate kinase-associated proteins (GKAP; also termed SAP-associated proteins, SAPAP1-4; Kim et al. 1997; Takeuchi et al. 1997; see Fig. 2).

GKAP/SAPAPs then interact with the PDZ domains of Shank (Boeckers et al. 1999b; Naisbitt et al. 1999; Yao et al. 1999), providing a rationale for the remote

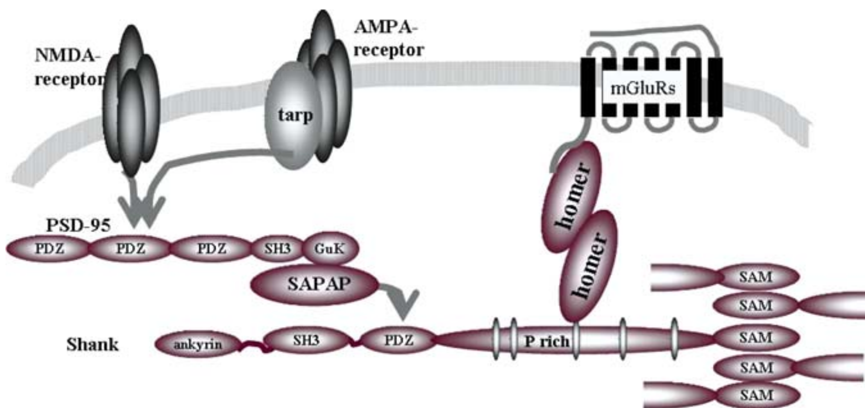

Fig. 2 Integration of different postsynaptic glutamatergic receptor systems through Shank and the attached proteins PSD-95, SAPAP and Homer. The ability of the Shank SAM domain to multimerize is indicated

position of Shank with respect to the postsynaptic plasma membrane as described by Valtschanoff and Weinberg (2001). G-protein coupled metabotropic glutamate receptors (mGluRs) are again indirectly linked to Shank; the intermediate Homer proteins have the ability to dimerize via a coiled-coil motif (Tu et al. 1999). Through additional Ena/VASP homology (EVH) domains, Homer binds to Pro-Pro-Xxx-Xxx-Phe sequences in the mGluR C-Terminal tails and Shank, respectively, thereby physically connecting both proteins. One consequence of this complex system of interactions is a tight connection between the metabotropic and ionotropic glutamate receptor systems of the PSD (Naisbitt et al. 1999; Tu et al. 1999; see Fig. 2). However, the relevance of the Homer/Shank interaction is not limited to the functional integration of cell surface receptors, as another proline-rich ligand motif for the Homer EVH domain is found in the ER-associated receptors for inositol trisphosphate (IP3-receptors). Sala et al. (2001, 2003, 2005) have shown that through this interaction ER cisternae are targeted to dendritic spines; the ability of Shank1 to enhance the functional maturation of dendritic spines is critically dependent on its interaction with Homer and the ability to recruit ER-type membranes to the spine.

The notion that the presence of Shank is crucial for the establishment of transmitter-dependent Ca-release from intracellular stores at the postsynaptic site is further supported by the observation that an isoform of phospholipase C (PLC-β3) binds to the PDZ domain of Shank2 (Hwang et al. 2005). Thus, with the exception of the heterotrimeric G-proteins, all components of mGluR/PLC-β3/IP3-R/Ca-signaling in spines are organized by the Shank/Homer scaffold function (Fig. 3).

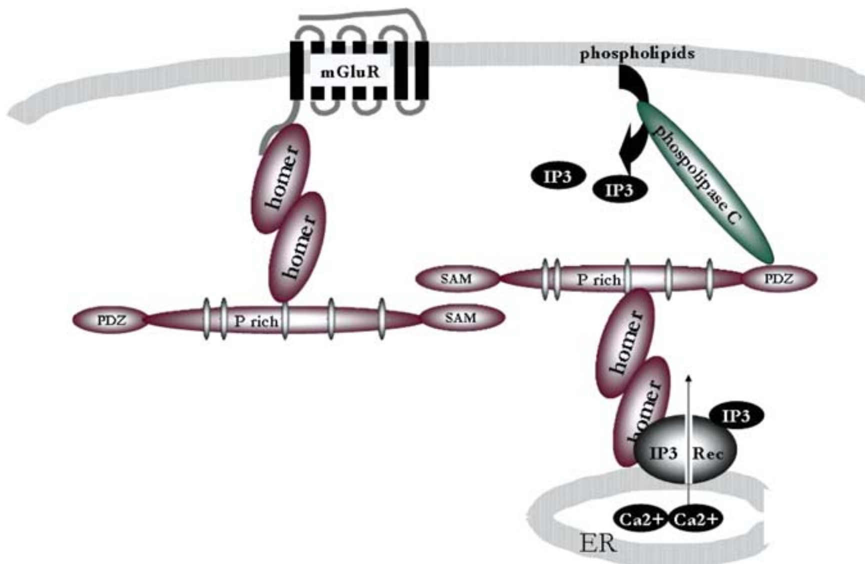

Fig. 3 Signaling complex for the activation of phospholipase C. Note that due to the dimeric nature of Homer, it could also bridge IP3 receptors and mGluR directly. Heterotrimeric G-proteins were omitted for clarity

Interestingly, the Shank/Homer complex may also be involved in the regulation of L-type Ca-channels, another downstream effector molecule for GPCR signaling. In spiny striatal neurons, channels containing the Cav1.3 subunit bind to the PDZ and SH3 domains of Shank. Modulation of channel activity by dopaminergic (D2) and muscarinergic (M1) receptors requires association of the channel with the Shank/Homer complex, as competitive blockers of the Shank PDZ domain, as well as the Homer EVH domain, prevent this type of regulation (Olson et al. 2005; Zhang et al. 2005). At present it is unclear whether the GPCRs involved are also physically associated with Shank proteins.

6 Molecular Interactions of Shank Scaffolds: Linkages to the Actin-Based Cytoskeleton

Shank proteins interact with several actin-binding proteins, including α-fodrin/spectrin (which binds to the N-terminal ankyrin repeat region; Boeckers et al. 2001), cortactin (Du et al. 1998), actin-binding protein 1 (Abp1; Qualmann et al. 2004) and the insulin receptor substrate of 53 kDa (IRSp53; Bockmann et al. 2002; Soltau et al. 2002). Cortactin, Abp1 and IRSp53 are attached to individual motifs in the proline-rich region of Shank proteins through their SH3 domains. IRSp53 also binds to the second PDZ domain of PSD-95 via its C-terminus, suggesting that it may provide a bridging function between Shank and PSD-95, similar to GKAP/SAPAP proteins (Soltau et al. 2004). As the cytoskeleton of dendritic spines is mostly formed by F-actin, it is expected that the interactions with F-actin-binding proteins link Shank and its associated proteins to the cytoskeleton of the spine (Fig. 4). However, this is not likely to represent a static anchoring function, as the actin filaments of spines are subject to actin treadmilling and are in fact highly dynamic in response to synaptic activity (Fischer et al. 1998, 2000). For IRSp53 and Abp1 it was shown that they translocate to the spine or the PSD after stimulation of NMDA receptors, whereas cortactin is removed from the spine under similar

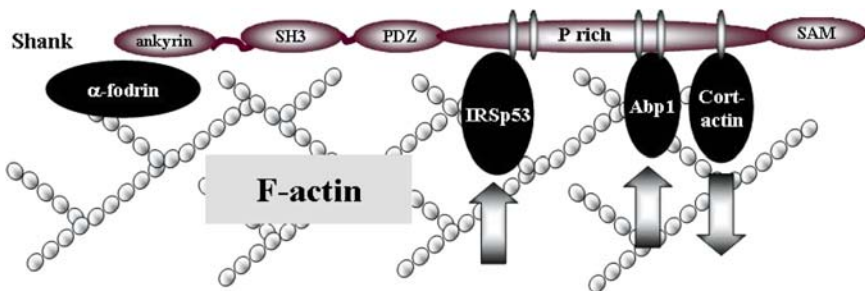

Fig. 4 Linkages to the actin cytoskeleton mediated by Shank-associated proteins. *Arrows* indicate proteins that translocate to or from the spine in response to synaptic activity

stimulating conditions (Hering and Sheng 2003). The interaction of IRSp53 with PSD proteins is in fact regulated by several signaling pathways; the small GTPase cdc42 binds to a CRIB-motif in IRSp53, thus enabling access of a segment of the proline-rich region of Shank to the SH3 domain of IRSp53 (Soltau et al. 2002). In addition, activation of protein kinase C is required for the translocation of IRSp53 to the PSD (Hori et al. 2005). Thus, these Shank-associated F-actin binding proteins might be involved in activity-dependent rearrangements of the actin network that are required for changes in spine structure and eventually contribute to synaptic plasticity. However, it remains unclear at present exactly what kind of restructuring of F-actin occurs; cortactin contains a motif that recruits the Arp2/3 complex to existing actin filaments, leading to the nucleation of new actin branches (e.g., Weaver et al. 2002). Similarly, IRSp53 may activate WAVE2 in dendrites (Choi et al. 2005), which also contributes to actin nucleation via Arp2/3 (Miki et al. 2000). However, interaction of IRSp53 with the actin capping protein Eps8 activates its own actin-bundling activity. Whereas the synergistic actin-bundling activity of the Eps8/IRSp53 complex causes formation of filopodia in fibroblasts (Disanza et al. 2006), it is not yet known how these different actin regulatory activities affect dendritic morphology. With respect to Shank, it also remains unclear whether Shank is required to localize actin regulators (thus performing a scaffolding function “only”) or whether Shank proteins themselves become part of a signaling chain.

7 Molecular Interactions of Shank Scaffolds: Signaling Proteins

Several interactors of the Shank PDZ domains have been suggested to play a role in morphogenic signaling within spines. Most notable, the guanine nucleotide exchange factor βPIX is attached to Shank via this type of interaction, involving the C-terminal PDZ ligand motif of PIX and an additional leucine zipper, also located in the C-terminal region of βPIX (Park et al. 2003). βPIX is an exchange factor for rho GTPases, in particular for cdc42 and rac. Both GTPases have been implicated in the localized restructuring of actin networks in neurons and other cell types. Rac has been implicated in the generation of dendritic spines; one downstream effector for rac (and cdc42), the p21-activated protein kinase (PAK1-3), also interacts with βPIX and can be targeted to the postsynaptic density through the Shank/βPIX complex (Park et al. 2003). In addition, the interaction between IRSp53 and Shank (see above) is dependent on prior activation of IRSp53 by cdc42 (Soltau et al. 2002). Thus, it is conceivable that Shank and associated proteins form a signalosome complex for rho-type GTPases. Though it is not known what the final signaling output of this complex may be, a role in the structural maturation of spines is likely.

The concept that Shank establishes a signaling platform at the synapse is further supported by recent findings by Proepper et al. (2007), who identified an interaction of a proline-rich segment of Shank2 and Shank3 (but not Shank1) with the SH3 domain of the Abelson tyrosine kinase interacting protein 1 (Abi-1). In non-neuronal

cell types, Abi-1 has been mainly implicated in signaling from receptor tyrosine kinases to the activation of rac, and furthermore to restructuring of the actin cytoskeleton. Abi-1 is localized to the PSD through interaction with Shank, and depletion of Abi-1 by RNA interference interferes with spine maturation and synapse formation, while in parallel allowing for excessive formation of dendritic branches. This may be ascribed to known actin-regulatory functions of Abi-1, which are tethered to locations of active synaptogenesis by Shank. Furthermore, once the PSD has been established, Abi-1 appears to assume a new type of signaling function as it travels from the PSD to the nucleus after synaptic stimulation of NMDA receptors. Within the nucleus, Abi-1 is involved in transcriptional regulation, suggesting that Abi-1 constitutes one of the cellular messengers turning a short-term synaptic stimulus into long-term changes in gene expression (Proepper et al. 2007).

We have identified an interaction of the SH3 domain of Shank1 and Shank3 with the PSD scaffold protein densin-180; in the PSD, densin-180 can interact additionally with the Ca-/calmodulin-dependent kinase II and α-actinin (Strack et al. 2000; Robison et al. 2005). Interestingly, the N-terminal leucine-rich repeat region of densin-180 contributes to neuronal morphology by generating excessively branched dendrites in overexpression experiments. The effects of densin overexpression suggest that it is involved in signaling via δ-catenin, which also contributes strongly to dendrite branching when overexpressed (Martinez et al. 2003). Similar to Shank, δ-catenin interacts with the C-terminal region of densin (Izawa et al. 2002) and appears to act synergistically with densin on branching (unpublished data). One aspect of how Shank interferes with the effect of densin-180 on branching may be that it competitively blocks interaction of δ-catenin with densin. So far, it is however unclear how this is transduced to the N-terminal leucine-rich repeat region of densin, which is the main effector region regulating neuronal morphology. Densin is recruited into PSD-type clusters when Shank is coexpressed in neurons, again confirming the role of Shank in the maturation of the postsynapse (Quitsch et al. 2005).

For some interaction partners of Shank, their role at the synapse remains somewhat enigmatic at present. Sharpin binds to the ankyrin repeat region of Shank proteins and may add to the formation of Shank-containing protein clusters at the PSD, as it contains an N-terminal dimerization motif. However, sharpin expression is not limited to neurons, and even in neurons substantial amounts of the protein are found in non-synaptic compartments where it is likely to fulfill a so far unknown function (Lim et al. 2001).

Another potentially very interesting contact occurs between dynamin-2 and a sequence element within the proline-rich region of Shank1 and Shank2 containing multiple serine residues (Okamoto et al. 2001). Dynamin isoforms are GTPases that are known as essential elements of the endocytotic machinery. Dynamin-2 tethered to the PSD via Shank may play a role in the endocytosis of ionotropic glutamate receptors of the AMPA- or NMDA-type (Scott et al. 2004). However, no direct function for dynamin-2 in these processes has so far been demonstrated.

8 Dynamic Regulation of PSD Scaffold Proteins at the Synapse

One of the most intriguing aspects concerning the PSD is the question when and where it is assembled, and which molecular signals trigger complex assembly at the correct point in time and space. An array of modern imaging techniques has been unleashed on this complex of questions, using fluorescent protein expression constructs of PSD proteins in fluorescence recovery, photoactivation and other time-lapse video microscopy applications. Unfortunately the results of these studies are so far conflicting; experiments performed by Ziv and colleagues argue that formation of postsynaptic clusters of GFP-labeled PSD-95 occurs after the formation of a detectable presynaptic active zone (Friedman et al. 2000). Shank and other prominent PSD proteins gradually co-accumulate within these postsynaptic clusters, apparently from diffuse cytoplasmic stores. As the rate of incorporation into these complexes is similar for several of the postsynaptic proteins studied, Bresler et al. (2004) have argued that PSD assembly occurs locally in the dendrite by stepwise addition of individual components to the complex. This is in remarkable contrast to the presynaptic cytomatrix of the active zone, which travels through axons in the form of preassembled transport packages. Only few of these packages or quanta are required for the formation of functional synapses (Shapira et al. 2003).

An alternative view to the gradual assembly of postsynaptic complexes has been provided recently by Gerrow et al. (2006); they observed mobile, non-synaptic clusters containing PSD-95, GKAP/SAPAP and Shank proteins traveling through the dendrite, apparently on their way to nascent or existing synapses. Assembly or clustering of these complexes should then occur before the establishment of axonal contact, probably in the cell body. At present it is not easily possible to reconcile the differences between these diverging views; however, differences in the experimental setup, such as the age of neuronal cultures and the use of different expression constructs, may complicate the analysis of these experimental findings.

Once incorporated into the PSD, one might assume that due to its multiple interactions with other proteins scaffold proteins Shank would be present in the PSD for a rather long period of time. However, this would be inconsistent with the requirements for rapid structural rearrangements of synapses that accompany synaptic plasticity. Indeed, using photoactivatable versions of Shank3 in live imaging experiments, Tsuriel et al. (2006) have observed that Shank3 may be released from individual PSDs and redistributed to other PSDs in the vicinity within a relatively short time frame (in the 10–30-min range). Rapid turnover of Shank is enhanced by synaptic activity and depends on the presence of F-actin in spines (Kuriu et al. 2006), suggesting a role of the various F-actin binding proteins attached to the proline-rich region of Shank in remodeling of the PSD complex.

The case of Shank proteins illustrates that the delivery of scaffold proteins to the PSD complex may be rather complicated. mRNAs coding for all three Shank isoforms have been detected not only in neuronal cell bodies, but also in dendrites

(Boeckers et al. 2004), suggesting the possibility that Shank proteins are synthesized locally in dendrites on ribosomes that have been detected at the base of dendritic spines (Steward and Levy 1982). Dendritic transport of the Shank1 mRNA depends on a targeting element in its 3′ untranslated region. Consistent with the assumption that the localization of mRNAs serves a function in locally restricting the synthesis of a protein, Shank1 translation is strongly inhibited by sequences in the 5′ UTR (unpublished data); thus, Shank1 synthesis would be blocked during transport of the mRNA to its dendritic destination, and translation would only be initiated by a local stimulus (which currently remains to be elucidated). Shank protein produced locally "on demand" might provide a driving force for maturation of the dendritic spine and assembly of the PSD complex.

The number of other mRNAs coding for PSD proteins that are localized in dendrites is rather limited; the most notable example is the α-subunit of the calmodulin-dependent protein kinase (αCaMKII); dendritic presence of this mRNA is required for synaptic plasticity, as detected in long-term potentiation paradigms (Miller et al. 2002). Of the multidomain structural proteins of the PSD, only the mRNA coding for the SAPAP/GKAP isoform 3 (SAPAP3) has been found in hippocampal dendrites (Kindler et al. 2004; Welch et al. 2004). As mentioned above, SAPAP/GKAP proteins are physically associated with Shank. Both Shank and SAPAPs are among those proteins that are degraded in periods of strong synaptic activity due to activation of the ubiquitin/proteasome system (Ehlers 2003). Thus, Shank and SAPAP may be co-regulated during protein synthesis from dendritically targeted mRNAs as well as during regulated degradation.

Shank1 deficient mice have been reported recently to exhibit smaller dendritic spines and impaired long term memory, further supporting the relevance of shank proteins for synapse function (Hung et al., 2008).

References

Baron MK, Boeckers TM, Vaida B, Faham S, Gingery M, Sawaya MR, Salyer D, Gundelfinger ED, Bowie JU (2006) An architectural framework that may lie at the core of the postsynaptic density. Science 311:531–535

Bockmann J, Kreutz MR, Gundelfinger ED, Boeckers TM (2002) ProSAP/Shank postsynaptic density proteins interact with insulin receptor tyrosine kinase substrate IRSp53. J Neurochem 83:1013–1017

Boeckers TM, Kreutz MR, Winter C, Zuschratter W, Smalla KH, Sanmarti-Vila L, Wex H, Langnaese K, Bockmann J, Garner CC, Gundelfinger ED (1999a) Proline-rich synapse-associated protein-1/cortactin binding protein 1 (ProSAP1/CortBP1) is a PDZ-domain protein highly enriched in the postsynaptic density. J Neurosci 19:6506–6518

Boeckers TM, Winter C, Smalla KH, Kreutz MR, Bockmann J, Seidenbecher C, Garner CC, Gundelfinger ED (1999b) Proline-rich synapse-associated proteins ProSAP1 and ProSAP2 interact with synaptic proteins of the SAPAP/GKAP family. Biochem Biophys Res Commun 264:247–252

Boeckers TM, Mameza MG, Kreutz MR, Bockmann J, Weise C, Buck F, Richter D, Gundelfinger ED, Kreienkamp HJ (2001) Synaptic scaffolding proteins in rat brain. Ankyrin repeats of the

multidomain Shank protein family interact with the cytoskeletal protein alpha-fodrin. J Biol Chem 276:40104–40112

Boeckers TM, Segger-Junius M, Iglauer P, Bockmann J, Gundelfinger ED, Kreutz MR, Richter D, Kindler S, Kreienkamp HJ (2004) Differential expression and dendritic transcript localization of Shank family members: identification of a dendritic targeting element in the 3 untranslated region of Shank1 mRNA. Mol Cell Neurosci 26:182–190

Boeckers TM, Liedtke T, Spilker C, Dresbach T, Bockmann J, Kreutz MR, Gundelfinger ED (2005) C-terminal synaptic targeting elements for postsynaptic density proteins ProSAP1/Shank2 and ProSAP2/Shank3. J Neurochem 92:519–524

Bonaglia MC, Giorda R, Borgatti R, Felisari G, Gagliardi C, Selicorni A, Zuffardi O (2001) Disruption of the ProSAP2 gene in a t(12;22)(q24.1;q13.3) is associated with the 22q13.3 deletion syndrome. Am J Hum Genet 69:261–268

Bresler T, Shapira M, Boeckers T, Dresbach T, Futter M, Garner CC, Rosenblum K, Gundelfinger ED, Ziv NE (2004) Postsynaptic density assembly is fundamentally different from presynaptic active zone assembly. J Neurosci 24:1507–1520

Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Bredt DS, Nicoll RA (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. Nature 408:936–943

Chen X, Vinade L, Leapman RD, Petersen JD, Nakagawa T, Phillips TM, Sheng M, Reese TS (2005) Mass of the postsynaptic density and enumeration of three key molecules. Proc Natl Acad Sci USA 102:11551–11556

Choi J, Ko J, Racz B, Burette A, Lee JR, Kim S, Na M, Lee HW, Kim K, Weinberg RJ, Kim E (2005) Regulation of dendritic spine morphogenesis by insulin receptor substrate 53, a downstream effector of Rac1 and Cdc42 small GTPases. J Neurosci 25:869–879

Disanza A, Mantoani S, Hertzog M, Gerboth S, Frittoli E, Steffen A, Berhoerster K, Kreienkamp HJ, Milanesi F, Di Fiore PP, Ciliberto A, Stradal TE, Scita G (2006) Regulation of cell shape by Cdc42 is mediated by the synergic actin-bundling activity of the Eps8-IRSp53 complex. Nat Cell Biol 8:1337–1347

Du Y, Weed SA, Xiong WC, Marshall TD, Parsons JT (1998) Identification of a novel cortactin SH3 domain-binding protein and its localization to growth cones of cultured neurons. Mol Cell Biol 18:5838–5851

Durand CM, Betancur C, Boeckers TM, Bockmann J, Chaste P, Fauchereau F, Nygren G, Rastam M, Gillberg IC, Anckarsater H, Sponheim E, Goubran-Botros H, Delorme R, Chabane N, Mouren-Simeoni MC, de Mas P, Bieth E, Roge B, Heron D, Burglen L, Gillberg C, Leboyer M, Bourgeron T (2007) Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. Nat Genet 39:25–27

Ehlers MD (2003) Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. Nat Neurosci 6:231–242

Fischer M, Kaech S, Knutti D, Matus A (1998) Rapid actin-based plasticity in dendritic spines. Neuron 20:847–854

Fischer M, Kaech S, Wagner U, Brinkhaus H, Matus A (2000) Glutamate receptors regulate actin-based plasticity in dendritic spines. Nat Neurosci 3:887–894

Friedman HV, Bresler T, Garner CC, Ziv NE (2000) Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. Neuron 27:57–69

Gerrow K, Romorini S, Nabi SM, Colicos MA, Sala C, El-Husseini A (2006) A preformed complex of postsynaptic proteins is involved in excitatory synapse development. Neuron 49:547–562

Gundelfinger ED, Boeckers TM, Baron MK, Bowie JU (2006) A role for zinc in postsynaptic density asSAMbly and plasticity? Trends Biochem Sci 31:366–373

Hering H, Sheng M (2003) Activity-dependent redistribution and essential role of cortactin in dendritic spine morphogenesis. J Neurosci 23:11759–11769

Hori K, Yasuda H, Konno D, Maruoka H, Tsumoto T, Sobue K (2005) NMDA receptor-dependent synaptic translocation of insulin receptor substrate p53 via protein kinase C signaling. J Neurosci 25:2670–2681

Hung AY, Futai K, Sala C, Valtschanoff JG, Ryu J, Woodworth MA, Kidd FL, Sung CC, Miyakawa T, Bear MF, Weinberg RJ, Sheng M (2008) Smaller dendritic spines, weaker synaptic transmission, but enhanced spatial learning in mice lacking Shank1. J Neurosci 28:1697–1708

Hung AY, Sala C, Valtschanoff JG, Ryu J, Burgoon M, Miyakawa T, Weinberg RJ, Sheng M (2005) Functional role of the Shank1 postsynaptic scaffold protein in synapse structure and learning and memory. Program no. 501.9. Washington, DC: Society for Neuroscience. Online

Hwang JI, Kim HS, Lee JR, Kim E, Ryu SH, Suh PG (2005) The interaction of phospholipase C-beta3 with Shank2 regulates mGluR-mediated calcium signal. J Biol Chem 280:12467–12473

Im YJ, Lee JH, Park SH, Park SJ, Rho SH, Kang GB, Kim E, Eom SH (2003) Crystal structure of the Shank PDZ-ligand complex reveals a class I PDZ interaction and a novel PDZ-PDZ dimerization. J Biol Chem 278:48099–48104

Izawa I, Nishizawa M, Ohtakara K, Inagaki M (2002) Densin-180 interacts with delta-catenin/neural plakophilin-related armadillo repeat protein at synapses. J Biol Chem 277:5345–5350

Jan HH, Chen IT, Tsai YY, Chang YC (2002) Structural role of zinc ions bound to postsynaptic densities. J Neurochem 83:525–534

Kim E, Naisbitt S, Hsueh YP, Rao A, Rothschild A, Craig AM, Sheng M (1997) GKAP, a novel synaptic protein that interacts with the guanylate kinase-like domain of the PSD-95/SAP90 family of channel clustering molecules. J Cell Biol 136:669–678

Kim JY, Han W, Namkung W, Lee JH, Kim KH, Shin H, Kim E, Lee MG (2004) Inhibitory regulation of cystic fibrosis transmembrane conductance regulator anion-transporting activities by Shank2. J Biol Chem 279:10389–10396

Kindler S, Rehbein M, Classen B, Richter D, Boeckers TM (2004) Distinct spatiotemporal expression of SAPAP transcripts in the developing rat brain: a novel dendritically localized mRNA. Brain Res Mol Brain Res 126:14–21

Kornau HC, Schenker LT, Kennedy MB, Seeburg PH (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science 269:1737–1740

Kreienkamp HJ, Zitzer H, Gundelfinger ED, Richter D, Boeckers TM (2000) The calcium-independent receptor for alpha-latrotoxin from human and rodent brains interacts with members of the ProSAP/SSTRIP/Shank family of multidomain proteins. J Biol Chem 275:32387–32390

Kuriu T, Inoue A, Bito H, Sobue K, Okabe S (2006) Differential control of postsynaptic density scaffolds via actin-dependent and -independent mechanisms. J Neurosci 26:7693–7706

Lee JH, Richter W, Namkung W, Kim KH, Kim E, Conti M, Lee MG (2007) Dynamic regulation of cystic fibrosis transmembrane conductance regulator by competitive interactions of molecular adaptors. J Biol Chem 282:10414–10422

Lim S, Naisbitt S, Yoon J, Hwang JI, Suh PG, Sheng M, Kim E (1999) Characterization of the Shank family of synaptic proteins. Multiple genes, alternative splicing, and differential expression in brain and development. J Biol Chem 274:29510–29518

Lim S, Sala C, Yoon J, Park S, Kuroda S, Sheng M, Kim E (2001) Sharpin, a novel postsynaptic density protein that directly interacts with the Shank family of proteins. Mol Cell Neurosci 17:385–397

Martinez MC, Ochiishi T, Majewski M, Kosik KS (2003) Dual regulation of neuronal morphogenesis by a delta-catenin-cortactin complex and Rho. J Cell Biol 162:99–111

McWilliams RR, Gidey E, Fouassier L, Weed SA, Doctor RB (2004) Characterization of an ankyrin repeat-containing Shank2 isoform (Shank2E) in liver epithelial cells. Biochem J 380:181–191

Miki H, Yamaguchi H, Suetsugu S, Takenawa T (2000) IRSp53 is an essential intermediate between Rac and WAVE in the regulation of membrane ruffling. Nature 408:732–735

Miller S, Yasuda M, Coats JK, Jones Y, Martone ME, Mayford M (2002) Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. Neuron 36:507–519

Naisbitt S, Kim E, Tu JC, Xiao B, Sala C, Valtschanoff J, Weinberg RJ, Worley PF, Sheng M (1999) Shank, a novel family of postsynaptic density proteins that binds to the NMDA receptor/PSD-95/GKAP complex and cortactin. Neuron 23:569–582
Okamoto PM, Gamby C, Wells D, Fallon J, Vallee RB (2001) Dynamin isoform-specific interaction with the Shank/ProSAP scaffolding proteins of the postsynaptic density and actin cytoskeleton. J Biol Chem 276:48458–48465
Olson PA, Tkatch T, Hernandez-Lopez S, Ulrich S, Ilijic E, Mugnaini E, Zhang H, Bezprozvanny I, Surmeier DJ (2005) G-protein-coupled receptor modulation of striatal CaV1.3 L-type Ca2+ channels is dependent on a Shank-binding domain. J Neurosci 25:1050–1062
Park E, Na M, Choi J, Kim S, Lee JR, Yoon J, Park D, Sheng M, Kim E (2003) The Shank family of postsynaptic density proteins interacts with and promotes synaptic accumulation of the beta PIX guanine nucleotide exchange factor for Rac1 and Cdc42. J Biol Chem 278:19220–19229
Proepper C, Johannsen S, Liebau S, Dahl J, Vaida B, Bockmann J, Kreutz MR, Gundelfinger ED, Boeckers TM (2007) Abelson interacting protein 1 (Abi-1) is essential for dendrite morphogenesis and synapse formation. Embo J 1397–1409
Qualmann B, Boeckers TM, Jeromin M, Gundelfinger ED, Kessels MM (2004) Linkage of the actin cytoskeleton to the postsynaptic density via direct interactions of Abp1 with the ProSAP/Shank family. J Neurosci 24:2481–2495
Quitsch A, Berhorster K, Liew CW, Richter D, Kreienkamp HJ (2005) Postsynaptic Shank antagonizes dendrite branching induced by the leucine-rich repeat protein densin-180. J Neurosci 25:479–487
Robison AJ, Bass MA, Jiao Y, MacMillan LB, Carmody LC, Bartlett RK, Colbran RJ (2005) Multivalent interactions of calcium/calmodulin-dependent protein kinase II with the postsynaptic density proteins NR2B, densin-180, and alpha-actinin-2. J Biol Chem 280:35329–35336
Romorini S, Piccoli G, Jiang M, Grossano P, Tonna N, Passafaro M, Zhang M, Sala C (2004) A functional role of postsynaptic density-95-guanylate kinase-associated protein complex in regulating Shank assembly and stability to synapses. J Neurosci 24:9391–9404
Roussignol G, Ango F, Romorini S, Tu JC, Sala C, Worley PF, Bockaert J, Fagni L (2005) Shank expression is sufficient to induce functional dendritic spine synapses in aspiny neurons. J Neurosci 25:3560–3570
Sala C, Piech V, Wilson NR, Passafaro M, Liu G, Sheng M (2001) Regulation of dendritic spine morphology and synaptic function by Shank and homer. Neuron 31:115–130
Sala C, Futai K, Yamamoto K, Worley PF, Hayashi Y, Sheng M (2003) Inhibition of dendritic spine morphogenesis and synaptic transmission by activity-inducible protein Homer1a. J Neurosci 23:6327–6337
Sala C, Roussignol G, Meldolesi J, Fagni L (2005) Key role of the postsynaptic density scaffold proteins Shank and homer in the functional architecture of Ca2+ homeostasis at dendritic spines in hippocampal neurons. J Neurosci 25:4587–4592
Schuetz G, Rosario M, Grimm J, Boeckers TM, Gundelfinger ED, Birchmeier W (2004) The neuronal scaffold protein Shank3 mediates signaling and biological function of the receptor tyrosine kinase Ret in epithelial cells. J Cell Biol 167:945–952
Scott DB, Michailidis I, Mu Y, Logothetis D, Ehlers MD (2004) Endocytosis and degradative sorting of NMDA receptors by conserved membrane-proximal signals. J Neurosci 24:7096–7109
Shapira M, Zhai RG, Dresbach T, Bresler T, Torres VI, Gundelfinger ED, Ziv NE, Garner CC (2003) Unitary assembly of presynaptic active zones from Piccolo-Bassoon transport vesicles. Neuron 38:237–252
Shi SH, Cheng T, Jan LY, Jan YN (2004) The immunoglobulin family member dendrite arborization and synapse maturation 1 (Dasm1) controls excitatory synapse maturation. Proc Natl Acad Sci USA 101:13346–13351
Soltau M, Richter D, Kreienkamp HJ (2002) The insulin receptor substrate IRSp53 links postsynaptic Shank1 to the small G-protein cdc42. Mol Cell Neurosci 21:575–583

Soltau M, Berhorster K, Kindler S, Buck F, Richter D, Kreienkamp HJ (2004) Insulin receptor substrate of 53 kDa links postsynaptic Shank to PSD-95. J Neurochem 90:659–665
Steward O, Levy WB (1982) Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. J Neurosci 2:284–291
Strack S, Robison AJ, Bass MA, Colbran RJ (2000) Association of calcium/calmodulin-dependent kinase II with developmentally regulated splice variants of the postsynaptic density protein densin-180. J Biol Chem 275:25061–25064
Sugiyama Y, Kawabata I, Sobue K, Okabe S (2005) Determination of absolute protein numbers in single synapses by a GFP-based calibration technique. Nat Methods 2:677–684
Takeuchi M, Hata Y, Hirao K, Toyoda A, Irie M, Takai Y (1997) SAPAPs. A family of PSD-95/SAP90-associated proteins localized at postsynaptic density. J Biol Chem 272:11943–11951
Tobaben S, Sudhof TC, Stahl B (2000) The G protein-coupled receptor CL1 interacts directly with proteins of the Shank family. J Biol Chem 275:36204–36210
Tsuriel S, Geva R, Zamorano P, Dresbach T, Boeckers T, Gundelfinger ED, Garner CC, Ziv NE (2006) Local sharing as a predominant determinant of synaptic matrix molecular dynamics. PLoS Biol 4:e271
Tu JC, Xiao B, Naisbitt S, Yuan JP, Petralia RS, Brakeman P, Doan A, Aakalu VK, Lanahan AA, Sheng M, Worley PF (1999) Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. Neuron 23:583–592
Uemura T, Mori H, Mishina M (2004) Direct interaction of GluRdelta2 with Shank scaffold proteins in cerebellar Purkinje cells. Mol Cell Neurosci 26:330–341
Valtschanoff JG, Weinberg RJ (2001) Laminar organization of the NMDA receptor complex within the postsynaptic density. J Neurosci 21:1211–1217
Weaver AM, Heuser JE, Karginov AV, Lee WL, Parsons JT, Cooper JA (2002) Interaction of cortactin and N-WASp with Arp2/3 complex. Curr Biol 12:1270–1278
Welch JM, Wang D, Feng G (2004) Differential mRNA expression and protein localization of the SAP90/PSD-95-associated proteins (SAPAPs) in the nervous system of the mouse. J Comp Neurol 472:24–39
Yao I, Hata Y, Hirao K, Deguchi M, Ide N, Takeuchi M, Takai Y (1999) Synamon, a novel neuronal protein interacting with synapse-associated protein 90/postsynaptic density-95-associated protein. J Biol Chem 274:27463–27466
Zhang H, Maximov A, Fu Y, Xu F, Tang TS, Tkatch T, Surmeier DJ, Bezprozvanny I (2005) Association of CaV1.3 L-type calcium channels with Shank. J Neurosci 25:1037–1049
Zitzer H, Honck HH, Bachner D, Richter D, Kreienkamp HJ (1999a) Somatostatin receptor interacting protein defines a novel family of multidomain proteins present in human and rodent brain. J Biol Chem 274:32997–33001
Zitzer H, Richter D, Kreienkamp HJ (1999b) Agonist-dependent interaction of the rat somatostatin receptor subtype 2 with cortactin-binding protein 1. J Biol Chem 274:18153–18156

Scaffolding Proteins in Cardiac Myocytes

N.L. Chudasama, S.O. Marx, and S.F. Steinberg(✉)

Contents

1 Stimulus-Specific or Localized cAMP-PKA Signaling in Cardiomyocytes 302
1.1 Compartmentalization of Individual Gs-Coupled GPCRs to Membrane Subdomains 303
1.2 The Role of PDZ-Domain-Containing Scaffolds 303
1.3 The Role of β-Arrestin and AKAPs 306
2 Scaffolding Functions for the Cardiac Z-Disc 308
3 Scaffolds That Regulate PKC Signaling in Cardiomyocytes 309
4 Scaffolds That Influence Ion Channel Function 311
4.1 SCN5A (Voltage-Dependent Na^+ Channel) 311
4.2 L-Type Ca^{2+} Channel ($Ca_v1.2$) 312
4.3 Ryanodine Receptor (RyR2) 314
4.4 KCNQ1 315
5. Scaffolds That Regulate Cardiac Hypertrophy 315
5.1 mAKAP-PKA-PDE4D3 Complexes 315
5.2 AKAP-Lbc and the Regulation of Rho and PKD 317
6 Conclusion 319
References 319

Abstract Post-translational modification, such as protein phosphorylation, plays a critical role to reversibly amplify and modulate signaling pathways. Since kinases and phosphatases have broad substrate recognition motifs, compartmentalization and localization of signaling complexes are required to achieve specific signals. Scaffolds are proteins that associate with two or more binding partners and function to enhance the efficiency and/or specificity of cellular signaling pathways. The identification of scaffolding proteins that control the tempo and/or spatial organization of signaling pathways in cells has benefited enormously from recent technological advances that allow for the detection of protein–protein interactions, including in vivo in intact cells. This review will focus on scaffolding proteins that nucleate multi-protein complexes (and could represent novel entry points into signaling pathways that might be amenable to therapeutic manipulation) in cardiomyocytes.

S.F. Steinberg
Departments of Pharmacology, College of Physicians and Surgeons, Columbia University, 630 West, 168 Street, New York, NY 10032, USA
sfs1@columbia.edu

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,

© Springer-Verlag Berlin Heidelberg 2008

1 Stimulus-Specific or Localized cAMP-PKA Signaling in Cardiomyocytes

cAMP constitutes an important intracellular second messenger molecule that is formed in response to the activation of many cell-surface G-protein-coupled receptors (GPCRs). In theory, cAMP is a freely diffusible second messenger that can act over long distances in cells. However, experimental results from over 25 years ago provided the first clues that cAMP/protein kinase A (PKA) pathways are compartmentalized in cells. Recognizing that the PKA regulatory subunit is expressed as structurally and functionally distinct RI and RII isoforms (forming the PKA-RI and PKA-RII holoenzymes), Brunton and colleagues showed that β-adrenergic receptors (βARs) activate particulate PKA-RII and induce changes in cardiac contractility, whereas prostaglandin E_2 (which stimulates a different GPCR) activates soluble PKA-RI and regulates glycogen metabolism, but does not influence cardiac contractile function. Subsequent studies identified specificity at each step of the signal transduction pathway, including evidence that: (1) Gs-coupled GPCRs differ drastically in their ability to increase cAMP accumulation in cardiomyocytes. Certain GPCRs (such as the β_2AR) increase cAMP accumulation when heterologously overexpressed in model cell types, but do not detectably elevate cAMP in certain cardiomyocyte preparations (Steinberg and Brunton 2001). Gs-coupled βARs also differ in their susceptibility to inhibitory input from muscarinic acetylcholine receptors (Aprigliano et al. 1997). (2) cAMP generated in response to GPCR activation does not necessarily gain access to all available PKA. (3) PKA tends to act in an agonist-specific manner to phosphorylate selective cellular targets. (4) cAMP signals can be terminated by phosphodiesterase (PDE) enzymes in a stimulus-specific manner.

Direct proof that cAMP signals are compartmentalized in cells is relatively recent and has resulted from technological advances that permit real-time imaging of cAMP gradients and compartments in cells. The Fischmeister laboratory used whole-cell patch-clamp recordings of Ca^{2+} transients at two physically distinct sites on a single isolated frog ventricular myocyte as a strategy to discriminate calcium channel regulation by local versus global pools of cAMP (Jurevicius and Fischmeister 1996). They showed that βARs activate a local population of calcium channels, whereas forskolin (a pharmacologic activator of adenylyl cyclase) activates calcium channels at both local and distal sites in the cell. Zaccolo and Pozzan (2002) took advantage of live cell imaging techniques to show that βAR-dependent cAMP signals are localized by PDE enzymes, leading to the activation of a specific sub-population of PKA molecules in cardiomyocytes.

While cAMP can act through several cellular effectors, including PKA, cyclic-nucleotide-gated ion channels, phosphodiesterases, and guanine nucleotide-exchange proteins activated by cAMP (EPACs), cAMP signaling via PKA (historically viewed as the primary cAMP effector) has been studied most intensively. PKA exists as a tetramer consisting of two catalytic subunits that are held inactive by two regulatory (R) subunits. While PKA activation in theory could lead to the phosphorylation of a

wide array of cellular proteins, PKA activity is regulated by A-kinase anchoring proteins (AKAPs) in cells. AKAPs position the PKA holoenzyme at specific subcellular microdomains where it can sense localized spikes in cAMP and gain access to specific cellular target substrates. AKAPs also function as multivalent scaffolds to assemble PKA in multi-protein complexes with other signaling kinases, phosphatases, and phosphodiesterases. By exerting bi-directional controls on both the propagation and termination of cAMP-initiated signals, AKAPs ensure a high degree of spatial and temporal control of cAMP signaling. This section summarizes current notions regarding mechanisms (involving AKAPs and other cellular scaffolds) that localize Gs-coupled receptors and the cAMP/PKA signaling pathway in cardiomyocytes.

1.1 Compartmentalization of Individual Gs-Coupled GPCRs to Membrane Subdomains

There is compelling evidence that β_1 and β_2ARs activate distinct G protein/effector pathways and evoke distinct regulatory controls that lead to subtype-specific differences in the regulation of cardiac contraction and hypertrophic growth programs (Steinberg and Brunton 2001). Functional differences between β_1 and β_2AR signaling have been attributed in part to subtype-specific targeting to distinct membrane compartments. β_2ARs are detected exclusively in caveolae/lipid raft membranes and egress from this compartment upon activation, whereas β_1ARs are detected in both caveolae and non-caveolae membranes and do not undergo a detectable translocation upon activation (Rybin et al. 2000). βAR subtype segregation to different intracellular compartments provides a mechanism to simultaneously facilitate βAR interactions with certain signaling partners, while restricting interactions with others. While caveolin-3 (a protein that oligomerizes and drives the formation of caveolae) contains an interaction motif that anchors and regulates the activity of many lipid-modified signaling proteins in caveolae, caveolin expression is not required for βAR localization to caveolae. Hence, this section will focus on other scaffolding proteins that interact with various components of the cAMP generating machinery and might contribute to specificity in the cAMP signaling pathway in cardiomyocytes.

1.2 The Role of PDZ-Domain-Containing Scaffolds

Recent studies have exposed βAR C-terminal domain interactions with PDZ-domain-containing scaffolds as a mechanism that controls βAR targeting in cells (Fig. 1). The extreme C-termini of β_1 and β_2 ARs conform to type-1 PDZ-domain-binding sequences (S/T-X-ϕ, where "ϕ" indicates a hydrophobic amino acid and "X" indicates any amino acid); these βAR subtypes interact with different sets of PDZ-domain-containing proteins. The β_1AR-ESKV motif associates with structurally

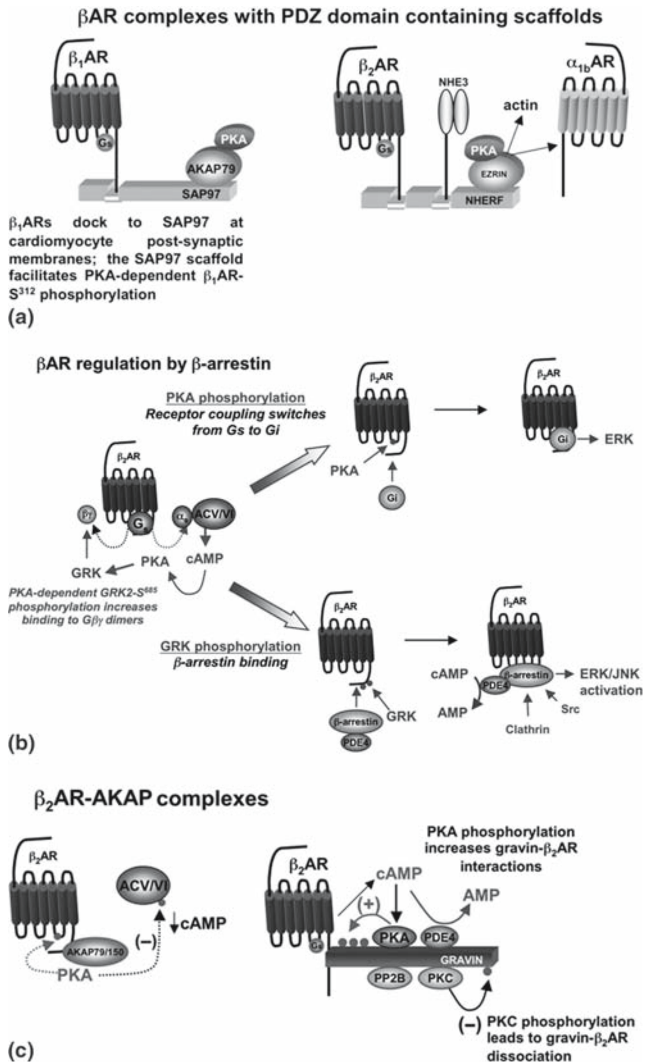

Fig. 1 βARs complex with three types of scaffolds. **(a)** β_1 and β_2ARs interact with PDZ-domain-containing scaffolding proteins. SAP97 links β_1ARs to the AKAP79-PKA complex, whereas NHERF links β_2ARs to the Na^+–H^+ exchanger 3 (NHE3) and the actin-associated protein ezrin. An interaction between ezrin and a membrane-proximal portion of the α_{1b}-AR C-tail is required for α_{1b}-AR recycling to the plasma membrane. (**b**) The initial βAR signal via the cAMP/PKA pathway is followed by a subsequent activation of the ERK cascade in many cell types. Two mechanisms have been identified: PKA phosphorylation switches βAR coupling from Gs to Gi. β-arrestin docks to GRK-phosphorylated βARs (and other GPCRs) and functions to interdict signaling by G proteins, to recruit PDE4, and to assemble signaling proteins that regulate growth/apoptosis signaling pathways. (**c**) An association between the βAR C-terminus and AKAP79/150 or gravin contributes to the spatial and temporal control of signaling through the PKA pathway (See text for details).

related membrane-associated guanylate kinase-like (MAGUK) PDZ family proteins, including postsynaptic density protein 95 (PSD-95), membrane-associated guanylate kinase-like protein inverted-2 (MAGI-2), MAGI-3, and SAP97, as well as GIPC (GAIP-interacting protein, carboxyl terminus), and cystic fibrosis transmembrane conductance regulator-associated ligand (CAL, a protein primarily involved in vesicular transport in the Golgi) (Hu et al. 2000; Xu et al. 2001; Hu et al. 2003; He et al. 2004, 2006; Gardner et al. 2007). PSD-95 is reported to inhibit β_1AR internalization, whereas MAGI-2 exerts a diametrically opposite effect to promote β_1AR internalization, indicating that individual MAGUK proteins regulate β_1AR signaling in a highly individualized manner (Hu et al. 2000; Xu et al. 2001). PSD-95 physically links β_1ARs to effectors, such as the *N*-methyl-D-aspartate (NMDA) class of glutamate receptor channels in neuronal synapses (a mechanism that is important in neurons, but not necessarily cardiomyocytes), whereas CAL redirects β_1ARs to the Golgi (Hu et al. 2000). In contrast, MAGI-3 (which is abundant in cardiac tissue; Wu et al. 2000) impairs β_1AR coupling to the Gi-ERK pathway, but it does not grossly alter β_1AR-dependent cAMP generation or agonist-dependent β_1AR internalization (Hu et al. 2003; He et al. 2006). Finally, SAP97 has been implicated as a scaffold that assembles AKAP79 and PKA in complexes with β_1ARs (Fig. 1a); β_1AR binding to SAP79 facilitates PKA-dependent β_1AR phosphorylation at S^{312} in the third intracellular loop and is required for β_1AR recycling following activation (Gardner et al. 2007). The observation that the WT-β_1AR couples exclusively to Gs and does not undergo internalization, whereas the β_1AR-PDZ mutant couples to both Gs and Gi and internalizes upon agonist exposure, has been taken as evidence that PDZ-domain-mediated interactions contribute to the control of β_1AR signaling in cardiomyocytes (Xiang et al. 2002). The additional observation that SAP97 co-localizes with cadherins (cell adhesion molecules that maintain the stability of cell–cell junctions), β-catenin (which links cadherins to the actin cytoskeleton), and β_1ARs at specialized postsynaptic membranes formed following cardiomyocyte sympathetic innervation suggests a mechanism for rapid responses to released neurotransmitters. However, the precise mechanism that localizes β_1ARs to postsynaptic membranes remains uncertain, since a C-terminal PDZ-binding motif mutation does not disrupt β_1AR localization to synapses (Shcherbakova et al. 2007).

β_2ARs interact with a different set of PDZ-domain-containing proteins, namely the Na^+–H^+ Exchanger Regulatory Factor proteins NHERF-1 and NHERF-2 [a protein also known as Ezrin-Radixin-Moesin (ERM)-Binding Protein-50 (EBP50) that also binds to cortical actin through its ERM domain, Fig. 1, right (Hall, Premont et al. 1998; Hall et al. 1998a)]. β_2AR interactions with NHERF proteins are increased by agonists and lead to enhanced Na^+–H^+ exchanger type 3 (NHE3) activity and β_2AR recycling to surface membranes following agonist-promoted internalization (Hall et al. 1998b). In keeping with the notion that PDZ domain interactions are regulated by phosphorylations at the extreme C-terminus position −2 S/T residue, β_1AR-PSD-95 and β_2AR-NHERF-1 interactions are disrupted by G protein-coupled receptor kinase 5 (GRK5) phosphorylation of the βAR (Hu et al. 2002; Cao et al. 1999). β_2ARs that harbor point mutations in the DSLL sequence

undergo normal agonist-dependent internalization, but they do not recycle to the plasma membrane. The β_2AR-DSLL sequence can also function as a transplantable sorting signal to reroute other GPCRs (that lack this extreme C-terminal sequence and are degraded following clathrin-dependent endocytosis) to a rapid recycling pathway (Gage et al. 2001).

The NHERF-binding protein ezrin plays an important role in the regulation of the actin cytoskeleton (controlling cell shape and cell adhesion/motility). Recent studies implicate ezrin as an AKAP (with greater affinity for PKA-RI, relative to RII) that modulates signaling pathways (Dransfield et al. 1997; Carlson et al. 2006). There is evidence that ezrin is a non-GPCR substrate for GRK2; GRK2-dependent ezrin-T^{567} phosphorylation stabilizes the active conformation that internalizes in association with β_2-ARs and contributes to the control of this process (Cant and Pitcher, 2005). Ezrin also directly interacts with a polyarginine sequence (R371–R378) in the membrane-proximal portion of the α_{1b}-AR C-tail, and this interaction between ezrin and the α_{1b}-AR is required for α_{1b}-AR recycling to the plasma membrane (although not internalization, Fig. 1a, Stanasila et al. 2006).

Collectively, these results identify functionally important interactions between βARs and a number of PDZ domain scaffolds that impact importantly on specific aspects of βAR signaling/desensitization. Insofar as PDZ domain scaffolding proteins tend to be expressed in a highly tissue- and cell-specific manner, these results provide a potential mechanism to explain tissue- and cell-specific differences in βAR signaling and trafficking that have been described in the literature. Moreover, these results suggest that clinical interventions designed to alter PDZ domain protein expression (or PDZ domain protein interactions with βARs or other binding partners) may have clinically important consequences that could be exploited for therapeutic advantage.

1.3 The Role of β-Arrestin and AKAPs

The traditional model of βAR activation focuses on the role of the heterotrimeric Gs protein to activate the cAMP pathway. βAR activation also leads to the liberation of Gβγ dimers that contribute to the recruitment of GRKs to the membrane, leading to phosphorylation of agonist-activated βARs (Fig. 1b). β-arrestin then binds GRK-phosphorylated βARs and acts both to sterically interdict further G protein signaling and to promote βAR desensitization. However, β-arrestins also act as scaffolds for components of the membrane trafficking machinery that internalize GPCRs from the surface membrane (including clathrin, AP-2, *N*-ethylmaleimide–sensitive factor (NSF), ARF6, ARNO, and Mdm2) and for signaling proteins (such as Src, JNK3, ASK1, ERK1/2, and PDE4D3) that provide for G protein-independent activation of growth/apoptosis regulatory pathways (Luttrell et al. 1999; McDonald et al. 2000; Miller et al. 2000).

AKAPs constitute another class of βAR-interacting scaffold that assembles components of the βAR signaling machinery at membranes and functionally

regulates both the formation and hydrolysis of cAMP (Fig. 1c). AKAPs comprise a family of structurally diverse proteins that contain an amphipathic helix that binds the PKA regulatory subunit (R) with high affinity as well as targeting motif that directs the AKAP-PKA complex to a specific subcellular location. Individual AKAPs display distinct binding affinities for RII, providing a mechanism to dynamically regulate PKA-dependent phosphorylation of individual substrates in cells. β_2ARs are recovered from both tissue and cell preparations in complexes with two distinct AKAPs, AKAP79/150 and AKAP250/gravin (Fraser et al. 2000; Shih et al. 1999; Tao et al. 2003). β_2ARs constitutively interact with AKAP79/150, providing a mechanism for forced proximity with PKA, PKC, and PP2B (which also interact with AKAP79/150) and β_2AR phosphorylation by PKA. This phosphorylation is believed to switch β_2AR-G protein coupling from the Gαs-cAMP pathway to a Gi pathway that activates ERK (Fig. 1b, Fraser et al. 2000). Although AKAP79/150 does not directly interact with GRK2, AKAP79/150 is required for PKA-dependent phosphorylation of GRK2 (at S^{685}), which increases GRK2 affinity for G$\beta\gamma$ subunits, leading to enhanced GRK2 translocation to membranes and GRK2-dependent β_2AR phosphorylation (which creates a docking site for β-arrestin and leads to βAR desensitization, Fig. 1b, Cong et al. 2001). However, β-arrestin (localized to the β_2AR-AKAP79/150 complex) also recruits the cAMP-specific phosphodiesterase enzyme PDE4D5, which functions locally to attenuate cAMP generation/PKA activation and prevent the PKA-dependent β_2AR switch to Gi-ERK (Perry et al. 2002a; Wallace et al. 2005). In neonatal cardiomyocytes, PDE4D activity limits signaling through the β_2AR-/PKA-dependent pathway that increases contraction (Xiang et al. 2005). In contrast, β_1AR-dependent regulation of cardiomyocyte contraction is not influenced by PDE4D (presumably due to the relatively low affinity of β_1ARs for β-arrestin and a relatively inefficient recruitment of β-arrestin-PDE4D complexes). While all PDE4 family members bind β-arrestin through a highly conserved site in the catalytic portion of the enzyme, PDE4D5 is preferentially recruited (relative to other PDE4 isoforms) to agonist-activated β_2ARs due to the presence of a unique N-terminal β-arrestin-binding site that allows for a second point of contact between these proteins (Perry et al. 2002). Of note, the PDE4D5 N-terminal region that binds β-arrestin also interacts with RACK1 (a WD repeat protein that acts as a scaffold for PKC). Since these are mutually exclusive binding interactions, the ability of PDE4D5 to toggle between β-arrestin and RACK1 is predicted to dictate signaling specificity (Yarwood et al. 1999; Bolger et al. 2003). Finally, there is evidence that AKAP79/150 interacts with both adenylyl cyclase V (ACV) and ACVI enzymes and assembles a local negative feedback loop to terminate cAMP signals by facilitating PKA-dependent AC-V-S^{676}/AC-VI-S^{674} phosphorylation (which inhibits cAMP generation, Fig. 1c, Bauman et al. 2006).

AKAP250/gravin is the second scaffold that nucleates β_2ARs-PKA-PKC-PP2B complexes (Fig. 1c, Shih et al. 1999). AKAP250/gravin binding has been mapped to residues R329-L413 in the C-terminal cytoplasmic tail of the β_2-AR; the β_2-AR-AKAP79/150-binding site has not been identified (Fan et al. 2001). While β_2-ARs constitutively interact with AKAP250/gravin in resting cells,

agonist activation strengthens βAR-AKAP250/gravin interactions as a result of PKA-dependent phosphorylation of AKAP250/gravin at S^{696}, S^{698}, and S^{772} (the β_2-AR docking site on AKAP250/gravin, Tao et al. 2003). AKAP250/gravin then inducibly associates with GRK2, β-arrestin, and clathrin; it functions as a mobile scaffold to internalize along with agonist-activated β_2ARs and control agonist-dependent βAR desensitization, sequestration, and recycling/resensitization (Lin et al. 2000; Fan et al. 2001). The recent observation that AKAP250/gravin also associates with PDE4D isoforms could suggest that this scaffold plays yet an additional role to terminate subplasmalemmal cAMP signals, although a specific role for AKAP250/gravin in the dynamic control of cAMP signals localized to the plasma membrane has not been established (Willoughby et al. 2006). Collectively, these studies indicate that β_2ARs associate with at least two distinct AKAPs (which assemble different subsets of second messenger-activated kinases and protein phosphatases); these βAR-AKAP complexes may reside in physically distinct subcellular compartments and differentially regulate cAMP signaling events that differ in time and space.

2 Scaffolding Functions for the Cardiac Z-Disc

The role of the sarcomeric Z disc in the assembly and three-dimensional organization of the sarcomere and the generation and transmission of force are well established. However, the large number of muscle-specific cardiac Z disc components recently identified has fueled interest in the cardiac Z disc as a mechanotransducer that senses and transmits signals arising from the force of normal cardiac contraction as well as the excessive stretch during volume or pressure overload. While a detailed discussion of the mechanosensing properties of the Z disc are beyond the scope of this review, this section will briefly discuss recent studies that implicate the cardiac Z disc as a scaffold for various signaling enzymes.

α-Actinin is a major constituent of Z discs that crosslinks actin and titin molecules from neighboring sarcomeres. Calsarsin is an α-actinin-binding protein that that also interacts with γ-filamin, T-Cap/telethonin, Cypher and the Ca^{2+}-sensitive phosphatase calcineurin at the cardiac Z disc (Frey et al. 2000). Calsarcin-1 is reported to negatively modulate calcineurin activity. Calsarcin-1-deficient mice develop an exaggerated hypertrophic response to calcineurin activation or pressure overload (but not other stimuli, Frey et al. 2004). These results indicate that calsarcin-1 is uniquely positioned to couple muscle activity to the regulation of calcineurin signaling and that calsarcin-1 deficiency sensitizes muscle cells to calcineurin signaling and selectively modulates the evolution of only certain pathological hypertrophy pathways. While the precise cellular targets of Z disc-associated calcineurin have not been identified, there is evidence that NFATc (one member of the Nuclear Factor of Activated T-Cells gene family) localizes to sarcomeric Z lines in unstimulated adult skeletal muscle fibers and translocates to the nucleus with certain forms of electrical stimulation (Liu et al. 2001). Studies to determine

whether calcium-dependent calcineurin activation might also lead to NFAT dephosphorylation in cardiomyocytes (providing a mechanism to unmask NFAT nuclear localization signals and increase of NFAT transcriptional activity in the nucleus) have not yet been published.

Obscurin is another Z disc protein that interacts with titin, the giant elastic filamentous sarcomeric protein that acts as a molecular ruler during sarcomeric assembly and maintains normal cardiac muscle cell length during contraction/relaxation cycles. The C-terminal region of obscurin contains both an IQ motif that is a binding site for calmodulin and a Rho-GEF domain that could activate Rho family small G proteins, suggesting an important heretofore unrecognized signaling function for obscurin in the sarcomere (Young et al. 2001)

Finally, Z discs have been identified as PKC isoform signaling domains. The mechanisms that localize and regulate PKC signaling at Z discs are discussed in the section that follows.

3 Scaffolds That Regulate PKC Signaling in Cardiomyocytes

PKCs localize to distinct intracellular sites via interactions with scaffolding proteins. The PKC-anchoring protein that has received most attention is the family of membrane-associated Receptors for Activated C Kinase (RACKs), molecular scaffolds that localize individual PKCs to distinct membrane microdomains in close proximity with their allosteric activators and unique intracellular substrates. RACK proteins share a seven-WD40-motif repeat structure, similar to the protein–protein-binding motifs found in heterotrimeric G-protein β subunits. To date, proteins with characteristics of RACKs (i.e. proteins that selectively/saturably bind only the active conformation of the cognate PKC isoform and recruit the enzyme in an active conformation to a specific membrane compartment) have been identified for PKCβ (RACK1) and PKCε (RACK2 or β'-COP; Mackay and Mochly-Rosen 2001; Schechtman and Mochly-Rosen 2001; Csukai et al. 1997). While PKCδ-binding proteins have been identified, the identity of the bona fide PKCδ RACK remains uncertain (Robles-Flores et al. 2002; Kheifets et al. 2006).

The current model holds that cells express a unique RACK (with a distinct subcellular localization) for each PKC isoform and that PKC-RACK interactions dictate isoform-specific cellular responses. According to this model, each PKC isoform contains both a RACK-binding sequence (that anchors the PKC to the RACK protein) and a sequence that mimics the PKC-binding site on the respective RACK protein (termed a ψRACK sequence). The RACK-binding site and the ψRACK sequence participate in an intramolecular interaction that maintains the enzyme in an inactive conformation at rest; this interaction must be disrupted for PKC activation. This model predicts that peptides based upon RACK-binding sites will competitively inhibit PKC docking to its specific membrane RACK and act as PKC isoform-selective translocation inhibitors. Similarly, peptides based upon ψRACK sequences will bind to the PKC-RACK-binding site, interfere with auto-

inhibitory (ψRACK sequence-RACK-binding site) intramolecular interactions, and destabilize the inactive “closed” conformation of the enzyme (i.e. act as allosteric PKC activators). Modulators of PKC translocation based upon PKC interactions with RACK scaffolds have been developed and offer certain theoretical advantages to the use of PKC overexpression strategies (that disturb the natural stoichiometry of a given PKC isoform to its upstream activators or downstream substrates) or conventional PKC activity inhibitors (which generally lack isoform specificity). However, there is ample evidence that PKCs interact with other anchoring proteins (such as AKAPs, caveolins, and various cytoskeletal proteins, see below) that are shown not be influenced by RACK-based translocation inhibitor peptides. Moreover, it is important to note that RACK functions are not limited to the control of PKC. Rather, RACK proteins have been implicated as scaffolds that organize signaling complexes involving Src family kinases, heterotrimeric G protein βγ subunits, dynamin, integrins, STAT1, the receptor protein tyrosine phosphatase PTPμ, and PDE4D5 (McCahill et al. 2002). The notion that peptides designed to modulate PKC-RACK binding may exert “off-target” actions on other RACK-based signaling pathways generally also is not considered.

Other true scaffolds that anchor PKC include AKAPs (which are discussed in the previous section) and caveolins. Caveolins are a family of three related isoforms that drive formation of caveolae, sphingolipid-/cholesterol-enriched buoyant detergent-resistant membranes that form flask-like invaginations of the plasma membrane. These morphologically distinct membrane structures (which biochemically resemble lipid rafts) form platforms that concentrate signaling complexes and play an important role in signal transduction pathways (note: the precise relationship between lipid rafts and caveolae remains the focus of considerable controversy; details are discussed by Patel et al. in a separate chapter in this volume). Caveolin-1 and the muscle-specific caveolin-3 (but not caveolin-2) are reported to physically interact with PKCα, PKCγ, and PKCζ (but not PKCε; Oka et al. 1997; Lin, Zhou et al. 2003), and there is evidence that caveolin-1 binding decreases PKCα and PKCζ activity. Caveolin-PKC interactions have been mapped to the caveolin scaffolding domain (a short membrane proximal region of the caveolin amino terminus that serves as a protein docking motif) and a caveolin interacting motif (ψ*X*ψ*XXXX*ψ or ψ*XXXX*ψ*XX*ψ, where ψ is a hydrophobic residue) found at similar locations in the kinase domains of PKCα and PKCζ. These results have been taken as evidence that caveolin-bound PKC represents a pool of membrane-associated enzyme that is inhibited at rest and is poised for activation (Oka et al. 1997). However, a role for caveolin proteins as scaffolds or regulators of PKC isoforms in caveolae membranes has never been directly established (and other mechanisms that might anchor/regulate PKCs at this destination also have been considered). cPKCs and nPKCs are recovered at rest or following agonist-activation in the caveolae fraction of several cell types (Muller et al. 1995). We previously demonstrated that caveolae isolated from resting neonatal rat cardiomyocytes contain little to no phorbol ester-sensitive PKC isoforms and that PKCα, PKCδ, and PKCε accumulate in cardiomyocyte caveolae in response to PMA (Rybin et al. 1999).

Finally, there is evidence that PKC localization is influenced by cytoskeletal and structural proteins. Several laboratories have reported that PKCε localizes to sarcomeric Z-discs, but the role of β'-COP as a Z disc scaffold is uncertain, since β'-COP has been characterized as a RACK that anchors activated PKCε to the Golgi in cardiomyocytes (Csukai et al. 1997). While mutagenesis studies map Z line binding to the PKCε-V1 domain (a region of PKCε that contains the RACK-binding site), more detailed studies suggest that PKCε anchors to Z lines via a mechanism that requires multiple points of contact (which is not necessarily restricted to the RACK-binding motif and may require both protein–protein and protein-lipid interactions, Robia et al. 2005). In this context, it is important to note that other components of the Z-disc with PKC-binding activity have been identified. Cypher is a striated muscle-specific protein with an N-terminal PDZ domain that interacts with α-actinin and a LIM-domain that binds PKCs (Zhou et al. 1999). CapZ is a capping protein that binds and anchors the barbed-end actin filaments. There is evidence that CapZ (rather than RACK1) targets PKCβII to the myofilaments and plays a key role in the transduction of PKC-dependent signals at Z-discs (Pyle et al. 2006); transgenic mice with decreased CapZ protein expression exhibit increased myofilament calcium sensitivity, a defect in PKC binding to cardiac myofilaments, and impaired PKC-dependent regulation of myofilament function (Pyle et al. 2002). Finally, actin (a sarcomeric protein that also constitutes a major component of the cytoskeletal network of non-muscle cells) interacts with a conformationally hidden actin-binding motif that lies in the PKC-ε regulatory region, between the two cysteine-rich C1 domain DAG/PMA binding sites. This region is exposed upon PKCε activation, leading to a PKCε-actin binding (an interaction that stabilizes PKCε in a catalytically active conformation, Prekeris et al. 1996).

4 Scaffolds That Influence Ion Channel Function

Ion channels form macromolecular complexes with a wide variety of proteins, modulating their function, localization and/or degradation. In the cardiovascular system, several unique ion-channel macromolecular complexes have been identified, which may enable the development of drugs that target specific ion channels. We will focus on evidence supporting the importance of several ion channel macromolecular complexes in modulating channel function in normal and cardiovascular diseases.

4.1 SCN5A (Voltage-Dependent Na^+ Channel)

Phase 0 (the “upstroke”) of the cardiac myocyte action potential is mediated by fast-activating voltage-gated sodium channels, which are preferentially localized near gap junctions (Kucera et al. 2002). The α subunit, $Na_v1.5$, is encoded by the gene *SCN5A*. The structure of this protein is described by four heterologous

domains (DI-DIV), each consisting of six transmembrane segments (S1–S6). Not surprisingly, a nine-base-pair deletion in this linker resulting in a triplet residue loss (ΔKPQ) is known to confer sustained sodium influx and subsequently delayed repolarization during the action potential, and it is one of the principally implicated mutations in the long QT syndrome variant LQT3 (named for all mutations in the *third* gene experimentally linked to the syndrome, *SCN5A*) (Kass and Moss 2006; Moss and Kass 2005). In contrast, reduced $Na_v1.5$ function is a cause of Brugada syndrome (BrS) (Antzelevitch and Fish 2006). βAR-mediated activation of PKA leads to an increased number of functional $Na_v1.5$ channels expressed at the membrane via phosphorylation at consensus sites S^{483}, S^{571}, and S^{593} in the DI-DII cytoplasmic linker (Zhou et al. 2002). In addition to the PKA-regulation of the Na sodium, SCN5A associates with calmodulin (CaM) and one of its effectors–Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Wagner et al. 2006). CaM binds to an isoleucine-glutamine ("IQ-") motif on the C-terminus of $Na_v1.5$ and promotes slow inactivation in response to activation by rapid Ca^{2+} cytosolic influx in the early action potential (Adams et al. 1990).

4.2 L-Type Ca^{2+} Channel ($Ca_v1.2$)

Phase 2 of the action potential, or the "plateau," refers to the period when K^+ efflux potassium channels are electrostatically balanced by persistent Na^+ influx from Na_v channels and Ca^{2+} influx from L-type voltage-dependent calcium channels (L-VDCC) as the process of excitation-contraction (EC) coupling begins. EC coupling proliferates as the initial increase in $[Ca^{2+}]_i$ induces sarcoplasmic reticular "calcium-induced calcium release (CICR)" through the cardiac ryanodine receptor, RyR2, in a feed-forward mechanism that eventually generates the actin-myosin contraction of the myocyte (Bodi et al. 2005). Cardiac L-VDCCs are heterotetrameric assemblies of α_1, α_2/δ, and β subunits.

The calcium-calmodulin complex has been shown to be a critical regulator of channel activation that binds to the C-terminus of $Ca_v1.2$ at an IQ motif. CaM constitutively binds the C-terminus of $Ca_v1.2$, changes conformation when bound to calcium, and subsequently causes inactivation of $Ca_v1.2$ during the action potential (Pitt et al. 2001). AKAP15/18 (which was independently cloned by the Catterall and Scott laboratories to be a 15- or 18-kDa protein; Gray et al. 1998; Fraser et al. 1998) is a lipid-modified protein that controls PKA-dependent mechanisms at the plasma membrane (Fig. 2). Early studies demonstrated that L-type Ca^{2+} channel regulation by PKA requires the presence of an AKAP, showing that submembrane targeting of PKA by AKAP facilitates channel phosphorylation (at both S^{1928} on the pore-forming α_{1c} subunit and on multiple sites on the accessory β_2 subunit), modifications that increase channel-open probability, increase inward calcium current, and prolong the plateau phase of the action potential (Gao et al. 1997; Gray et al. 1997). Similarly, β-AR stimulation of L-type Ca^{2+} channel activity in cardiomyocytes requires AKAP recruitment of PKA and could be

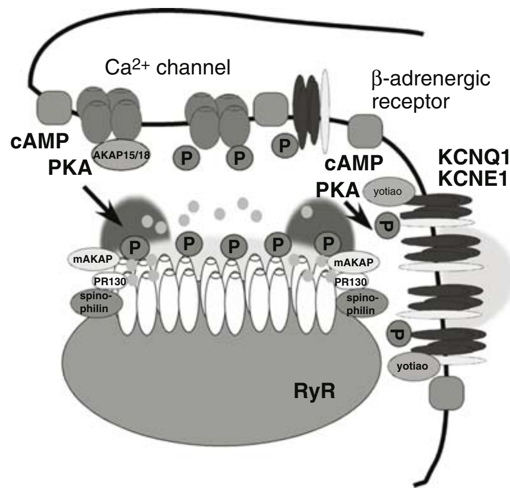

Fig. 2 Schematic representation of $Ca_v1.2$, KCNQ1, and RyR macromolecular complexes. PKA phosphorylates several cardiovascular ion channels, including $Ca_v1.2$, KCNQ1, and RyR, which associate with the PKA-binding scaffolding proteins AKAP15/18, yotiao, and mAKAP, respectively. KCNQ1 also associates with PP1 by binding yotiao. RyR2 binds to the PP1-binding protein spinophilin and to the PP2A-binding protein PR130

abrogated by microinjection of Ht31 in cardiomyocytes (Gao et al. 1997). Subsequent studies implicated AKAP15/18 as a scaffold that plays a role in potentiating cAMP-/PKA-dependent Ca^{2+} currents in HEK293 cells (Fraser et al. 1998). Biochemical and electrophysiological experiments have been used to map an AKAP15/18-L-type calcium-channel-binding interaction to amino acids 25–54 in the AKAP and leucine zipper motif in the C-terminus of the L-type calcium channel α_1-subunit (Hulme et al. 2002). Based upon this information, the Catterall laboratory has used a synthetic peptide based upon the AKAP15/18 calcium-channel-binding domain to inhibit voltage-dependent potentiation of L-type calcium-channel function in skeletal myotubes. This peptide also has been used to disrupt β-AR-dependent regulation of calcium-channel function in cardiomyocytes (Hulme et al. 2002, 2003). However, a RII-binding peptide termed Ht31 (since it was originally isolated from human thyroid; Carr et al. 1992) that is based upon the RII-binding portion of AKAP-Lbc (which is widely used to inhibit AKAP-RII interactions since it has a relatively high affinity for RII, relative to other AKAPs) also can be used to inhibit PKA-dependent potentiation of L-type Ca^{2+} channel function (Johnson et al. 1994). These results emphasize the importance of AKAP15/18 in L-type Ca^{2+} channel regulation by the small, localized increases in cAMP that typically are associated with GPCR activation, suggesting that AKAP15/18-dependent mechanisms might be targeted for therapeutic advantage. Finally, while most of the literature has focused on AKAP15/18 interactions with L-type calcium channels, there is some evidence that AKAP15/18 can also interact

with other ion channels (sodium channel, delayed rectifier potassium channels), β_2-ARs, and/or calcineurin (PP2B; Sacchetto et al. 2001), although the putative AKAP15/18 interactions with β_2-ARs or PP2B are based upon studies using subcellular fractionation techniques and may not necessarily reflect a direct protein–protein interaction. The role of AKAP15/18 to regulate other components of the β-AR complex deserves further study.

Recent work has also shown that the formation of a $Ca_v1.2$ macromolecular complex is spatially facilitated by the formation of caveolar complexes stabilized by caveolin-3 that can contain $Ca_v1.2$, β_2AR (not β_1AR), $G_{\alpha s}$, adenylyl cyclase, PKA, and PP2A all on the same plasma membrane microdomain (Balijepalli et al. 2006). In contrast to heart, in hippocampal neurons, AKAP79 co-targets PKA as well as the Ca^{2+}/CaM-activated phosphatase calcineurin (CaN) to the channel and allows for the two parallel pathways to adjust response. CaN dominantly suppresses PKA phosphorylation and subsequent channel activation in direct response to pulse frequency (not amplitude) of $Ca_v1.2$ current. In addition to effects on $Ca_v1.2$, CaN was also shown to dephosphorylate NFATc4 and allow its nuclear translocation, whereas inhibition of PKA binding to AKAP79 also partially increased translocation, suggesting that the PKA/CaN axis can also modulate nuclear transduction of calcium signaling (Oliveria et al. 2007).

4.3 Ryanodine Receptor (RyR2)

The rapid calcium influx of phase 2 of the cardiac myocyte action potential incites CICR from intracellularly sequestered, sarcoplasmic reticular calcium stores via (predominantly) ryanodine receptor (RyR)-mediated release. RyRs are the largest ion channels characterized to date: homotetramers consisting of four monomers that are each ~560 kDa and have large cytoplasmic domains (Wehrens and Marks, 2005; Santonastasi and Wehrens, 2007). These cytoplasmic domains also serve as the junctions at which diffusible regulatory proteins, such as FKBP12.6 (otherwise known as “calstabin2”), can potently modulate channel function. Calstabin2 binding to RyR2 can be physiologically regulated by PKA phosphorylation. In response to sympathetic stimulation, activation of the cAMP-PKA pathway leads to dissociation of FKBP12.6 from the complex, which provides a mechanism for modulating Ca^{2+} release from the SR. In failing hearts, PKA hyperphosphorylation of RyR leads to FKBP12.6 dissociation and abnormal channel function (Marx et al. 2000). Administration of metoprolol reverses the hyperphosphorylation, restoring the normal stoichiometry of the RyR macromolecular complex and normal channel function (Reiken et al. 2001).

RyR2 phosphorylation is potently modulated by macromolecular complexes (Fig. 2). These macromolecular complexes are formed by scaffolding proteins anchored at cytoplasmic domain LIZ. Through LIZ binding with the anchoring proteins spinophilin, PR130, and mAKAP, the phosphorylation state of RyR can be tuned by PP1, PP2A, and PKA/phosphodiesterase PDE4D3, respectively (Marx

et al. 2001b; Lehnart et al. 2005). In disease states such as heart failure, the amount of PP1, PP2A, and PDE4D3 associated with mAKAP is reduced, potentially accounting for the PKA hyperphosphorylation of RyR2 (Marx et al. 2000, 2001; Lehnart et al. 2005). The rebinding of calstabin2 to RyR2 is an important target for heart failure and arrhythmias. Administration of K201, also termed JTV519 (a derivative of the 1,4-benzothiazepine), can promote this rebinding and has been shown to reduce heart failure and arrhythmias in several animal models (Wehrens et al. 2004, 2006; Yano et al. 2003).

4.4 KCNQ1

Phase 3 of the action potential occurs as the L-type calcium current dissipates, and the membrane potential is predominantly repolarized by fast and slow components of the delayed rectifier potassium current. KCNQ1 is the channel responsible for the slow component (I_{Ks}) and was also the first protein linked to the long QT syndrome: LQT1 (Kass and Moss 2006; Moss and Kass 2005). The channel is thought to exist as a complex of four pore-forming KCNQ1 monomers (each with six transmembrane domains and a pore-forming region) and at least two accessory KCNE1 subunits that confer slower activation/deactivation kinetics, rightward shift of voltage-dependent activation, and increase in conductance.

Sympathetic stimulation of β-AR slows deactivation and causes a leftward shift in the voltage-dependent activation of I_{Ks} through PKA phosphorylation of N-terminal S^{27} proximal to the first transmembrane domain of KCNQ1 (Kurokawa et al. 2003). Yotiao is an AKAP that binds to a C-terminal LIZ in KCNQ1 and forms the scaffold of a macromolecular complex that colocalizes protein phosphatase 1 (PP1) and PKA in another example of bidirectional regulation (Marx et al. 2002) (Fig. 2). A naturally occurring mutation within the KCNQ1 LIZ motif (G589D) is associated with reduced PKA-mediated regulation of KCNQ1 (Marx et al. 2002). Pharmacologic βAR blockade confers a survival benefit in KCNQ1-associated long QT syndrome variants versus those with LQT3 (SCN5A mutations) (Priori et al. 2004).

5 Scaffolds That Regulate Cardiac Hypertrophy

5.1 mAKAP-PKA-PDE4D3 Complexes

Several AKAPs have been implicated in the coordination of cardiac hypertrophy. The muscle-specific AKAP (mAKAP, the 255-kDa muscle A-kinase anchoring protein) is a multivalent scaffold that binds PDE4D3 (and other signaling proteins, see below), is tethered to the nuclear envelope (through a protein–protein interaction with the integral nuclear membrane protein nesprin-1α), and plays a crucial

role to synchronize PKA and PDE4D3 activity in cardiomyocytes (Fig. 3; Pare et al. 2005; Dodge-Kafka et al. 2005; Dodge et al. 2001). PDE4D3 accelerates local cAMP breakdown and tonically prevents a cAMP increase to a threshold level that would activate mAKAP-tethered PKA in resting cardiomyocytes. A βAR agonist-dependent rise in cAMP/PKA leads to PDE4D3 phosphorylation at S^{54} (which induces a two- to three-fold increase in PDE activity) and S^{13} (which increases PDE4D3 binding affinity for mAKAP), setting up a negative feedback loop that restores cAMP to low basal levels (Sette and Conti 1996; Carlisle et al. 2004). Experiments with fluorescent reporters of PKA activity provide compelling evidence that PDE4D3 plays a critical role to terminate signaling by AKAP-anchored PKA (Dodge-Kafka et al. 2005).

While the synergistic actions of PKA and PDE4D3 lead to localized pulses of cAMP at the nuclear membrane, their actions are counterbalanced by Extracellular signal-Regulated Kinase 5/big mitogen-activated protein kinase 1 (ERK5/BMK1), a cytokine-activated serine/threonine kinase that has been implicated in the induction of cardiac hypertrophy as well as in antiapoptotic mechanisms in vitro in cardiomyocytes cultures (during oxidative stress or during overexpression of the constitutively active form of MEK5α) and cardioprotection in vivo in the ischemic/reperfused intact heart (Suzaki et al. 2002; Cameron et al. 2004). ERK5 indirectly anchors to mAKAP (through PDE4D3) and phosphorylates PDE4D3 at S^{579}, leading to suppressed PDE activity (Hoffmann et al. 1999). cAMP acts reciprocally to

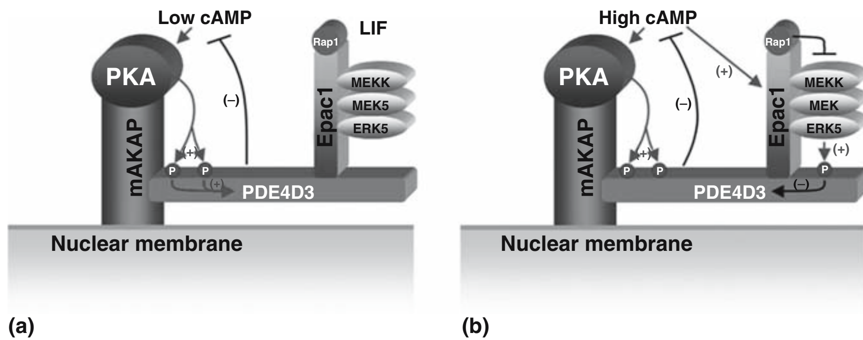

Fig. 3 mAKAP binds PKA, anchors PDE4D3, and serves as an adaptor to recruit Epac1 and ERK5. **(a)** A modest rise in cAMP activates PKA (but not Epac1), leading to the phosphorylation of PDE4D3 at S^{13} (which increases PDE4D3 affinity for mAKAP) and S^{54} (which increase PDE4D3 activity). These PKA-dependent phosphorylations limit a local increase in cAMP and restrict mAKAP-anchored PKA activity. cAMP does not activate Epac1; there is no Epac1-dependent suppression of ERK5. The cytokine- (LIF-) dependent pathway leading to mobilization of the MEKK-MEK5-ERK5 pathway and cardiac hypertrophy is intact under these conditions. **(b)** Stimuli that activate ERK5 lead to PDE4D3-S^{579} phosphorylation and decreased PDE4D3 activity, leading to a local rise in cAMP and activation of Epac1. At high cAMP, activation of Epac1 and Rap1 suppresses ERK5 and abrogates ERK5-dependent inhibition of PDE4D3, permitting cAMP to return to low basal levels. The effect of the cAMP-Epac1-Rap1 pathway to inhibit ERK5 also suppresses LIF-dependent cardiac hypertrophy. mAKAP dephosphorylation by PP2A (which also is identified in mAKAP complexes) also contributes to the local control

inhibit mAKAP-anchored ERK5. Importantly, this inhibitory effect of cAMP is mediated through a PKA-independent mechanism involving Epac1 (a cAMP-dependent-GEF for the small Ras-related GTP binding proteins Rap1 and Rap2), which also localizes to the nuclear membrane and is recruited by PDE4D3 to the mAKAP complex (Dodge-Kafka et al. 2005). The ramifications of heart failure-dependent changes in ERK5 activation and its control by the cAMP-Epac1 signaling pathway have not been examined in any detail, but may be significant, given evidence that human heart failure is characterized by high levels of cAMP and decreased ERK5 expression, which would effectively prevent cardioprotection via this pathway (Takeishi et al. 2001).

Finally, it is worth noting that the presence of three cAMP effectors (with markedly different affinities for cAMP) on a single mAKAP scaffold might provide a mechanism to influence the timing of cAMP signals at the nuclear membrane. PKA (which is responsive to nM cAMP) is predicted to be activated more rapidly and persistently than PDE4D3 or Epac1 (which bind cAMP with much lower micromolar affinity (Dodge-Kafka et al. 2005).

5.2 AKAP-Lbc and the Regulation of Rho and PKD

AKAP-Lbc is the second AKAP that is abundant in cardiac tissue and has been implicated in hypertrophic signaling pathways involving Rho and PKD (Fig. 4). AKAP-Lbc is the full-length form of the PKA-binding fragment Ht31 that was first isolated from human thyroid and has been widely used as a prototype to study the structural basis of PKA-AKAP interactions (and the physiologic importance of AKAP-mediated PKA anchoring in physiologic responses). AKAP-Lbc is a large 320-kDa modular protein with an N-terminal regulatory region that contains ankyrin repeats, a leucine zipper motif, sites for PKA and 14-3-3 binding and a C1 domain (homologous to the highly conserved compact DAG/PMA-binding site that was first identified in PKCs), followed by adjacent DH and PH domains [a characteristic structural feature of Rho guanine nucleotide exchange factors (GEFs)] and a second regulatory region that binds PKD at the C-terminus. AKAP-Lbc Rho-GEF activity is increased by GPCRs that couple to the heterotrimeric G_{12} proteins, $G\alpha_{12}$ overexpression, or by serum (Diviani et al. 2001, 2004). Recent studies implicate AKAP-Lbc in α_1-AR-dependent activation of Rho and induction of the hypertrophic phenotype in neonatal cardiomyocyte cultures (Appert-Collin et al. 2007). The observation that chronic α_1-AR activation leads to increased AKAP-Lbc expression (which augments α_1-AR-dependent activation of Rho) has been taken as tentative evidence that dynamic changes in AKAP-Lbc expression might amplify the early molecular events that contribute to the pathogenesis of cardiac hypertrophy/failure (i.e. that AKAP-Lbc might constitute a rather attractive target for therapeutic intervention).

AKAP-Lbc-Rho-GEF activity is autoinhibited in the basal state through intramolecular interactions with N- and/or C-terminal sequences; a truncation form

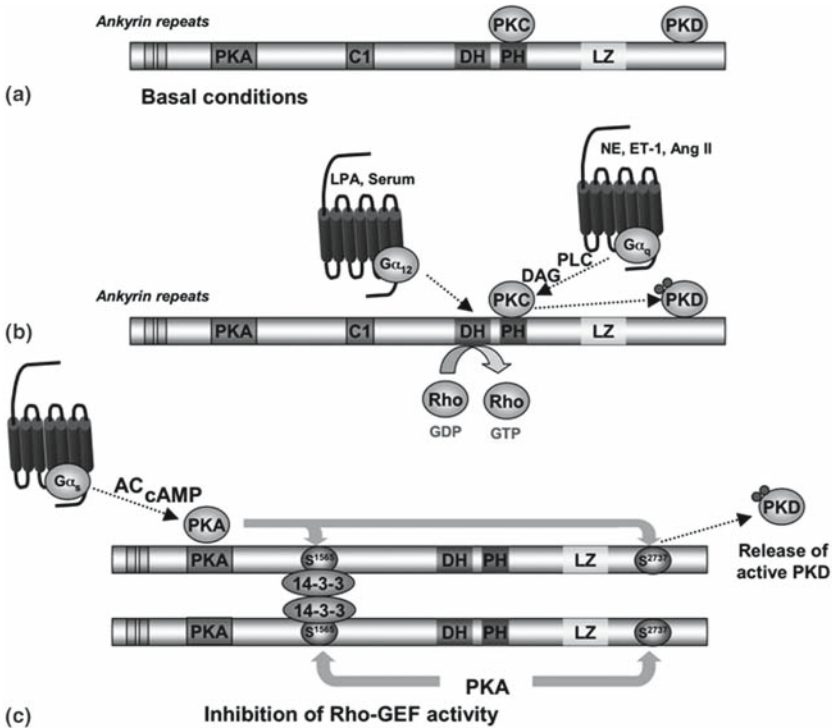

Fig. 4 mAKAP binds PKA, anchors PDE4D3, and serves as an adaptor to recruit Epac1 and ERK5. (**a**) Schematic representation and protein domain organization of AKAP-Lbc, which includes two ankyrin repeats, a PKA-binding domain, a C1 domain, and both Dbl (DH) and pleckstrin (PH) homology domains. (**b**) Various Gα12-coupled receptors enhance the Rho-GEF activity of AKAP-Lbc, leading to increased GDP-GTP exchange on RhoA and the activation of downstream effectors that remodel the actin cytoskeleton and influence gene transcription. AKAP-Lbc also localizes PKD with its upstream activator PKC (which interacts with the AKAP-Lbc-PH domain). (**c**) PKA anchored to AKAP-Lbc executes phosphorylations that inhibit AKAP-Lbc-Rho GEF activity and release active PKD from the AKAP-Lbc complex

of AKAP-Lbc encoding the DH- and PH domains without regulatory sequences (termed Onco-Lbc) is an oncogene in NIH 3T3 cells (Sterpetti et al. 1999). Recent studies implicate leucine zipper-mediated AKAP-Lbc homo-oligomerization and PKA-dependent phosphorylation of AKAP-Lbc at S^{1565} as a mechanism that decreases AKAP-Lbc-Rho-GEF activity (Baisamy et al. 2005; Diviani et al. 2004). While the AKAP-Lbc-***R***RHS***^{1565}***WG***P***GK site diverges somewhat from a canonical 14-3-3 binding site [RSXpSXP or RXXXpSXP], it nevertheless mediates 14-3-3 protein binding. Since 14-3-3 proteins typically assume a dimeric structure, 14-3-3 binding promotes AKAP-Lbc homo-dimerization and imposes a conformational constraint that inhibits Rho-GEF activity (Baisamy et al. 2005). In this manner, AKAP-Lbc anchored PKA antagonizes Rho signaling in cells.

AKAP-Lbc also has been implicated in the activation of PKD, a serine/threonine kinase that has recently emerged as a biologically important mediator of cardiac hypertrophy/remodeling. PKD phosphorylates class II HDACs (effectively neutralizing HDAC5's repressive effects on MEF2-dependent transcription and inhibiting HDAC5's antihypertrophic actions), cardiac troponin I, and CREB (Vega et al. 2004; Haworth et al. 2004; Harrison et al. 2006). PKD activation is via translocation to membranes and PKC-dependent phosphorylation of S^{744}/S^{748} in the activation loop. There is recent evidence that the AKAP-Lbc-PH domain binds certain PKC isoforms (PKCα and PKCη) (Carnegie et al. 2004) and that AKAP-Lbc coordinates PKC-dependent PKD activation in cells (Carnegie et al. 2004). PKA contributes to the activation of AKAP-Lbc-anchored PKD by phosphorylating AKAP-Lbc at S^{2737} in the PKD-binding region that releases newly phosphorylated PKD to the cytosol.

6 Conclusion

The role of molecular scaffolds in the regulation of signaling pathways is a relatively new concept that offers a host of heretofore unanticipated therapeutic approaches for the therapy of cardiovascular disorders. This concept has been reinforced by recent evidence that genetic abnormalities of scaffolding proteins may underlie certain cardiac disorders. For example, recent studies identify a polymorphism in a human AKAP gene that leads to marked differences in this protein's affinity for PKA and is associated with cardiac rhythm disturbances, including a decreased PR interval on the ECG, an increase in basal heart rate, and decreased heart rate variability (a finding that is characteristic of decreased vagal tone and has been viewed as a predictor of sudden cardiac death; Tingley et al. 2007; Kammerer et al., 2003). Modulation of proteins that control important signaling pathways in the heart provides an alternate therapeutic strategy to influence the incidence and/or progression of various cardiac disorders.

Acknowledgments This work was supported by U.S.P.H.S.-N.H.L.B.I. grants HL-77860 (SFS), HL-74161 (SFS) and HL-68093 (SOM), and American Heart Association Established Investigator Award (SOM).

References

Adams BA, Tanabe T, Mikami A, Numa S, Beam KG (1990) Intramembrane charge movement restored in dysgenic skeletal muscle by injection of dihydropyridine receptor cDNAs. Nature 346:569–572

Antzelevitch C, Fish JM (2006) Therapy for the Brugada syndrome. Handbook Exp Pharmacol 305–330

Appert-Collin A, Cotecchia S, Nenniger-Tosato M, Pedrazzini T, Diviani D (2007) The A-kinase anchoring protein (AKAP)-Lbc-signaling complex mediates α_1-adrenergic receptor-induced cardiomyocyte hypertrophy. Proc Natl Acad Sci USA 104:10140–10145

Aprigliano O, Rybin VO, Pak E, Robinson RB, Steinberg SF (1997) β_1- and β_2-Adrenergic receptors exhibit differing susceptibility to muscarinic accentuated antagonism. Am J Physiol 272: H2726–H2735

Baisamy L, Jurisch N, Diviani D (2005) Leucine zipper-mediated homo-oligomerization regulates the Rho-GEF activity of AKAP-Lbc. J Biol Chem 280:15405–15412

Balijepalli RC, Foell JD, Hall DD, Hell JW, Kamp TJ (2006) Localization of cardiac L-type Ca^{2+} channels to a caveolar macromolecular signaling complex is required for β_2-adrenergic regulation. Proc Natl Acad Sci USA 103:7500–7505

Bauman AL, Soughayer J, Nguyen BT, Willoughby D, Carnegie GK, Wong W, Hoshi N, Langeberg LK, Cooper DM, Dessauer CW, Scott JD (2006) Dynamic regulation of cAMP synthesis through anchored PKA-adenylyl cyclase V/VI complexes. Mol Cell 23: 925–931

Bodi I, Mikala G, Koch SE, Akhter SA, Schwartz A (2005) The L-type calcium channel in the heart: the beat goes on. J Clin Invest 115:3306–3317

Bolger GB, McCahill A, Huston E, Cheung YF, McSorley T, Baillie GS, Houslay MD (2003) The unique amino-terminal region of the PDE4D5 cAMP phosphodiesterase isoform confers preferential interaction with beta-arrestins. J Biol Chem 278:49230–49238

Cameron SJ, Itoh S, Baines CP, Zhang C, Ohta S, Che W, Glassman M, Lee JD, Yan C, Yang J, Abe J (2004) Activation of big MAP kinase 1 (BMK1/ERK5) inhibits cardiac injury after myocardial ischemia and reperfusion. FEBS Lett 566:255–260

Cant SH, Pitcher JA (2005) G protein-coupled receptor kinase 2-mediated phosphorylation of ezrin is required for G protein-coupled receptor-dependent reorganization of the actin cytoskeleton. Mol Biol Cell 16:3088–3099

Cao TT, Deacon HW, Reczek D, Bretscher A, von Zastrow M (1999) A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β_2-adrenergic receptor. Nature 401:286–290

Carlisle Michel JJ, Dodge KL, Wong W, Mayer NC, Langeberg LK, Scott JD (2004) PKA-phosphorylation of PDE4D3 facilitates recruitment of the mAKAP signalling complex. Biochem J 381:587–592

Carlson CR, Lygren B, Berge T, Hoshi N, Wong W, Tasken K, Scott JD (2006) Delineation of type I protein kinase A-selective signaling events using an RI anchoring disruptor. J Biol Chem 281:21535–21545

Carnegie GK, Smith FD, McConnachie G, Langeberg LK, Scott JD (2004) AKAP-Lbc nucleates a protein kinase D activation scaffold. Mol Cell 15:889–899

Carr DW, Hausken ZE, Fraser ID, Stofko-Hahn RE, Scott JD (1992) Association of the type II cAMP-dependent protein kinase with a human thyroid RII-anchoring protein. Cloning and characterization of the RII-binding domain. J Biol Chem 267:13376–13382

Cong M, Perry SJ, Lin FT, Fraser ID, Hu LA, Chen W, Pitcher JA, Scott JD, Lefkowitz RJ (2001) Regulation of membrane targeting of the G protein-coupled receptor kinase 2 by protein kinase A and its anchoring protein AKAP79. J Biol Chem 276:15192–15199

Csukai M, Chen CH, De Matteis MA, Mochly-Rosen D (1997) The coatomer protein β'-COP, a selective binding protein (RACK) for protein kinase Cε. J Biol Chem 272:29200–29206

Diviani D, Soderling J, Scott JD (2001) AKAP-Lbc anchors protein kinase A and nucleates $G\alpha_{12}$-selective Rho-mediated stress fiber formation. J Biol Chem 276:44247–44257.

Diviani D, Abuin L, Cotecchia S, Pansier L (2004) Anchoring of both PKA and 14-3-3 inhibits the Rho-GEF activity of the AKAP-Lbc signaling complex. EMBO J 23:2811–2820

Dodge KL, Khouangsathiene S, Kapiloff MS, Mouton R, Hill EV, Houslay MD, Langeberg LK, Scott JD (2001) mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module. EMBO J 20:1921–1930

Dodge-Kafka KL, Soughayer J, Pare GC, Carlisle Michel JJ, Langeberg LK, Kapiloff MS, Scott JD (2005) The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. Nature 437:574–578

Dransfield DT, Bradford AJ, Smith J, Martin M, Roy C, Mangeat PH, Goldenring JR (1997) Ezrin is a cyclic AMP-dependent protein kinase anchoring protein. EMBO J 16:35–43

Fan G, Shumay E, Wang H, Malbon CC (2001) The scaffold protein gravin (cAMP-dependent protein kinase-anchoring protein 250) binds the β_2-adrenergic receptor via the receptor cytoplasmic Arg-329 to Leu-413 domain and provides a mobile scaffold during desensitization. J Biol Chem 276:24005–24014

Fraser ID, Tavalin SJ, Lester LB, Langeberg LK, Westphal AM, Dean RA, Marrion NV, Scott JD (1998) A novel lipid-anchored A-kinase anchoring protein facilitates cAMP-responsive membrane events. EMBO J 17:2261–2272

Fraser ID, Cong M, Kim J, Rollins EN, Daaka Y, Lefkowitz RJ, Scott JD (2000) Assembly of an A kinase-anchoring protein-β_2-adrenergic receptor complex facilitates receptor phosphorylation and signaling. Curr Biol 10:409–412

Frey N, Richardson JA, Olson EN (2000) Calsarcins, a novel family of sarcomeric calcineurin-binding proteins. Proc Natl Acad Sci USA 97:14632–14637

Frey N, Barrientos T, Shelton JM, Frank D, Rutten H, Gehring D, Kuhn C, Lutz M, Rothermel B, Bassel-Duby R, Richardson JA, Katus HA, Hill JA, Olson EN (2004) Mice lacking calsarcin-1 are sensitized to calcineurin signaling and show accelerated cardiomyopathy in response to pathological biomechanical stress. Nat Med 10:1336–1343

Gage RM, Kim KA, Cao TT, von Zastrow M (2001) A transplantable sorting signal that is sufficient to mediate rapid recycling of G protein-coupled receptors. J Biol Chem 276:44712–44720

Gao T, Yatani A, Dell'Acqua ML, Sako H, Green SA, Dascal N, Scott JD, Hosey MM (1997) cAMP-dependent regulation of cardiac L-type Ca^{2+} channels requires membrane targeting of PKA and phosphorylation of channel subunits. Neuron 19:185–196

Gardner LA, Naren AP, Bahouth SW (2007) Assembly of an SAP97-AKAP79-cAMP-dependent protein kinase scaffold at the type 1 PSD-95/DLG/ZO1 motif of the human β_1-adrenergic receptor generates a receptosome involved in receptor recycling and networking. J Biol Chem 282:5085–5099

Gray PC, Tibbs VC, Catterall WA, Murphy BJ (1997) Identification of a 15-kDa cAMP-dependent protein kinase-anchoring protein associated with skeletal muscle L-type calcium channels. J Biol Chem 272:6297–6302

Gray PC, Johnson BD, Westenbroek RE, Hays LG, Yates JR III, Scheuer T, Catterall WA, Murphy BJ (1998) Primary structure and function of an A kinase anchoring protein associated with calcium channels. Neuron 20:1017–1026

Hall RA, Ostedgaard LS, Premont RT, Blitzer JT, Rahman N, Welsh MJ, Lefkowitz RJ (1998a) A C-terminal motif found in the β_2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na^+/H^+ exchanger regulatory factor family of PDZ proteins. Proc Natl Acad Sci USA 95:8496–8501

Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, Claing A, Stoffel RH, Barak LS, Shenolikar S, Weinman EJ, Grinstein S, Lefkowitz RJ (1998b) The β_2-adrenergic receptor interacts with the Na^+/H^+-exchanger regulatory factor to control Na^+/H^+ exchange. Nature 392:626–630

Harrison BC, Kim MS, van Rooij E, Plato CF, Papst PJ, Vega RB, McAnally JA, Richardson JA, Bassel-Duby R, Olson EN, McKinsey TA (2006) Regulation of cardiac stress signaling by protein kinase D1. Mol Cell Biol 26:3875–3888

Haworth RS, Cuello F, Herron TJ, Franzen G, Kentish JC, Gautel M, Avkiran M (2004) Protein kinase D is a novel mediator of cardiac troponin I phosphorylation and regulates myofilament function. Circ Res 95:1091–1099

He J, Bellini M, Xu J, Castleberry AM, Hall RA (2004) Interaction with cystic fibrosis transmembrane conductance regulator-associated ligand (CAL) inhibits β_1-adrenergic receptor surface expression. J Biol Chem 279:50190–50196

He J, Bellini M, Inuzuka H, Xu J, Xiong Y, Yang X, Castleberry AM, Hall RA (2006) Proteomic analysis of β_1-adrenergic receptor interactions with PDZ scaffold proteins. J Biol Chem 281:2820–2827

Hoffmann R, Baillie GS, MacKenzie SJ, Yarwood SJ, Houslay MD (1999) The MAP kinase ERK2 inhibits the cyclic AMP-specific phosphodiesterase HSPDE4D3 by phosphorylating it at Ser^{579}. EMBO J 18:893–903

Hu LA, Tang Y, Miller WE, Cong M, Lau AG, Lefkowitz RJ, Hall RA (2000) β_1-adrenergic receptor association with PSD-95. Inhibition of receptor internalization and facilitation of β_1-adrenergic receptor interaction with *N*-methyl-*D*-aspartate receptors. J Biol Chem 275:38659–38666
Hu LA, Chen W, Premont RT, Cong M, Lefkowitz RJ (2002) G protein-coupled receptor kinase 5 regulates β_1-adrenergic receptor association with PSD-95. J Biol Chem 277:1607–1613
Hu LA, Chen W, Martin NP, Whalen EJ, Premont RT, Lefkowitz RJ (2003) GIPC interacts with the b_{1-}adrenergic receptor and regulates β_1-adrenergic receptor-mediated ERK activation. J Biol Chem 278:26295–26301
Hulme JT, Ahn M, Hauschka SD, Scheuer T, Catterall WA (2002) A novel leucine zipper targets AKAP15 and cyclic AMP-dependent protein kinase to the C terminus of the skeletal muscle Ca^{2+} channel and modulates its function. J Biol Chem 277:4079–4087
Hulme JT, Lin TW, Westenbroek RE, Scheuer T, Catterall WA (2003) Beta-adrenergic regulation requires direct anchoring of PKA to cardiac CaV1.2 channels via a leucine zipper interaction with A kinase-anchoring protein 15. Proc Natl Acad Sci USA 100:13093–13098
Johnson BD, Scheuer T, Catterall WA (1994) Voltage-dependent potentiation of L-type Ca^{2+} channels in skeletal muscle cells requires anchored cAMP-dependent protein kinase. Proc Natl Acad Sci USA 91:11492–11496
Jurevicius J, Fischmeister R (1996) cAMP compartmentation is responsible for a local activation of cardiac Ca^{2+} channels by β-adrenergic agonists. Proc Natl Acad Sci USA 93:295–299
Kammerer S, Burns-Hamuro LL, Ma Y, Hamon SC, Canaves JM, Shi MM, Nelson MR, Sing CF, Cantor CR, Taylor SS, Braun A (2003) Amino acid variant in the kinase binding domain of dual-specific A kinase-anchoring protein 2: a disease susceptibility polymorphism. Proc Natl Acad Sci USA 100:4066–4071
Kass RS, Moss AJ (2006) Mutation-specific pharmacology of the long QT syndrome. Handbook Exp Pharmacol 287–304
Kheifets V, Bright R, Inagaki K, Schechtman D, Mochly-Rosen D (2006) Protein kinase C delta (δPKC)-annexin V interaction: a required step in δPKC translocation and function. J Biol Chem 281:23218–23226
Kucera JP, Rohr S, Rudy Y (2002) Localization of sodium channels in intercalated disks modulates cardiac conduction. Circ Res 91:1176–1182
Kurokawa J, Chen L, Kass RS (2003) Requirement of subunit expression for cAMP-mediated regulation of a heart potassium channel. Proc Natl Acad Sci USA 100:2122–2127
Lehnart SE, Wehrens XH, Reiken S, Warrier S, Belevych AE, Harvey RD, Richter W, Jin SL, Conti M, Marks AR (2005) Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias. Cell 123:25–35
Lin F, Wang H, Malbon CC (2000) Gravin-mediated formation of signaling complexes in β_2-adrenergic receptor desensitization and resensitization. J Biol Chem 275:19025–19034
Lin D, Zhou J, Zelenka PS, Takemoto DJ (2003) Protein kinase Cγ regulation of gap junction activity through caveolin-1-containing lipid rafts. Invest Ophthalmol Vis Sci 44: 5259–5268
Liu Y, Cseresnyes Z, Randall WR, Schneider MF (2001) Activity-dependent nuclear translocation and intranuclear distribution of NFATc in adult skeletal muscle fibers. J Cell Biol 155:27–39
Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ (1999) β-Arrestin-dependent formation of β_2 adrenergic receptor-Src protein kinase complexes. Science 283:655–661
Mackay K, Mochly-Rosen D (2001) Localization, anchoring, and functions of protein kinase C isozymes in the heart. J Mol Cell Cardiol 33:1301–1307
Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosemblit N, Marks AR (2000) PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. Cell 101:365–376
Marx SO, Reiken S, Hisamatsu Y, Gaburjakova M, Gaburjakova J, Yang YM, Rosemblit N, Marks AR (2001) Phosphorylation-dependent regulation of ryanodine receptors: a novel role for leucine/isoleucine zippers. J Cell Biol 153:699–708

Marx SO, Kurokawa J, Reiken S, Motoike H, D'Armiento J, Marks AR, Kass RS (2002) Requirement of a macromolecular signaling complex for beta adrenergic receptor modulation of the KCNQ1-KCNE1 potassium channel. Science (New York, NY) 295:496–499

McCahill A, Warwicker J, Bolger GB, Houslay MD, Yarwood SJ (2002) The RACK1 scaffold protein: a dynamic cog in cell response mechanisms. Mol Pharmacol 62:1261–1273

McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ, Lefkowitz RJ (2000) Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. Science 290:1574–1577

Miller WE, Maudsley S, Ahn S, Khan KD, Luttrell LM, Lefkowitz RJ (2000) β-Arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of b-arrestin1-dependent targeting of c-SRC in receptor endocytosis. J Biol Chem 275:11312–11319

Moss AJ, Kass RS (2005) Long QT syndrome: from channels to cardiac arrhythmias. J Clin Invest 115:2018–2024

Muller G, Ayoub M, Storz P, Rennecke J, Fabbro D, Pfizenmaier K (1995) PKC-ζ is a molecular switch in signal transduction of TNF-alpha, bifunctionally regulated by ceramide and arachidonic acid. EMBO J 14:1961–1969

Oka N, Yamamoto M, Schwencke C, Kawabe JI, Ebina T, Ohno S, Couet J, Lisanti MP, Ishikawa Y (1997) Caveolin interaction with protein kinase C: isoenzyme-dependent regulation of kinase activity by the caveolin scaffolding domain peptide. J Biol Chem 272:33416–33421

Oliveria SF, Dell'Acqua ML, Sather WA (2007) AKAP79/150 anchoring of calcineurin controls neuronal L-type Ca^{2+} channel activity and nuclear signaling. Neuron 55:261–275

Pare GC, Easlick JL, Mislow JM, McNally EM, Kapiloff MS (2005) Nesprin-1α contributes to the targeting of mAKAP to the cardiac myocyte nuclear envelope. Exp Cell Res 303:388–399

Perry SJ, Baillie GS, Kohout TA, McPhee I, Magiera MM, Ang KL, Miller WE, McLean AJ, Conti M, Houslay MD, Lefkowitz RJ (2002) Targeting of cyclic AMP degradation to beta 2-adrenergic receptors by beta-arrestins. Science 298:834–836

Pitt GS, Zuhlke RD, Hudmon A, Schulman H, Reuter H, Tsien RW (2001) Molecular basis of calmodulin tethering and Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels. J Biol Chem 276:30794–30802

Prekeris R, Mayhew MW, Cooper JB, Terrian DM (1996) Identification and localization of an actin-binding motif that is unique to the epsilon isoform of protein kinase C and participates in the regulation of synaptic function. J Cell Biol 132:77–90

Priori SG, Napolitano C, Schwartz PJ, Grillo M, Bloise R, Ronchetti E, Moncalvo C, Tulipani C, Veia A, Bottelli G, Nastoli J (2004) Association of long QT syndrome loci and cardiac events among patients treated with beta-blockers. JAMA 292:1341–1344

Pyle WG, La Rotta G, de Tombe PP, Sumandea MP, Solaro RJ (2006) Control of cardiac myofilament activation and PKC-bII signaling through the actin capping protein, CapZ. J Mol Cell Cardiol 41:537–543

Pyle WG, Hart MC, Cooper JA, Sumandea MP, de Tombe PP, Solaro RJ (2002) Actin capping protein: an essential element in protein kinase signaling to the myofilaments. Circ Res 90:1299–1306

Reiken S, Gaburjakova M, Gaburjakova J, He Kl KL, Prieto A, Becker E, Yi Gh GH, Wang J, Burkhoff D, Marks AR (2001) Beta-adrenergic receptor blockers restore cardiac calcium release channel (ryanodine receptor) structure and function in heart failure. Circulation 104:2843–2848

Robia SL, Kang M, Walker JW (2005) Novel determinant of PKC-ε anchoring at cardiac Z-lines. Am J Physiol Heart Circ Physiol 289:H1941–H1950

Robles-Flores M, Rendon-Huerta E, Gonzalez-Aguilar H, Mendoza-Hernandez G, Islas S, Mendoza V, Ponce-Castaneda MV, Gonzalez-Mariscal L, Lopez-Casillas F (2002) p32 (C1qBP) is a general protein kinase C (PKC)-binding protein. J Biol Chem 277:5247–5255

Rybin VO, Xu X, Steinberg SF (1999) Activated protein kinase C isoforms target to cardiomyocyte caveolae. Circ Res 84:980–988

Rybin VO, Xu X, Lisanti MP, Steinberg SF (2000) Differential targeting of β-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae: A mechanism to functionally regulate the cAMP signaling pathway. J Biol Chem 275:41447–41457

Sacchetto R, Damiani E, Margreth A (2001) Clues to calcineurin function in mammalian fast-twitch muscle. J Muscle Res Cell Motil 22:545–559

Santonastasi M, Wehrens XH (2007) Ryanodine receptors as pharmacological targets for heart disease. Acta Pharmacologica Sinica 28:937–944

Schechtman D, Mochly-Rosen D (2001) Adaptor proteins in protein kinase C-mediated signal transduction. Oncogene 20:6339–6347

Sette C, Conti M (1996) Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. Involvement of serine 54 in the enzyme activation. J Biol Chem 271:16526–16534

Shcherbakova OG, Hurt CM, Xiang Y, Dell'Acqua ML, Zhang Q, Tsien RW, Kobilka BK (2007) Organization of beta-adrenoceptor signaling compartments by sympathetic innervation of cardiac myocytes. J Cell Biol 176:521–533

Shih M, Lin F, Scott JD, Wang HY, Malbon CC (1999) Dynamic complexes of β_2-adrenergic receptors with protein kinases and phosphatases and the role of gravin. J Biol Chem 274:1588–1595

Stanasila L, Abuin L, Diviani D, Cotecchia S (2006) Ezrin directly interacts with the α_{1b}-adrenergic receptor and plays a role in receptor recycling. J Biol Chem 281:4354–4363

Steinberg SF, Brunton LL (2001) Compartmentalization of G-protein-mediated signal transduction components in the heart. Ann Rev Pharm Tox 41:751–773

Sterpetti P, Hack AA, Bashar MP, Park B, Cheng SD, Knoll JH, Urano T, Feig LA, Toksoz D (1999) Activation of the Lbc Rho exchange factor proto-oncogene by truncation of an extended C terminus that regulates transformation and targeting. Mol Cell Biol 19:1334–1345

Suzaki Y, Yoshizumi M, Kagami S, Koyama AH, Taketani Y, Houchi H, Tsuchiya K, Takeda E, Tamaki T (2002) Hydrogen peroxide stimulates c-Src-mediated big mitogen-activated protein kinase 1 (BMK1) and the MEF2C signaling pathway in PC12 cells: potential role in cell survival following oxidative insults. J Biol Chem 277:9614–9621

Takeishi Y, Huang Q, Abe J, Glassman M, Che W, Lee JD, Kawakatsu H, Lawrence EG, Hoit BD, Berk BC, Walsh RA (2001) Src and multiple map kinase activation in cardiac hypertrophy and congestive heart failure under chronic pressure-overload: comparison with acute mechanical stretch. J Mol Cell Cardiol 33:1637–1648

Tao J, Wang HY, Malbon CC (2003) Protein kinase A regulates AKAP250 (gravin) scaffold binding to the β_2-adrenergic receptor. EMBO J 22:6419–6429

Tingley WG, Pawlikowska L, Zaroff JG, Kim T, Nguyen T, Young SG, Vranizan K, Kwok PY, Whooley MA, Conklin BR (2007) Gene-trapped mouse embryonic stem cell-derived cardiac myocytes and human genetics implicate AKAP10 in heart rhythm regulation. Proc Natl Acad Sci USA 104:8461–8466

Vega RB, Harrison BC, Meadows E, Roberts CR, Papst PJ, Olson EN, McKinsey TA (2004) Protein kinases C and D mediate agonist-dependent cardiac hypertrophy through nuclear export of histone deacetylase 5. Mol Cell Biol 24:8374–8385

Wagner S, Dybkova N, Rasenack EC, Jacobshagen C, Fabritz L, Kirchhof P, Maier SK, Zhang T, Hasenfuss G, Brown JH, Bers DM, Maier LS (2006) Ca^{2+}/calmodulin-dependent protein kinase II regulates cardiac Na^+ channels. J Clin Invest 116:3127–3138

Wallace DA, Johnston LA, Huston E, MacMaster D, Houslay TM, Cheung YF, Campbell L, Millen JE, Smith RA, Gall I, Knowles RG, Sullivan M, Houslay MD (2005) Identification and characterization of PDE4A11, a novel, widely expressed long isoform encoded by the human PDE4A cAMP phosphodiesterase gene. Mol Pharmacol 67:1920–1934

Wehrens XH (2006) Structural determinants of potassium channel blockade and drug-induced arrhythmias. Handbook Exp Pharmacol 123–157

Wehrens XHT, Marks AR (2005) Ryanodine receptors: structure, function, and dysfunction in clinical disease. Springer, New York

Wehrens XH, Lehnart SE, Reiken SR, Deng SX, Vest JA, Cervantes D, Coromilas J, Landry DW, Marks AR (2004) Protection from cardiac arrhythmia through ryanodine receptor-stabilizing protein calstabin2. Science (New York, NY) 304:292–296

Willoughby D, Wong W, Schaack J, Scott JD, Cooper DM (2006) An anchored PKA and PDE4 complex regulates subplasmalemmal cAMP dynamics. EMBO J 25:2051–2061

Wu Y, Dowbenko D, Spencer S, Laura R, Lee J, Gu Q, Lasky LA (2000) Interaction of the tumor suppressor PTEN/MMAC with a PDZ domain of MAGI3, a novel membrane-associated guanylate kinase. J Biol Chem 275:21477–21485

Xiang Y, Devic E, Kobilka B (2002) The PDZ binding motif of the Beta 1 adrenergic receptor modulates receptor trafficking and signaling in cardiac myocytes. J Biol Chem 277:33783–33790

Xiang Y, Naro F, Zoudilova M, Jin SL, Conti M, Kobilka B (2005) Phosphodiesterase 4D is required for β_2-adrenoceptor subtype-specific signaling in cardiac myocytes. Proc Natl Acad Sci USA 102:909–914

Xu J, Paquet M, Lau AG, Wood JD, Ross CA, Hall RA (2001) β_1-Adrenergic receptor association with the synaptic scaffolding protein membrane-associated guanylate kinase inverted-2 (MAGI-2). Differential regulation of receptor internalization by MAGI-2 and PSD- 95. J Biol Chem 276:41310–41317

Yano M, Kobayashi S, Kohno M, Doi M, Tokuhisa T, Okuda S, Suetsugu M, Hisaoka T, Obayashi M, Ohkusa T, Kohno M, Matsuzaki M (2003) FKBP12.6-mediated stabilization of calcium-release channel (ryanodine receptor) as a novel therapeutic strategy against heart failure. Circulation 107:477–484

Yarwood SJ, Steele MR, Scotland G, Houslay MD, Bolger GB (1999) The RACK1 signaling scaffold protein selectively interacts with the cAMP-specific phosphodiesterase PDE4D5 isoform. J Biol Chem 274:14909–14917

Young P, Ehler E, Gautel M (2001) Obscurin, a giant sarcomeric Rho guanine nucleotide exchange factor protein involved in sarcomere assembly. J Cell Biol 154:123–136

Zaccolo M, Pozzan T (2002) Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. Science 295:1711–1715

Zhou Q, Ruiz-Loz1no P, Martone ME, Chen J (1999) Cypher, a striated muscle-restricted PDZ and LIM domain-containing protein, binds to a-actinin-2 and protein kinase C. J Biol Chem 274:19807–19813

Zhou J, Shin HG, Yi J, Shen W, Williams CP, Murray KT (2002) Phosphorylation and putative ER retention signals are required for protein kinase A-mediated potentiation of cardiac sodium current. Circ Res 91:540–546

Proline-Rich Sequence Recognition Domains (PRD): Ligands, Function and Inhibition

C. Freund(✉), H.-G. Schmalz, J. Sticht, and R. Kühne(✉)

Contents

1 Small-Molecule Modulators of Protein–Protein Interactions (PPI) . . . 408
2 Adaptor Domains as Key Components of PPI-Mediated Signal Complex Formation . . . 408
3 Properties of the PPII Helix and Principles of PRM Recognition . . . 409
4 Molecular Features of PRD . . . 410
5 Recognition Codes . . . 412
6 Regulation of the Binding Function of PRDs . . . 413
7 PRD:PRM Complexes as Targets for Pharmacological Intervention . . . 414
7.1 SH3 Domains . . . 415
7.2 WW Domains . . . 417
7.3 GYF Domain . . . 417
7.4 EVH1 Domains . . . 418
7.5 UEV Domains . . . 419
8 Ligand Design for PRDs . . . 419
References . . . 422

Abstract Low-affinity protein–protein interactions (PPI) between domains of modular proteins and short, solvent-exposed peptide sequences within their binding partners play an essential role in intracellular signaling. An important class of PPIs comprises proline-rich motifs (PRM) that are specifically recognized by PRM-binding domains (PRD). Aromatic side chains of the PRDs define the binding pockets that often recognize individual proline residues, while flanking sequences mediate specificity. Several of these PRM:PRD interactions are associated with cellular malfunction, cancer or infectious diseases. Thus, the design of PRM:PRD inhibitors by using structure-based molecular modeling as well as peptidomimetic approaches and high-throughput screening strategies is of great pharmacological interest. In this chapter we describe the molecular basis of PRM:PRD interactions, highlight their functional role in certain cellular processes and give an overview of recent strategies of inhibitor design.

C. Freund and R. Kühne
Protein Engineering, Molecular Modeling Group, FU and FMP Berlin,
Robert-Rössle-Str. 10, 13125 Berlin, Germany
freund@fmp-berlin.de, kuehne@fmp-berlin.de

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,

© Springer-Verlag Berlin Heidelberg 2008

1 Small-Molecule Modulators of Protein–Protein Interactions (PPI)

To date, only ~1% of the roughly 30,000 unique protein sequences encoded by the human genome have been targeted successfully by small-molecule drugs. Furthermore, these protein targets fall into few structural or functional families, e.g. enzymes and G-protein-coupled receptors (Bleicher et al. 2003). Typically, the natural substrates for these successfully targeted proteins are themselves small organic molecules. In contrast, there has been notably little success in developing small molecule modulators of proteins whose natural ligands are other proteins (Arkin and Wells 2004). Therefore, such protein targets were commonly considered "non-druggable." A major reason for the difficulty in mimicking protein–protein interactions (PPI) is the fact that many PPIs are characterized by flat and rather large binding interfaces, displaying little concavity. Especially for non-enzymatic proteins, these surfaces can be almost devoid of characteristic cavities that are well suited for interference with small-molecule ligands.

However, in 1995 a landmark study based on alanine scanning mutagenesis of human growth hormone and its receptor demonstrated that the main contribution to the binding energy derives from contacts with a small fraction of amino acids (hotspots) constituting the protein–protein interface (Clackson and Wells 1995). For the first time it seemed feasible for small molecules to access such hotspots, and it was subsequently shown that small-molecule inhibition of protein–protein interactions is possible (Fischer and Lane 2004; He et al. 2005; Oltersdorf et al. 2005). Another strategy is exemplified by binding of a small molecule to the cytokine IL-2 which induces changes in the protein surface via low-energy rearrangement of some hotspot side chains, thereby interfering with receptor binding of the cytokine (Arkin et al. 2003). In these cases, the discovery of small-molecule inhibitors of PPIs has depended on applied screening methods rather than on *in silico* approaches based on the target structure. On the other hand, a large number of complex structures of PPI sites with peptides have been solved in recent years (for reviews see Lim 1996; Ilsley et al. 2002; Harris and Lim 2001). Analysis of these complexes in combination with the SPOT synthesis techniques (for review see Frank 2002) can help extract information about both the hotspot region and the important molecular interactions. This allows the use of virtual screening methods and rational design in the development of small molecule peptidomimetics. In summary, despite the above-mentioned advances, the development of small molecule modulators of PPIs is a great scientific challenge that requires close collaboration between protein and organic chemistry, assay development, screening, and rational drug design.

2 Adaptor Domains as Key Components of PPI-Mediated Signal Complex Formation

The potential to interfere with PPIs in multi-protein signaling complexes is of great importance both for the understanding of cell physiology and for the development of novel disease treatments. Modular proteins involved in signal transduction utilize

highly conserved non-catalytic adaptor domains that mediate PPIs during the formation of multi-protein signaling complexes. In general, these adaptor domains must fulfill certain requirements to exert their binding function. Firstly, the structural properties of the domain have to ensure the incorporation into a limited set of macromolecular complexes. The limited specificity of the adaptor domains may well be complemented by additional contacts of surrounding PPIs (Zarrinpar et al. 2003a). Secondly, the interactions have to be reversible to allow the dissociation of the complex as soon as a certain stimulus occurs or is removed. The requirement for relatively high off rates implies that PPIs are often characterized by modest affinities in the low micromolar to low millimolar range. Recognition of short, solvent-exposed peptide sequences mostly fulfills this prerequisite. Correspondingly, many adaptor domains have been shown to bind to small synthetic peptides containing a characteristic core motif, flanked by residues that are responsible for a certain degree of specificity. A large number of different families of PPI domains have been described. In many cases, the domains recognize target core motifs containing phosphorylated residues. Paradigmatic examples are the Src-homology 2 (SH2) and phosphotyrosine-binding (PTB) domains which bind core sequence motifs containing phosphorylated tyrosines (Vetter and Zhang 2002). Among others, forkhead-associated (FHA) domains are often utilized by eukaryotic proteins for the recognition of phosphoserine- and phosphothreonine-containing peptides (Williams, Bernstein et al. 2005). Another well-known family of protein-interaction domains is the postsynaptic density/disc-large/ZO1 (PDZ) family that recognizes and binds the extreme C-terminal sequence of their binding partners (Dev 2004). An important class of PPI modules binds to solvent-exposed regions within their respective target proteins containing proline-rich motifs (PRMs). These PRM-recognition domains (PRDs) are especially abundant in higher eukaryotes and are particularly interesting in the context of multi-component signaling events that govern cellular behavior in its full complexity. The functional context of PRDs, their recognition codes and the regulation of PRD-binding function will be described in the following. Subsequently, efforts to target the respective interaction sites by small organic compounds will be discussed.

3 Properties of the PPII Helix and Principles of PRM Recognition

A common property of all PRMs is that they preferentially form a left-handed poly-proline type-II (PPII) helix with an overall shape resembling a triangular prism (Kay et al. 2000). This structural element has a helical pitch of 9.3 Å, three residues per turn, and Φ and Ψ angles centered around −75° and 145°, respectively (Bochicchio and Tamburro 2002; Cubellis et al. 2005). The PPII helix is characterized by a complete lack of main-chain hydrogen bonding patterns that are commonly used to identify other secondary structures, such as α-helices or β-sheets. This property is the reason for problems in distinguishing PPII helices from unfolded conformations using NMR spectroscopy. However, other spectroscopic techniques, such as CD spectroscopy, indicate that the PPII helix is the

common conformation of the unbound state (Tiffany and Krimm 1968). Although proline is common in PPII helices, PPII helices without proline were found in 46% of the total identified proteins taken from the HOMSTRAD database (Mizuguchi et al. 1998; de Bakker et al. 2001). Most amino acids are accepted in the context of the PPII helix, though glycine and aromatic amino acids have low propensities to be part of this structure (Cubellis et al. 2005). Results of a theoretical study shed light on the forces stabilizing the PPII helix (Sreerama and Woody 1999). A PPII, an α-helix and a β-strand were the starting conformations for a molecular dynamics study of an $(Ala)_8$-peptide in water. Only the α-helix remained intact during the simulation, while the others showed more flexibility due to the lack of stabilizing intramolecular hydrogen bonds. The PPII conformation was found to be populated twice as frequently as the β-strand. In fact, for both conformations the water molecules form hydrogen bonds to the peptide backbone and to one another, thus constituting a bridge connecting two backbone atoms of the peptide. Interestingly, only in the case of the PPII conformations were water molecules able to connect two consecutive oxygen atoms of the backbone. This clearly suggests that water can be important in stabilizing the PPII structure. It was shown that PPII helices disrupt the structural arrangement of water molecules to a smaller degree than α-helices and β-sheets. Thus, PPII helix formation appears to be entropically driven (Kentsis et al. 2004; Mezei et al. 2004). The tendency of the PPII structure to form favorable contacts with water is potentially responsible for the finding that PPII helices within proteins are frequently solvent-exposed (Adzhubei and Sternberg 1993). Taking into account that PPII conformers contain an increased number of non-satisfied hydrogen bond donors and acceptors in comparison to α-helices and β-strands, the PPII helix is well suited to take part in protein–protein interactions.

4 Molecular Features of PRD

The signaling domains that recognize PRMs that have been identified so far seem to have evolved specifically to exploit the special features of the amino acid proline. The signaling domains known to interact with PRMs are the SH3 (Varmus et al. 1989), the WW (Bork and Sudol 1994), the EVH1 (Niebuhr et al. 1997), the GYF (Nishizawa et al. 1998, Freund et al., 1999) and the UEV domain (Garrus et al., 2001; Pornillos et al., 2002a). In addition, profilin was found to bind to PRMs (Tanaka and Shibata 1985), aside from its property as an actin-interacting protein involved in cytoskeletal assembly (Carlson et al., 1977). This set of adaptor domains may not be complete, for example, the binding domain of prolyl-4-hydroxylase could be an additional member of the PRD family (Myllyharju and Kivirikko 1999). From the studies of these domains, several features of PRM recognition have emerged from the structures of PRD:PRM complexes (Musacchio et al. 1994a; Yu et al. 1994; Macias et al. 1996; Prehoda et al. 1999; Pornillos et al. 2002a; Freund et al. 2002). A set of conserved, often aromatic amino acid residues

defines the respective fold families, and the aromatic side chains form at least one stacked pair of an aromatic cradle that represents the proline-binding pocket. The structural comparison shows that while GYF and WW domains converge on a single pocket, SH3, EVH1 and UEV mediate PRM recognition via two neighboring surface depressions. A detailed structural comparison has been described in a number of excellent reviews and will not be discussed here (Mayer 2001; Zarrinpar et al. 2003a; Li 2005; Kay et al. 2000; Sudol 1996; Ball et al. 2005; Kofler and Freund 2006). Despite the convergence in ligand binding, it has to be noted that the folds of the various domains are quite different. They all represent stable scaffolds that are compatible with the formation of the aromatic cradle, and nature has exploited these scaffolds early on in evolution since all of the domains exist in *S. cerevisiae*. It is interesting in this respect that scaffold amplification is observed for SH3 and WW domains, which contribute to the assembly of many protein complexes in higher eukaryotes. In contrast, the occurrence of GYF domains has remained relatively constant. For example, there are three characterized GYF domains containing proteins each in *S. cerevisiae* and *H. sapiens*. The anticipated function of GYF domains in splicing-associated processes is therefore likely to be conserved throughout evolution, while several functional clusters are associated with SH3 and WW domains.

Whereas most members of the PRDs converge on the recognition of at least two prolines in the PRM, there is considerable variety in the accommodation of residues flanking the proline-rich core motif. Interestingly, variations occur within the respective fold families as well as between folds. Consequently, the array of ligands bound by one fold family member might be more similar to the member of another fold family than to a member of its own family. For example, an early finding suggested that the SH3 domain of Abl and the WW domain of FBP11 bind to similar PRM in vitro (Bedford et al. 1997), and evidence is mounting that such "shared targets" exist in vivo (Kofler and Freund 2006). How then is specificity obtained in PRM recognition? A certain degree of specificity is often introduced by charge complementarity. Charged residues flanking the proline-binding pocket within the domain preferably select ligands with opposite charge. For example, the Fyn-SH3 domain contains a very acidic RT loop (a feature shared by most SH3 domains) that leads to the preferred ligand signatures RxxPxxP and PxxPxR, dependent on the two pseudosymmetric orientations of the peptide (Cesareni et al. 2002). Similarly, the GYF domain of CD2BP2 favors positive-charged ligands over neutral or negatively charged ligands. Correspondingly, Fyn-SH3 and CD2BP2-GYF were shown to bind to the same positively charged PRM in the CD2 cytoplasmic tail with low, but comparable affinity (Freund et al. 2002). While sequence recognition outside the proline pocket(s) still allows a whole set of different peptides to bind to the individual domains, it clearly reduces sequence space considerably by deselection based on steric hindrance and charge repulsion. The power of negative selection was shown in a paper from the Lim group which showed that within a given organism an SH3 domain with almost exclusive ligand specificity could be observed (Zarrinpar et al. 2003b). However, such specificity can normally not be observed at the level of recognition rules alone since other

powerful factors, such as compartmentalization, avidity and multiple additional interactions contribute to specificity in vivo. However, molecular promiscuity also seems to be inherent to many PRD and thereby allows the recruitment of the respective proteins to different target sites within the cell. A common feature of most PRDs is that their N and C termini are located relatively close in space, thereby allowing the domains to be integrated into the respective full-length proteins with minimal disruption of the overall protein structure.

5 Recognition Codes

Since the early days of PRD identification, attempts have been made for the thorough identification of the recognition code of the respective domains. Phage display and peptide SPOT analysis have contributed most to defining the sequence space of PRDs, and refinement of this is still ongoing. Classification schemes are mainly based on ligand specificity and are supported by the structures of PRD-ligand complexes in many cases. We will not discuss this, since several excellent reviews have been written on this topic (Musacchio et al. 1994b; Karkkainen et al. 2006; Sudol 1996; Kay et al. 2000; Mayer 2001; Zarrinpar et al. 2003a; Li 2005). One interesting question raised in this context is whether the highest affinity binders really represent the physiological targets in vivo. For example, in the case of GYF domain binders, the PPGW motif was contained in a peptide derived from phage display selection, and subsequent analysis showed a higher affinity as compared to cognate ligands. As already discussed, additional factors come into play in vivo, and selection of the highest affinity binder might not be necessary in such cases. In addition, motif repetition has been shown to be a powerful mechanism to introduce threshold behavior into signaling circuits mediated by multiple phosphorylation sites (Nash et al. 2001). Whether similar molecular behavior can be elicited by multiple PRMs – possibly by indirect coupling to post-translational modifications – remains to be investigated. To account for avidity or cooperativity effects, motif repetition can be taken into account for database searches, as it was for the identification of natural peptide binders of GYF domains (Kofler et al. 2005). However, additional data need to complement the in vitro kursiv setzen selection data, and ideally the interaction should be verified in a cellular environment, e.g. by FRET experiments.

It should be noted that an increasing number of cognate ligands have been identified that contain only one or no proline. The first structure showing that SH3 domains are able to interact with a folded structure devoid of the canonical P-X-X-P motif came from the p53 binding protein 2 in complex with p53 (Gorina and Pavletich 1996). Another interesting example of such an interaction is the complex of the Gads SH3 domain bound to a peptide from SLP-76 (Harkiolaki et al. 2003; Liu et al. 2003). In this case, the ligand does not form a PPII helix; instead, one turn of a 3_{10} helix is formed by the R-X-X-K motif of the SLP-76 peptide. Along these lines, some of the domains were shown to be able to interact

with the composite-binding partner of other proteins. For example, the SH3 domain of Fyn additionally interacts with the SH2 domain of SAP by employing its RT loop and neighboring regions (Chan et al. 2003). PRM binding and SAP binding should therefore be competitive. For the GYF domain of CD2BP2, simultaneous and independent binding of PRMs and the "back-site" binding partner U5–15 K could be observed (Nielsen et al. 2007; Kofler and Freund, unpublished observations). This shows that additional binding events within the domain itself may already modulate the localization and function of PRD-containing proteins.

6 Regulation of the Binding Function of PRDs

Although the binding affinities for PRDs are usually in the μM range, proteins containing these domains might nevertheless contribute critically to protein complex formation. Many of these complexes become activated when a certain biological stimulus arrives and complex assembly needs to be suppressed prior to activation. Therefore, activation-induced unmasking or compartmentalization of the PRD binding site is frequently observed in proteins. While certain WW domains have evolved into p-Ser-dependent modules (Lu et al. 1999), it has also been observed that post-translational modifications adjacent to the PRM affect the binding of SH3 domains in vitro (Bedford et al. 2000). The compartmentalization of PRD-containing proteins will usually be assisted by domains other than PRDs, but it has also been observed that the PRD itself is absolutely required for the localization of certain proteins. Another possibility is that PRD:PRM interactions contribute to compartmentalization, but will be complemented or substituted by other, more specific interactions during the course of protein complex assembly. There are several examples of molecular complexes that rely on multiple domain interactions in conjunction with post-translational modifications. A well-described system is the NADPH oxidase, the catalytic and membrane-bound core of which consists of the gp91 and p22 subunits (Li 2005; Takeya et al. 2006). The cytosolic components p40, p47, p67 and Rac1 need to be recruited to the membrane for activation of the oxidase. A critical step in the assembly is the release of an autoinhibited conformation of the p47 protein. The two SH3 domains of this subunit are able to interact directly, forming a super-SH3 fold with a composite binding site that is masked by a basic C-terminal region comprising the motif RGAPPRRSS (Groemping et al. 2003). Upon stimulation of the cell, several serine residues in the polybasic region become phosphorylated, the auto-inhibited conformation is released, and binding of p47 to a classical PxxP motif within the membranous p22 subunit takes place. This example highlights several of the general mechanisms that are able to modulate the binding function of PRDs: (1) intramolecular interactions represent a means to prevent premature binding, (2) PRDs may form dimers with altered binding specificities, (3) sequences other than classical PxxP motifs are able to interact with the SH3 interaction site, and (4) post-translational modifications are able to couple indirectly to PRD scaffolding. Furthermore, the PX domain of p47 is also

necessary for membrane recruitment of the protein, nicely exemplifying the additive or even cooperative binding of modular domains within the context of multiprotein complexes.

Another paradigmatic example of enzyme regulation by SH3 domains is the members of the Src family of tyrosine kinases. These proteins can exist in two major forms: an inactive, auto-inhibited conformation that is stabilized by intramolecular interactions between the SH2 domain and a phosphotyrosine motif and between the SH3 domain and a proline-helix-forming linker segment between the SH2 and kinase domain (Xu et al. 1999). Dephosphorylation of the inhibitory tyrosine and intermolecular binding by the SH2 and SH3 domains leads to the formation of a fully active conformation that is able to phosphorylate several downstream targets. This “turned on by touch” behavior seems to be a remarkably common feature of tyrosine kinases in general, even in cases where binding partners are distinct and post-translational modifications differ (Boggon and Eck 2004). Targeting by SH3 domains and other adaptor domains is thereby unequivocally linked to the control of enzymatic activity.

7 PRD:PRM Complexes as Targets for Pharmacological Intervention

PRD:PRM interactions are widely utilized by nature in higher eukaryotes and have been implicated in diverse processes, such as tyrosine kinase receptor signaling (Komuro et al. 2003), rather: endosomal trafficking (Hurley and Emr 2006), cytoskeletal rearrangements (Dustin et al. 1998; Laurent et al. 1999), transcription (Goldstrohm et al. 2001) and splicing (Zhang et al. 2000; Kofler et al. 2004; Kofler et al. 2005; Laggerbauer et al. 2005; Nielsen et al. 2007). Some of the domains seem to participate in a more specialized and evolutionary conserved function. For example, evidence is mounting that the primary role of GYF-domain-containing proteins is associated with the processing of mRNA in the context of splicing, mRNA transport and degradation. For UEV domains a critical function in endosomal sorting has been shown, and viruses hijack the domain to exploit the protein’s function for the budding process. EVH1 domains are cytoskeleton-associated proteins that, for example, as part of the Ena/VASP proteins, promote the branching of actin filaments in lamellipodia (Bear et al. 2002). Contrary to GYF, UEV, and EVH1 domains, the SH3 and WW folds have experienced a more dramatic amplification during eukaryotic evolution (Zarrinpar et al. 2003). They are contained in a manifold of proteins and thereby seem to present almost universal scaffolds for the establishment of signal transduction networks. It is tempting to speculate that such differences in occurrence are due to the adaptability of the scaffold itself, but more systematic investigations of scaffold mutability in combination with ligand-array-based assays are needed to support such a hypothesis. Interestingly, the two fold families also play a role in the processes for which the

other PRDs are specialized. In many instances, WW domains are involved in nuclear function, such as splicing and transcription, and thereby they act in the same functional framework as GYF domains (Sudol et al. 2001). Additionally, WW domains are found in proteins binding to the actin cytoskeleton, and they play a targeting role within E3 ubiquitin ligases, such as Nedd4 (Staub et al. 1996; Ingham et al. 2004). SH3 domains are often found in proteins at the cytoskeleton-membrane interface, for example as dynamin-interacting proteins (Orth and McNiven 2003). The occurrence of SH3 domains in Src and many other tyrosine kinases, often in conjunction with the phosphotyrosine-binding SH2 domains, confines the enzymatic activity of these kinases to certain subscellular locales and a limited set of target proteins (Boggon and Eck 2004). Modulation of enzymatic activity by SH3 domain-mediated interactions within the context of macromolecular complexes is also observed for GTPases, such as dynamin (Scaife and Margolis 1997), and for NADPH oxidase (see discussion above).

In light of the many processes that involve PRD:PRM recognition, it was not surprising to find that ablation of these interactions by mutations are correlated with certain diseases. Liddle syndrome, a disease associated with hypertension, is caused by mutations in the PPxY motifs of the cytoplasmic domains of the amiloride epithelial sodium channel (Schild et al. 1996; Kanelis et al. 2001). The proline-rich region of huntingtin is targeted by SH3 (Qin et al. 2004) and WW domains (Holbert et al. 2001) and is likely to play a role in the progression of Huntington's disease pathogenesis. A missense mutation within the WW domain of the polyglutamine-tract-binding protein PQBP-1 leads to Golabi-Ito-Hall syndrome, a certain type of X-linked mental retardation (Lubs et al. 2006). Mutations within the EVH1 domain of WASP affect the binding to the proline-rich ligand of the WASP-interacting protein (Volkman et al. 2002) (WIP) and are causative for Wiskott-Aldrich syndrome, an X-linked recessive disorder leading to immunodeficiency, eczema and thrombocytopenia (Derry et al. 1994; Derry et al. 1995; Kolluri et al. 1995; Villa et al. 1995; Zhu et al. 1997). These examples show that PRD:PRM interactions are pivotal for the formation of certain molecular assemblies associated with cellular malfunction. Along these lines the question may be asked which PRD: PRM containing target couples are good candidates for pharmacological interference and are of potential significance for drug research. We select specific cases for each of the fold families and discuss the possible merits of such an inhibition in the respective molecular context (Fig. 1).

7.1 SH3 Domains

Protein tyrosine kinases of the Src family are essential for cell development, growth, replication, adhesion, motility and neuronal function, and they are overexpressed in a wide number of human cancers. In the brain, the SH3 domain of Fyn tyrosine kinase was shown to interact with microtubule-associated Tau protein (Lee et al. 1998). Tau

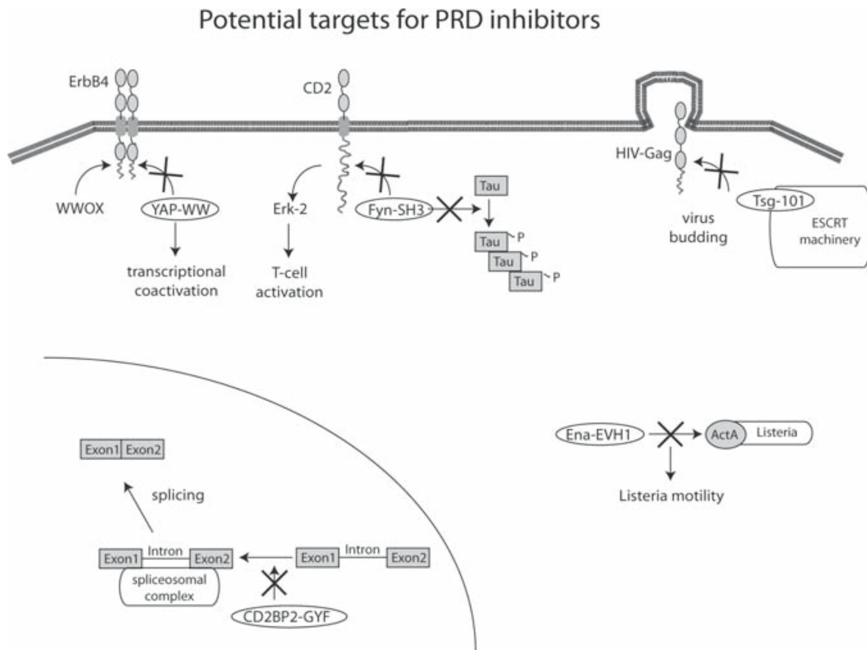

Fig. 1 Pharmacological interference with PRM:PRD interaction in the context of disease. Shown are examples of cellular PRM:PRD interactions for every PRD fold family that represent pharmacological targets for drug development

is the major component of neurofibrillary tangles formed during Alzheimer's disease, and it is linked genetically to frontotemporal dementias (FTDP-17). Disease-related mutations in the Tau protein negatively affect the ability of the protein to bind and stabilize microtubuli (Hong et al. 1998). The various splice variants of Tau bind with different affinities to Fyn-SH3, and tyrosine phosphorylation was hypothesized to modulate these affinities (Bhaskar et al. 2005). Brain-specific inhibition of Fyn kinase is suggested to alter the onset of neuronal diseases, such as Alzheimer's (Chin et al. 2005), and the current data are sufficiently persuasive to suggest that the Fyn-SH3:Tau interaction has an impact on the Fyn-mediated progression of the disease.

Association of Src kinase family members with the cytoplasmic tails of transmembrane receptors is another common theme for these proteins, and many of the targeted receptors are involved in growth and differentiation of cells. For example, many of the T-cell-receptor-associated adhesion and co-stimulatory molecules are targeted by Src kinases, and in several cases this recruitment is mediated or at least complemented by the SH3 domain. Examples are CD28 and CD2, two molecules expressed specifically in T cells and NK cells. Both molecules harbor cytoplasmic PRM sites that are recognized by essential signaling molecules, such as Grb2 or the tyrosine kinase Itk in the case of CD28 (Okkenhaug and Rottapel 1998; Kim et al. 1998) and Fyn in the case of CD2 (Lin et al. 1998; Fukai et al. 2000). Antibodies against both

of these molecules were shown to be effective in prolonged tolerance of transplants in cellular and animal models (Woodward et al. 1996; Rothstein and Sayegh 2003), and it is attractive to think of intracellular inhibition of the corresponding signaling pathways as a novel avenue of immune suppression. Fyn was shown to bind to several of the PRMs within the cytoplasmic domain of CD2. Interestingly, binding of the closely related Lck protein to the RPPPPGHR motif of the CD2 tail was much weaker and could be explained by a T97H exchange within the RT loop of the respective SH3 domain (Freund et al. 2002). The histidine in Lck forms an intramolecular hydrogen bond to aspartate 100, while the corresponding threonine residue in Fyn points towards the solvent. Thereby, a ligand arginine is able to insert into the specificity pocket of Fyn-SH3, but not Lck-SH3. This example shows that selective inhibition of SH3 domains may be achieved by exploiting small differences in the specificity determining regions of the respective SH3 domain.

7.2 WW Domains

ErbB-4 is the newest identified member of the epidermal growth-factor-receptor family and seems to be a good tumor marker for certain types of malignancies, such as prostate, lung or breast cancer (Starr et al. 2006; Ben et al. 2007; Junttila et al. 2005). The C-terminal fragment (CTF) of ErbB-4 is cleaved by γ-secretase and shuttles to the nucleus of stimulated cells (Ni et al. 2001). The two WW-domain-containing proteins WWOX and YAP (Yes kinase associated protein) were shown to compete for interaction with the PPXY motifs of the cytosplamic tail of the ErbB-4 receptor (Komuro et al. 2003; Aqeilan et al. 2005). YAP acts as a transcriptional coactivator, while WWOX, which was described as a tumor suppressor gene (Paige et al. 2001), binds to ErbB-4 in the cytoplasm, thereby acting as an inhibitor of CTF nuclear translocation. For future drug development, the challenge will be to develop an inhibitor that targets the WW domain of YES, but leaves the interaction of WWOX and ErbB-4 unaffected. Since both WW domains converge on the same recognition motifs, amino acids outside the conserved proline-binding pocket have to be addressed as differentiating epitopes for the binding of chemical moieties (see discussion below). The power of negative selection, for example, implemented as a pre-clearing step in the screening process, might be employed in order to gain predominant inhibition of the desired WW domain.

7.3 GYF Domain

The GYF domain has so far not yet been unambiguously assigned to certain disease states. Nevertheless, overexpression of the GYF-containing protein CD2BP2 was observed in chronic fatigue syndrome (Kaushik et al. 2005), and the same

protein was found to be overexpressed in estradiol-, 4-hydroxytamoxifen- and acolbifene-treated T47D breast cancer cells (Al Dhaheri et al. 2006). Independent of its possible role in these disease-related processes, GYF domain inhibition would be a valuable tool for deciphering its function within the spliceosome. Several proteins exist that are likely to be targeted by CD2BP2-GYF. A specific inhibitor in combination with pulldown experiments and proteomic analysis would enable the delineation of the relative contribution of PRM recognition to GYF domain function. Since the GYF domain contains a second, PRM-independent binding site for the U5 snRNP protein 15 K (Nielsen et al. 2007), site-specific blocking of the PRM pocket by peptides or small molecules is an effective strategy to achieve such de-convolution of a binary adaptor domain. Thereby, PRM recognition by the GYF domain could be a paradigmatic example of PRM recognition in the context of multiple binding events within a macromolecular complex.

7.4 EVH1 Domains

Listeria monocytogenes infections are a common cause of food poisoning, and an effective means to block their infectivity is highly desirable. These pathogens contain a protein called ActA that contains four EVH1 FPPPP consensus motifs and that effectively hijacks the EVH1-containing proteins Ena and VASP (Chakraborty et al. 1995). EVH1-mediated actin binding allows the bacterium to propel itself along the cell's actin cytoskeleton, leaving behind a so-called actin-rich "comet tail" and thus allowing the pathogen to move rapidly through a cell's cytoplasm. Deletion of the FPPP motifs in ActA or injection of FPPPP-containing peptide into the cytoplasm strongly reduces the motility of Listeria (Southwick and Purich 1994), and small-molecule mimetics of the FPPPP motif would represent an important step towards a Listeria-targeting drug.

Another interesting class of proteins containing EVH1 domains is the Homer proteins (Shiraishi-Yamaguchi and Furuichi 2007). These proteins are implicated in the formation of oligomeric structures of the post-synaptic density matrix and thereby contribute to synaptogenesis and receptor trafficking. In addition, Homer proteins are directly involved in controlling glutamate levels in the limbo-corticostriatal regions of the brain. Studies of Homer knock-out mice revealed involvement of these proteins in a number of behavioral abnormalities and neuropsychatric disorders, such as addiction, depression, epilepsy and schizophrenia (Szumlinski et al. 2006). Homer 2 knockout and overexpression studies in mice, for example, reveal a positive role of the protein in behavior leading to increased alcohol consumption (Szumlinski et al. 2005). The role of the EVH1 domain in the development of these diseases is not understood, but specific inhibition of Homer EVH1 domains would enable their function to be studied and may provide useful lead compounds for the development of drugs to be used in the context of neurobehavioral disorders.

7.5 UEV Domains

UEV (ubiquitin E2 variant) domains are homologs of ubiquitin E2 ligases that lack the active-site cysteine, but retain the capability of ubiquitin binding, which is often of functional importance. Human UEV1a as well as its yeast homolog Mms2, for example, are involved in polyubiquitin chain assembly (Hofmann and Pickart 1999; Deng et al. 2000; VanDemark et al. 2001). Interaction with proline-rich motifs, however, has only been described for the UEV domain of human Tsg101 (tumor-susceptibility gene 101) and its yeast homolog Vps23. PRM recognition of Tsg101-UEV plays a pivotal role in endosomal sorting (Williams and Urbe 2007; Hurley and Emr 2006; Babst 2005) and has recently been shown to be essential for the budding of HIV and other enveloped viruses (Pornillos et al. 2002b; Morita and Sundquist 2004; Bieniasz 2006). The P(T/S)AP motif of HIV-1 is called the L (late) domain, because it is essential for the late budding step of pinching off the plasma membrane (Gottlinger et al. 1991; Huang et al. 1995). This L-domain was shown to interact with Tsg101-UEV (Garrus et al. 2001; VerPlank et al. 2001), presumably recruiting the ESCRT machinery to the budding site at the plasma membrane. Thereby, viruses might mimic the function of the cellular protein Hrs that is assumed to recruit the ESCRT machinery to the endosomal membrane via a PSAP-UEV interaction (Pornillos et al. 2003). As Tsg101-UEV is essential for HIV replication, the Tsg-UEV/HIV-PTAP interaction is an attractive target for the development of antiviral compounds. These compounds should very specifically target the PRM recognition site within the UEV domain as is guaranteed by a proline-mimetic approach described by Liu and colleagues (2006). Such a strategy might make it possible to inhibit virus replication without interfering with other functions of Tsg101 such as cell proliferation and survival (Krempler et al. 2002; Wagner et al. 2003; Carstens et al. 2004). This is supported by the finding that overexpression of the N-terminal (UEV) domain of Tsg101 efficiently blocks HIV release, while overexpression of the C-terminal domain or full-length Tsg101 results in aberrant endsosomes (Demirov et al. 2002; Goila-Gaur et al. 2003).

8 Ligand Design for PRDs

A first strategy for PRM:PRD inhibition was based on the introduction of non-natural amino acids into the known “wild-type” peptides (Nguyen et al. 1998, 2000). They demonstrated that the second proline residue of the PxxP motif recognized by SH3 domains could be replaced with *N*-alkylated glycine derivatives to create ligands exhibiting enhanced affinities. Their strategy for inhibitor design can be outlined as follows: maintain the required hybrid Cα- and N-substituted scaffold, but vary side-chain identity along this scaffold to optimize complementarity. Using this strategy, they could not only enhance the affinity of the peptoid structures compared to wild type peptides, but also improve the selectivity within the family of SH3 domains. The replacement of prolines in the conserved

PxxP motif by N-substituted glycines was also successfully applied to peptides recognized by the class-1 EVH1 domain of human VASP (Zimmermann et al. 2003). It could be shown that the first proline of the PxxP motif of the ActA-derived peptide SFEFPPPPTEDEL can be replaced by an *N*-propylphenyl-substituted glycine without loss of binding. However, substitution of the last proline with this approach was not possible, indicating that successful replacement of prolines within a PPII helix is position-dependent. The ability of these structures to replace prolines depends mostly on their ability to reach additional binding sites on the domain surface. The relevance of epitope recognition was exemplified for the EVH1 domain (Zimmermann et al. 2003). It was found that the mutated peptide SFEAPPPPTEDEL is not able to bind to the class-1 EVH1 domain due to the F to A exchange at position 4. However, the binding could be restored by replacement of the first proline (position 5) with *N*-proylphenyl-glycine. In that case, the phenyl ring of the non-natural amino acid is able to reach the F_4 binding epitope of the wild type peptide.

For short proline-rich peptides, little or no ordered secondary structure is observed before binding takes place. The association of an unfolded peptide with PRD involves unfavorable binding entropy due to the loss of rotational freedom on forming a PPII helix. Despite the slight preference of unbound PRM peptide structures for forming a PPII helix, only a small fraction of peptides is available in a preformed PPII conformation. Thus, concepts were developed to stabilize the PPII helix by replacing residues within PRM by conformationally restricted PPII mimetics. However, as yet only few examples for isosteric PPII helix modules have been published. Several of those are shown in Fig. 2.

Compound A was synthesized recently as a potential PPII helix isostere (Bandur et al. 2005). The stereo-selective construction of a tri-substituted double bond in an

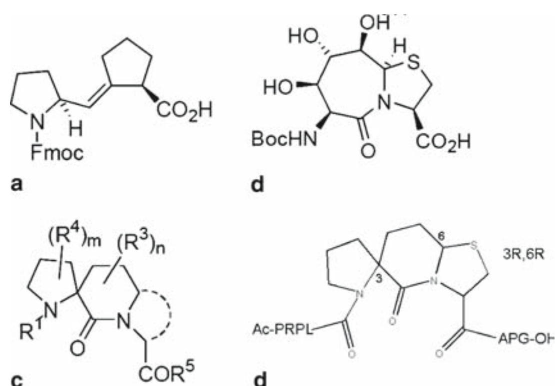

Fig. 2 Selection of known PPII mimetics

E-Pro–Pro isostere induces an anti-periplanar conformational lock that constrains the two rings in a PPII-typical orientation. However, this compound lacks the "central" carbonyl group that seems to be important for the binding to PRDs. Tremmel and Geyer (2002) described the synthesis and the structural analysis of a hexapeptide surrogate based on a trimer of scaffold B displaying all characteristic features of the PPII helix. The sugar-derived peptide mimetics have the amphiphilic character of Ser-Pro dipeptide units. Castelhano et al. (Witter et al. 1998) (WO 98/54208) developed a highly constrained spirolactam mimetic (compound C) of two prolines within the PLPPLP sequence of SH3-binding peptides. They also showed that incorporating a tricylic spirolactam scaffold into a p85-derived peptide (see compound D) instead of a PV-motif maintained the affinity of the wild-type peptide. The other diastereoisomers (3R,6S; 3S,6S; and 3S,6R) showed lower affinity. Lack of enhanced binding of compound D reflects the absence of entropy gain from constraining the structure. This is in agreement with a recently published study (Ruzza et al. 2006), in which specific proline positions of a PRM peptide corresponding to a sequence 394–403 of hematopoietic progenitor kinase (HPK-1) were replaced by 4(R)- or 4(S)-4-fluoro-L-proline. 4-Fluoro-substituted prolines are known to induce and stabilize the PPII helix in solution (Holmgren et al. 1999; Doi et al. 2005; Nishi et al. 2005). The propensity for a PPII helix was determined using a CD thermal analysis, and the affinity of the HPK-1 peptide to HS1-SH3 was measured. They found that none of the peptides with induced PPII helix was able to bind with higher affinity than the parent peptide. These results clearly show that the induction of a PPII helix in short peptides is not sufficient to increase the affinity towards an SH3 domain. However, the situation is different when peptides are incorporated into a protein scaffold (Golemi-Kotra et al. 2004). Schaepartz et al. used a miniature protein design strategy based on the avian pancreatic polypeptide (aPP). aPP consists of an eight-residue PPII helix linked through a type-I β-turn to a 20-residue helix characterized by stabilizing interactions between the PPII helix and the α-helix. The architecture of aPP enables the protein to display a solvent-exposed binding epitope at the α-helical or the PPII helical element of the structure. Using this strategy, it was possible to design aPP-based mini-proteins with nanomolar affinity to different proteins (Rutledge et al. 2003; Montclare and Schepartz 2003). The application of the "grafting" strategy on the PPII-part of aPP results in a mini-protein that binds with ten-fold higher affinity to Mena than the $ActA_{11}$ ligand from *Listeria monocytogenes*, the best previously known Mena ligand. The peptides forming the PPII helix in the mini-protein alone showed very weak affinity to Mena. Thus, stabilization of the PPII conformation within the context of a scaffold can improve binding affinity.

Recently, Lawrence and colleagues described a strategy to develop peptide/non-peptide chimeras that bind the SH3 domain of Fyn, a Src kinase family member, in a selective manner (Lawrence 2005; Li and Lawrence 2005). Starting with the consensus peptide RALPPLP coupled to a disulfide-linked Tentagel resin, certain positions flanking the PxxP motif were modified by introducing (L)-2,3,-diaminopropionic acid (DAP). The strategy behind this is based on the idea that modified residues adjacent to the core motif may contribute significantly to the binding

energy by engaging in non-covalent interactions with sub-sites of the domain that cannot be reached by natural amino acids. Using this approach, it was possible to develop a highly selective Fyn-SH3 inhibitor with an affinity in the nanomolar range. Utilizing the recognition of non-conserved sites of individual adaptor domains is a promising way of obtaining specificity. This can only be achieved by recruitment of additional hot spot areas for binding located on the domain surface and related to the non-conserved sequence segments within the domain family.

Another interesting case is the discovery of UCS15A (Sharma et al. 2001; Oneyama et al. 2002; Oneyama et al. 2003). This compound was selected from a screen for Src kinase signaling antagonists using a yeast-based high-throughput assay identifying compounds that are able to rescue the growth arrest of v-Src overexpressing strains. Surprisingly, it was found that although UCS15A was able to inhibit the tyrosine phosphorylation of two Src substrates in a dose-dependent manner, no in vitro inhibition of Src-kinase activity was observed. Follow-up experiments indicated that UCS15A was able to block the SH3-mediated protein–protein interaction for a number of typical SH3-mediated protein–protein interactions, such as cortactin-Zo1 and Grb2-Sos1. Further, it was shown that the compound binds to the PRM directly and not to SH3 domains. It is interesting to note that UCS15A contains many H-donating groups destined for interaction with exposed main-chain carbonyl oxygens of a PPII helix.

Using the mouse Tec kinase SH3 domain as a model system for structure-based ligand design, Inglis and co-workers (2004, 2006) have identified several simple heterocyclic compounds that selectively bind to the Tec-SH3 domain. They reported that 6-substituted 2-aminochinolines interact with the solvent-exposed Trp of the Tec-SH3 domain core motif. NMR chemical shift perturbation from [^{1}H,^{15}N]-HSQC experiments using ^{15}N-labeled Tec SH3 protein were analyzed to reveal K_D-values within the 125–300 μM range.

In summary, there are several strategies emerging to design peptide-based high-affinity ligands for PRDs. Key building blocks have been identified that might serve as potent leads for the design of second-generation compounds with improved affinities and selectivities.

References

Adzhubei AA, Sternberg MJ (1993) Left-handed polyproline II helices commonly occur in globular proteins. J Mol Biol 229:472–493

Al Dhaheri MH, Shah YM, Basrur V, Pind S, Rowan BG (2006) Identification of novel proteins induced by estradiol, 4-hydroxytamoxifen and acolbifene in T47D breast cancer cells. Steroids 71:966–978

Aqeilan RI, Donati V, Palamarchuk A, Trapasso F, Kaou M, Pekarsky Y, Sudol M, Croce CM (2005) WW domain-containing proteins, WWOX and YAP, compete for interaction with ErbB-4 and modulate its transcriptional function. Cancer Res 65:6764–6772

Arkin MR, Wells JA (2004) Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. Nat Rev Drug Discov 3:301–317

Arkin MR, Randal M, DeLano WL, Hyde J, Luong TN, Oslob JD, Raphael DR, Taylor L, Wang J, McDowell RS, Wells JA, Braisted AC (2003) Binding of small molecules to an adaptive protein-protein interface. Proc Natl Acad Sci USA 100:1603–1608
Babst M (2005) A protein's final ESCRT. Traffic 6:2–9
Ball LJ, Kuhne R, Schneider-Mergener J, Oschkinat H (2005) Recognition of proline-rich motifs by protein–protein-interaction domains. Angew Chem Int Ed Engl 44:2852–2869
Bandur NG, Harms K, Koert U (2005) First stereoselective synthesis of a pro-pro E-alkene dipeptide isostere. Synlett 773–776
Bear JE, Svitkina TM, Krause M, Schafer DA, Loureiro JJ, Strasser GA, Maly IV, Chaga OY, Cooper JA, Borisy GG, Gertler FB (2002) Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. Cell 109:509–521
Bedford MT, Chan DC, Leder P (1997) FBP WW domains and the Abl SH3 domain bind to a specific class of proline-rich ligands. EMBO J 16:2376–2383
Bedford MT, Frankel A, Yaffe MB, Clarke S, Leder P, Richard S (2000) Arginine methylation inhibits the binding of proline-rich ligands to Src homology 3, but not WW, domains. J Biol Chem 275:16030–16036
Ben Yosef R, Starr A, Karaush V, Loew V, Lev-Ari S, Barnea I, Lidawi G, Shtabsky A, Greif Y, Yarden Y, Vexler A (2007) ErbB-4 may control behavior of prostate cancer cells and serve as a target for molecular therapy. Prostate 67:871–880
Bhaskar K, Yen SH, Lee G (2005) Disease-related modifications in tau affect the interaction between Fyn and Tau. J Biol Chem 280:35119–35125
Bieniasz PD (2006) Late budding domains and host proteins in enveloped virus release. Virology 344:55–63
Bleicher KH, Bohm HJ, Muller K, Alanine AI (2003) Hit and lead generation: beyond high-throughput screening. Nat Rev Drug Discov 2:369–378
Bochicchio B, Tamburro AM (2002) Polyproline II structure in proteins: identification by chiroptical spectroscopies, stability, and functions. Chirality 14:782–792
Boggon TJ, Eck MJ (2004) Structure and regulation of Src family kinases. Oncogene 23:7918–7927
Bork P, Sudol M (1994) The WW domain: a signalling site in dystrophin? Trends Biochem Sci 19:531–533
Carlsson L, Nystrom LE, Sundkvist I, Markey F, Lindberg U (1977) Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. J Mol Biol 115:465–483
Carstens MJ, Krempler A, Triplett AA, Van Lohuizen M, Wagner KU (2004) Cell cycle arrest and cell death are controlled by p53-dependent and p53-independent mechanisms in Tsg101-deficient cells. J Biol Chem 279:35984–35994
Cesareni G, Panni S, Nardelli G, Castagnoli L (2002) Can we infer peptide recognition specificity mediated by SH3 domains? FEBS Lett 513:38–44
Chakraborty T, Ebel F, Domann E, Niebuhr K, Gerstel B, Pistor S, Temm-Grove CJ, Jockusch BM, Reinhard M, Walter U (1995) A focal adhesion factor directly linking intracellularly motile Listeria monocytogenes and Listeria ivanovii to the actin-based cytoskeleton of mammalian cells. EMBO J 14:1314–1321
Chan B, Lanyi A, Song HK, Griesbach J, Simarro-Grande M, Poy F, Howie D, Sumegi J, Terhorst C, Eck MJ (2003) SAP couples Fyn to SLAM immune receptors. Nat Cell Biol 5:155–160
Chin J, Palop JJ, Puolivali J, Massaro C, Bien-Ly N, Gerstein H, Scearce-Levie K, Masliah E, Mucke L (2005) Fyn kinase induces synaptic and cognitive impairments in a transgenic mouse model of Alzheimer's disease. J Neurosci 25:9694–9703
Clackson T, Wells JA (1995) A hot spot of binding energy in a hormone-receptor interface. Science 267:383–386
Cubellis MV, Caillez F, Blundell TL, Lovell SC (2005) Properties of polyproline II, a secondary structure element implicated in protein–protein interactions. Proteins 58:880–892

de Bakker PI, Bateman A, Burke DF, Miguel RN, Mizuguchi K, Shi J, Shirai H, Blundell TL (2001) HOMSTRAD: adding sequence information to structure-based alignments of homologous protein families. Bioinformatics 17:748–749
Demirov DG, Ono A, Orenstein JM, Freed EO (2002) Overexpression of the N-terminal domain of TSG101 inhibits HIV-1 budding by blocking late domain function. Proc Natl Acad Sci USA 99:955–960
Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ (2000) Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain Cell 103:351–361
Derry JM, Ochs HD, Francke U (1994) Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. Cell 79:635–644
Derry JM, Kerns JA, Weinberg KI, Ochs HD, Volpini V, Estivill X, Walker AP, Francke U (1995) WASP gene mutations in Wiskott-Aldrich syndrome and X-linked thrombocytopenia. Hum Mol Genet 4:1127–1135
Dev KK (2004) Making protein interactions druggable: targeting PDZ domains. Nat Rev Drug Discov 3:1047–1056
Doi M, Nishi Y, Uchiyama S, Nishiuchi Y, Nishio H, Nakazawa T, Ohkubo T, Kobayashi Y (2005) Collagen-like triple helix formation of synthetic (Pro-Pro-Gly)10 analogues: (4(S)-hydroxyprolyl-4(R)-hydroxyprolyl-Gly)10, (4(R)-hydroxyprolyl-4(R)-hydroxyprolyl-Gly)10 and (4(S)-fluoroprolyl-4(R)-fluoroprolyl-Gly)10. J Pept Sci 11:609–616
Dustin ML, Olszowy MW, Holdorf AD, Li J, Bromley S, Desai N, Widder P, Rosenberger F, van der Merwe PA, Allen PM, Shaw AS (1998) A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. Cell 94:667–677
Fischer PM, Lane DP (2004) Small-molecule inhibitors of the p53 suppressor HDM2: have protein–protein interactions come of age as drug targets? Trends Pharmacol Sci 25:343–346
Frank R (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports–principles and applications. J Immunol Methods 267:13–26
Freund C, Dotsch V, Nishizawa K, Reinherz EL, Wagner G (1999) The GYF domain is a novel structural fold that is involved in lymphoid signaling through proline-rich sequences. Nat Struct Biol 6:656–660
Freund C, Kuhne R, Yang H, Park S, Reinherz EL, Wagner G (2002) Dynamic interaction of CD2 with the GYF and the SH3 domain of compartmentalized effector molecules. EMBO J 21:5985–5995
Fukai I, Hussey RE, Sunder-Plassmann R, Reinherz EL (2000) A critical role for p59(fyn) in CD2-based signal transduction. Eur J Immunol 30:3507–3515
Garrus JE, von Schwedler UK, Pornillos OW, Morham SG, Zavitz KH, Wang HE, Wettstein DA, Stray KM, Cote M, Rich RL, Myszka DG, Sundquist WI (2001) Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell 107:55–65
Goila-Gaur R, Demirov DG, Orenstein JM, Ono A, Freed EO (2003) Defects in human immunodeficiency virus budding and endosomal sorting induced by TSG101 overexpression. J Virol 77:6507–6519
Goldstrohm AC, Albrecht TR, Sune C, Bedford MT, Garcia-Blanco MA (2001) The transcription elongation factor CA150 interacts with RNA polymerase II and the pre-mRNA splicing factor SF1. Mol Cell Biol 21:7617–7628
Golemi-Kotra D, Mahaffy R, Footer MJ, Holtzman JH, Pollard TD, Theriot JA, Schepartz A (2004) High affinity, paralog-specific recognition of the Mena EVH1 domain by a miniature protein. J Am Chem Soc 126:4–5
Gorina S, Pavletich NP (1996) Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. Science 274:1001–1005
Gottlinger HG, Dorfman T, Sodroski JG, Haseltine WA (1991) Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. Proc Natl Acad Sci USA 88:3195–3199
Groemping Y, Lapouge K, Smerdon SJ, Rittinger K (2003) Molecular basis of phosphorylation-induced activation of the NADPH oxidase. Cell 113:343–355

Harkiolaki M, Lewitzky M, Gilbert RJ, Jones EY, Bourette RP, Mouchiroud G, Sondermann H, Moarefi I, Feller SM (2003) Structural basis for SH3 domain-mediated high-affinity binding between Mona/Gads and SLP-76. EMBO J 22:2571–2582

Harris BZ, Lim WA (2001) Mechanism and role of PDZ domains in signaling complex assembly. J Cell Sci 114:3219–3231

He MM, Smith AS, Oslob JD, Flanagan WM, Braisted AC, Whitty A, Cancilla MT, Wang J, Lugovskoy AA, Yoburn JC, Fung AD, Farrington G, Eldredge JK, Day ES, Cruz LA, Cachero TG, Miller SK, Friedman JE, Choong IC, Cunningham BC (2005) Small-molecule inhibition of TNF-alpha. Science 310:1022–1025

Hofmann RM, Pickart CM (1999) Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. Cell 96:645–653

Holbert S, Denghien I, Kiechle T, Rosenblatt A, Wellington C, Hayden MR, Margolis RL, Ross CA, Dausset J, Ferrante RJ, Neri C (2001) The Gln-Ala repeat transcriptional activator CA150 interacts with huntingtin: neuropathologic and genetic evidence for a role in Huntington's disease pathogenesis. Proc Natl Acad Sci USA 98:1811–1816

Holmgren SK, Bretscher LE, Taylor KM, Raines RT (1999) A hyperstable collagen mimic. Chem Biol 6:63–70

Hong M, Zhukareva V, Vogelsberg-Ragaglia V, Wszolek Z, Reed L, Miller BI, Geschwind DH, Bird TD, McKeel D, Goate A, Morris JC, Wilhelmsen KC, Schellenberg GD, Trojanowski JQ, Lee VM (1998) Mutation-specific functional impairments in distinct tau isoforms of hereditary FTDP-17. Science 282:1914–1917

Huang M, Orenstein JM, Martin MA, Freed EO (1995) p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. J Virol 69:6810–6818

Hurley JH, Emr SD (2006) The ESCRT complexes: structure and mechanism of a membrane-trafficking network. Annu Rev Biophys Biomol Struct 35:277–298

Ilsley JL, Sudol M, Winder SJ (2002) The WW domain: linking cell signalling to the membrane cytoskeleton. Cell Signal 14:183–189

Ingham RJ, Gish G, Pawson T (2004) The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture. Oncogene 23:1972–1984

Inglis SR, Stojkoski C, Branson KM, Cawthray JF, Fritz D, Wiadrowski E, Pyke SM, Booker GW (2004) Identification and specificity studies of small-molecule ligands for SH3 protein domains. J Med Chem 47:5405–5417

Inglis SR, Jones RK, Booker GW, Pyke SM (2006) Synthesis of N-benzylated-2-aminoquinolines as ligands for the Tec SH3 domain. Bioorg Med Chem Lett 16:387–390

Junttila TT, Sundvall M, Lundin M, Lundin J, Tanner M, Harkonen P, Joensuu H, Isola J, Elenius K (2005) Cleavable ErbB4 isoform in estrogen receptor-regulated growth of breast cancer cells. Cancer Res 65:1384–1393

Kanelis V, Rotin D, Forman-Kay JD (2001) Solution structure of a Nedd4 WW domain-ENaC peptide complex. Nat Struct Biol 8:407–412

Karkkainen S, Hiipakka M, Wang JH, Kleino I, Vaha-Jaakkola M, Renkema GH, Liss M, Wagner R, Saksela K (2006) Identification of preferred protein interactions by phage-display of the human Src homology-3 proteome. EMBO Rep 7:186–191

Kaushik N, Fear D, Richards SC, McDermott CR, Nuwaysir EF, Kellam P, Harrison TJ, Wilkinson RJ, Tyrrell DA, Holgate ST, Kerr JR (2005) Gene expression in peripheral blood mononuclear cells from patients with chronic fatigue syndrome. J Clin Pathol 58:826–832

Kay BK, Williamson MP, Sudol M (2000) The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. FASEB J 14:231–241

Kentsis A, Mezei M, Gindin T, Osman R (2004) Unfolded state of polyalanine is a segmented polyproline II helix. Proteins 55:493–501

Kim HH, Tharayil M, Rudd CE (1998) Growth factor receptor-bound protein 2 SH2/SH3 domain binding to CD28 and its role in co-signaling. J Biol Chem 273:296–301

Kofler MM, Freund C (2006) The GYF domain. FEBS J 273:245–256

Kofler M, Heuer K, Zech T, Freund C (2004) Recognition sequences for the GYF domain reveal a possible spliceosomal function of CD2BP2. J Biol Chem 279:28292–28297
Kofler M, Motzny K, Freund C (2005) GYF Domain proteomics reveals interaction sites in known and novel target proteins. Mol Cell Proteomics 4:1797–1811
Kolluri R, Shehabeldin A, Peacocke M, Lamhonwah AM, Teichert-Kuliszewska K, Weissman SM, Siminovitch KA (1995) Identification of WASP mutations in patients with Wiskott-Aldrich syndrome and isolated thrombocytopenia reveals allelic heterogeneity at the WAS locus. Hum Mol Genet 4:1119–1126
Komuro A, Nagai M, Navin NE, Sudol M (2003) WW domain-containing protein YAP associates with ErbB-4 and acts as a co-transcriptional activator for the carboxyl-terminal fragment of ErbB-4 that translocates to the nucleus. J Biol Chem 278:33334–33341
Krempler A, Henry MD, Triplett AA, Wagner KU (2002) Targeted deletion of the Tsg101 gene results in cell cycle arrest at G1/S and p53-independent cell death. J Biol Chem 277:43216–43223
Laggerbauer B, Liu S, Makarov E, Vornlocher HP, Makarova O, Ingelfinger D, Achsel T, Luhrmann R (2005) The human U5 snRNP 52 K protein (CD2BP2) interacts with U5-102 K (hPrp6), a U4/U6.U5 tri-snRNP bridging protein, but dissociates upon tri-snRNP formation. RNA 11:598–608
Laurent V, Loisel TP, Harbeck B, Wehman A, Grobe L, Jockusch BM, Wehland J, Gertler FB, Carlier MF (1999) Role of proteins of the Ena/VASP family in actin-based motility of Listeria monocytogenes. J Cell Biol 144:1245–1258
Lawrence DS (2005) Signaling protein inhibitors via the combinatorial modification of peptide scaffolds. Biochim Biophys Acta 1754:50–57
Lee G, Newman ST, Gard DL, Band H, Panchamoorthy G (1998) Tau interacts with src-family non-receptor tyrosine kinases. J Cell Sci 111(Pt 21):3167–3177
Li SS (2005) Specificity and versatility of SH3 and other proline-recognition domains: structural basis and implications for cellular signal transduction. Biochem J 390:641–653
Li H, Lawrence DS (2005) Acquisition of Fyn-selective SH3 domain ligands via a combinatorial library strategy. Chem Biol 12:905–912
Lim WA (1996) Reading between the lines: SH3 recognition of an intact protein. Structure 4:657–659
Lin H, Hutchcroft JE, Andoniou CE, Kamoun M, Band H, Bierer BE (1998) Association of p59(fyn) with the T lymphocyte costimulatory receptor CD2. Binding of the Fyn Src homology (SH) 3 domain is regulated by the Fyn SH2 domain. J Biol Chem 273:19914–19921
Liu Q, Berry D, Nash P, Pawson T, McGlade CJ, Li SS (2003) Structural basis for specific binding of the Gads SH3 domain to an RxxK motif-containing SLP-76 peptide: a novel mode of peptide recognition. Mol Cell 11:471–481
Liu F, Stephen AG, Adamson CS, Gousset K, Aman MJ, Freed EO, Fisher RJ, Burke TR, Jr. (2006) Hydrazone- and hydrazide-containing N-substituted glycines as peptoid surrogates for expedited library synthesis: application to the preparation of Tsg101-directed HIV-1 budding antagonists. Org Lett 8:5165–5168
Lu PJ, Zhou XZ, Shen M, Lu KP (1999) Function of WW domains as phosphoserine- or phosphothreonine-binding modules. Science 283:1325–1328
Lubs H, Abidi FE, Echeverri R, Holloway L, Meindl A, Stevenson RE, Schwartz CE (2006) Golabi-Ito-Hall syndrome results from a missense mutation in the WW domain of the PQBP1 gene. J Med Genet 43:e30
Macias MJ, Hyvonen M, Baraldi E, Schultz J, Sudol M, Saraste M, Oschkinat H (1996) Structure of the WW domain of a kinase-associated protein complexed with a proline-rich peptide. Nature 382:646–649
Mayer BJ (2001) SH3 domains: complexity in moderation. J Cell Sci 114:1253–1263
Mezei M, Fleming PJ, Srinivasan R, Rose GD (2004) Polyproline II helix is the preferred conformation for unfolded polyalanine in water. Proteins 55:502–507
Mizuguchi K, Deane CM, Blundell TL, Overington JP (1998) HOMSTRAD: a database of protein structure alignments for homologous families. Protein Sci 7:2469–2471

Montclare JK, Schepartz A (2003) Miniature homeodomains: high specificity without an N-terminal arm. J Am Chem Soc 125:3416–3417
Morita E, Sundquist WI (2004) Retrovirus budding. Annu Rev Cell Dev Biol 20:395–425
Musacchio A, Saraste M, Wilmanns M (1994a) High-resolution crystal structures of tyrosine kinase SH3 domains complexed with proline-rich peptides. Nat Struct Biol 1:546–551
Musacchio A, Wilmanns M, Saraste M (1994b) Structure and function of the SH3 domain. Prog Biophys Mol Biol 61:283–297
Myllyharju J, Kivirikko KI (1999) Identification of a novel proline-rich peptide-binding domain in prolyl 4-hydroxylase. EMBO J 18:306–312
Nash P, Tang X, Orlicky S, Chen Q, Gertler FB, Mendenhall MD, Sicheri F, Pawson T, Tyers M (2001) Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. Nature 414:514–521
Nguyen JT, Turck CW, Cohen FE, Zuckermann RN, Lim WA (1998) Exploiting the basis of proline recognition by SH3 and WW domains: design of N-substituted inhibitors. Science 282:2088–2092
Nguyen JT, Porter M, Amoui M, Miller WT, Zuckermann RN, Lim WA (2000) Improving SH3 domain ligand selectivity using a non-natural scaffold. Chem Biol 7:463–473
Ni CY, Murphy MP, Golde TE, Carpenter G (2001) gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science 294:2179–2181
Niebuhr K, Ebel F, Frank R, Reinhard M, Domann E, Carl UD, Walter U, Gertler FB, Wehland J, Chakraborty T (1997) A novel proline-rich motif present in ActA of Listeria monocytogenes and cytoskeletal proteins is the ligand for the EVH1 domain, a protein module present in the Ena/VASP family. EMBO J 16:5433–5444
Nielsen TK, Liu S, Luhrmann R, Ficner R (2007) Structural basis for the bifunctionality of the U5 snRNP 52 K protein (CD2BP2). J Mol Biol 369:902–908
Nishi Y, Uchiyama S, Doi M, Nishiuchi Y, Nakazawa T, Ohkubo T, Kobayashi Y (2005) Different effects of 4-hydroxyproline and 4-fluoroproline on the stability of collagen triple helix. Biochemistry 44:6034–6042
Nishizawa K, Freund C, Li J, Wagner G, Reinherz EL (1998) Identification of a proline-binding motif regulating CD2-triggered T lymphocyte activation. Proc Natl Acad Sci USA 95:14897–14902
Okkenhaug K, Rottapel R (1998) Grb2 forms an inducible protein complex with CD28 through a Src homology 3 domain-proline interaction. J Biol Chem 273:21194–21202
Oltersdorf T, Elmore SW, Shoemaker AR, Armstrong RC, Augeri DJ, Belli BA, Bruncko M, Deckwerth TL, Dinges J, Hajduk PJ, Joseph MK, Kitada S, Korsmeyer SJ, Kunzer AR, Letai A, Li C, Mitten MJ, Nettesheim DG, Ng S, Nimmer PM, O'Connor JM, Oleksijew A, Petros AM, Reed JC, Shen W, Tahir SK, Thompson CB, Tomaselli KJ, Wang B, Wendt MD, Zhang H, Fesik SW, Rosenberg SH (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature 435:677–681
Oneyama C, Nakano H, Sharma SV (2002) UCS15A, a novel small molecule, SH3 domain-mediated protein–protein interaction blocking drug. Oncogene 21:2037–2050
Oneyama C, Agatsuma T, Kanda Y, Nakano H, Sharma SV, Nakano S, Narazaki F, Tatsuta K (2003) Synthetic inhibitors of proline-rich ligand-mediated protein–protein interaction: potent analogs of UCS15A. Chem Biol 10:443–451
Orth JD, McNiven MA (2003) Dynamin at the actin-membrane interface. Curr Opin Cell Biol 15:31–39
Paige AJ, Taylor KJ, Taylor C, Hillier SG, Farrington S, Scott D, Porteous DJ, Smyth JF, Gabra H, Watson JE (2001) WWOX: a candidate tumor suppressor gene involved in multiple tumor types. Proc Natl Acad Sci USA 98:11417–11422
Pornillos O, Alam SL, Davis DR, Sundquist WI (2002a) Structure of the Tsg101 UEV domain in complex with the PTAP motif of the HIV-1 p6 protein. Nat Struct Biol 9:812–817
Pornillos O, Garrus JE, Sundquist WI (2002b) Mechanisms of enveloped RNA virus budding. Trends Cell Biol 12:569–579

Pornillos O, Higginson DS, Stray KM, Fisher RD, Garrus JE, Payne M, He GP, Wang HE, Morham SG, Sundquist WI (2003) HIV Gag mimics the Tsg101-recruiting activity of the human Hrs protein. J Cell Biol 162:425–434

Prehoda KE, Lee DJ, Lim WA (1999) Structure of the enabled/VASP homology 1 domain-peptide complex: a key component in the spatial control of actin assembly. Cell 97:471–480

Qin ZH, Wang Y, Sapp E, Cuiffo B, Wanker E, Hayden MR, Kegel KB, Aronin N, DiFiglia M (2004) Huntingtin bodies sequester vesicle-associated proteins by a polyproline-dependent interaction. J Neurosci 24:269–281

Rothstein DM, Sayegh MH (2003) T-cell costimulatory pathways in allograft rejection and tolerance. Immunol Rev 196:85–108

Rutledge SE, Volkman HM, Schepartz A (2003) Molecular recognition of protein surfaces: high affinity ligands for the CBP KIX domain. J Am Chem Soc 125:14336–14347

Ruzza P, Siligardi G, Donella-Deana A, Calderan A, Hussain R, Rubini C, Cesaro L, Osler A, Guiotto A, Pinna LA, Borin G (2006) 4-Fluoroproline derivative peptides: effect on PPII conformation and SH3 affinity. J Pept Sci 12:462–471

Scaife RM, Margolis RL (1997) The role of the PH domain and SH3 binding domains in dynamin function. Cell Signal 9:395–401

Schild L, Lu Y, Gautschi I, Schneeberger E, Lifton RP, Rossier BC (1996) Identification of a PY motif in the epithelial Na channel subunits as a target sequence for mutations causing channel activation found in Liddle syndrome. EMBO J 15:2381–2387

Sharma SV, Oneyama C, Yamashita Y, Nakano H, Sugawara K, Hamada M, Kosaka N, Tamaoki T (2001) UCS15A, a non-kinase inhibitor of Src signal transduction. Oncogene 20:2068–2079

Shiraishi-Yamaguchi Y, Furuichi T (2007) The Homer family proteins. Genome Biol 8:206

Southwick FS, Purich DL (1994) Arrest of Listeria movement in host cells by a bacterial ActA analogue: implications for actin-based motility. Proc Natl Acad Sci USA 91:5168–5172

Sreerama N, Woody RW (1999) Molecular dynamics simulations of polypeptide conformations in water: A comparison of alpha, beta, and poly(pro)II conformations. Proteins 36:400–406

Starr A, Greif J, Vexler A, Ashkenazy-Voghera M, Gladesh V, Rubin C, Kerber G, Marmor S, Lev-Ari S, Inbar M, Yarden Y, Ben Yosef R (2006) ErbB4 increases the proliferation potential of human lung cancer cells and its blockage can be used as a target for anti-cancer therapy. Int J Cancer 119:269–274

Staub O, Dho S, Henry P, Correa J, Ishikawa T, McGlade J, Rotin D (1996) WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle's syndrome. EMBO J 15:2371–2380

Sudol M (1996) Structure and function of the WW domain. Prog Biophys Mol Biol 65:113–132

Sudol M, Sliwa K, Russo T (2001) Functions of WW domains in the nucleus. FEBS Lett 490:190–195

Szumlinski KK, Lominac KD, Oleson EB, Walker JK, Mason A, Dehoff MH, Klugmann M, Cagle S, Welt K, During M, Worley PF, Middaugh LD, Kalivas PW (2005) Homer2 is necessary for EtOH-induced neuroplasticity. J Neurosci 25:7054–7061

Szumlinski KK, Kalivas PW, Worley PF (2006) Homer proteins: implications for neuropsychiatric disorders. Curr Opin Neurobiol 16:251–257

Takeya R, Ueno N, Sumimoto H (2006) Regulation of superoxide-producing NADPH oxidases in nonphagocytic cells. Methods Enzymol, Regulators and Effectors of Small Gtpases: Rho Family 406:456–468

Tanaka M, Shibata H (1985) Poly(L-proline)-binding proteins from chick embryos are a profilin and a profilactin. Eur J Biochem 151:291–297

Tiffany ML, Krimm S (1968) Circular dichroism of poly-L-proline in an unordered conformation. Biopolymers 6:1767–1770

Tremmel P, Geyer A (2002) An oligomeric ser-pro dipeptide mimetic assuming the polyproline II helix conformation. J Am Chem Soc 124:8548–8549

VanDemark AP, Hofmann RM, Tsui C, Pickart CM, Wolberger C (2001) Molecular insights into polyubiquitin chain assembly: crystal structure of the Mms2/Ubc13 heterodimer. Cell 105:711–720

Varmus H, Hirai H, Morgan D, Kaplan J, Bishop JM (1989) Function, location, and regulation of the src protein-tyrosine kinase. Princess Takamatsu Symp 20:63–70

VerPlank L, Bouamr F, LaGrassa TJ, Agresta B, Kikonyogo A, Leis J, Carter CA (2001) Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). Proc Natl Acad Sci USA 98:7724–7729

Vetter SW, Zhang ZY (2002) Probing the phosphopeptide specificities of protein tyrosine phosphatases, SH2 and PTB domains with combinatorial library methods. Curr Protein Pept Sci 3:365–397

Villa A, Notarangelo L, Macchi P, Mantuano E, Cavagni G, Brugnoni D, Strina D, Patrosso MC, Ramenghi U, Sacco MG, (1995) X-linked thrombocytopenia and Wiskott-Aldrich syndrome are allelic diseases with mutations in the WASP gene. Nat Genet 9:414–417

Volkman BF, Prehoda KE, Scott JA, Peterson FC, Lim WA (2002) Structure of the N-WASP EVH1 domain-WIP complex: insight into the molecular basis of Wiskott-Aldrich Syndrome. Cell 111:565–576

Wagner KU, Krempler A, Qi Y, Park K, Henry MD, Triplett AA, Riedlinger G, Rucker EB III, Hennighausen L (2003) Tsg101 is essential for cell growth, proliferation, and cell survival of embryonic and adult tissues. Mol Cell Biol 23:150–162

Williams RL, Urbé S (2007) The emerging shape of the ESCRT machinery. Nat Rev Mol Cell Biol 8:355–368. Review.

Williams RS, Bernstein N, Lee MS, Rakovszky ML, Cui D, Green R, Weinfeld M, Glover JN (2005) Structural basis for phosphorylation-dependent signaling in the DNA-damage response. Biochem Cell Biol 83:721–727

Witter DJ, Famiglietti SJ, Cambier JC, Castelhano AL (1998) Design and synthesis of SH3 domain binding ligands: modifications of the consensus sequence XPpXP. Bioorg Med Chem Lett 8:3137–3142

Woodward JE, Qin L, Chavin KD, Lin J, Tono T, Ding Y, Linsley PS, Bromberg JS, Baliga P (1996) Blockade of multiple costimulatory receptors induces hyporesponsiveness: inhibition of CD2 plus CD28 pathways. Transplantation 62:1011–1018

Xu W, Doshi A, Lei M, Eck MJ, Harrison SC (1999) Crystal structures of c-Src reveal features of its autoinhibitory mechanism. Mol Cell 3:629–638

Yu H, Chen JK, Feng S, Dalgarno DC, Brauer AW, Schreiber SL (1994) Structural basis for the binding of proline-rich peptides to SH3 domains. Cell 76:933–945

Zarrinpar A, Bhattacharyya RP, Lim WA (2003a) The structure and function of proline recognition domains. Sci STKE 2003:RE8

Zarrinpar A, Park SH, Lim WA (2003b) Optimization of specificity in a cellular protein interaction network by negative selection. Nature 426:676–680

Zhang Y, Lindblom T, Chang A, Sudol M, Sluder AE, Golemis EA (2000) Evidence that dim1 associates with proteins involved in pre-mRNA splicing, and delineation of residues essential for dim1 interactions with hnRNP F and Npw38/PQBP-1. Gene 257:33–43

Zhu Q, Watanabe C, Liu T, Hollenbaugh D, Blaese RM, Kanner SB, Aruffo A, Ochs HD (1997) Wiskott-Aldrich syndrome/X-linked thrombocytopenia: WASP gene mutations, protein expression, and phenotype. Blood 90:2680–2689

Zimmermann J, Kuhne R, Volkmer-Engert R, Jarchau T, Walter U, Oschkinat H, Ball LJ (2003) Design of N-substituted peptomer ligands for EVH1 domains. J Biol Chem 278:36810–36818

Chemical Inhibition Through Conformational Stabilization of Rho GTPase Effectors

S.W. Deacon and J.R. Peterson(✉)

Contents

1 Introduction 432
 1.1 Small-Molecule Inhibitors as Biological Probes 432
 1.2 Chemical Genetics 433
 1.3 Chemical Genetics as a Tool to Dissect the Regulation of Rho GTPase Signaling 434
2 A Forward Chemical Genetic Screen Identifies Wiskostatin, a Small-Molecule Inhibitor of N-WASP 435
 2.1 Actin Polymerization Induced by PIP2 and Cdc42 435
 2.2 The Target of Wiskostatin is N-WASP 437
 2.3 Mechanism of N-WASP Inhibition by Wiskostatin 438
 2.4 Autoinhibition of Rho GTPase Effectors as a Target for Small-Molecule Inhibitors 440
3 p21-Activated Kinases as Targets for Allosteric Inhibition 441
 3.1 The Pak Family of Rho GTPase Effectors 441
 3.2 Autoinhibition of Pak1 442
 3.3 Relevance of an Allosteric Pak1 Inhibitor 443
 3.4 Endogenous Protein Inhibitors of Pak1 Bind the Autoregulatory Domain 445
 3.5 Screening Approaches to Identify Allosteric Group I Pak Inhibitors 446
4 DRFs as Targets for Allosteric Inhibition 447
 4.1 Formins Are a Family of Actin-Nucleating Proteins 447
 4.2 Diaphanous-Related Formins Are Rho GTPase Effectors That Undergo Autoinhibition 447
 4.3 Autoinhibition of mDia1 448
 4.4 Relevance of an Allosteric mDia1 Inhibitor 450
 4.5 Screening Approaches to Identify Allosteric mDia1 Inhibitors 451
5 Concluding Remarks/Perspective 452
References 453

J.R. Peterson
Division of Basic Science, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA
Jeffrey.Peterson@fccc.edu

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

Abstract The Rho family of small GTP-binding proteins can activate a large number of downstream effectors and participate in a wide variety of biological processes, including cell motility, membrane trafficking, cell polarity, gene transcription, and mitosis. Specific small-molecule inhibitors of individual effector proteins downstream of Rho GTPases would be powerful tools to elucidate the contributions of particular effectors to these processes. In this chapter we describe the identification of a chemical inhibitor of a Rho effector and scaffolding protein neural-Wiskott-Aldrich syndrome protein (N-WASP), and the discovery of its novel mechanism of action, stabilization of N-WASP's native autoinhibited conformation. Inasmuch as several other Rho GTPase effectors are regulated by autoinhibition, we discuss how this regulatory mechanism could be exploited by small molecules to develop highly specific inhibitors of other Rho GTPase effectors. We illustrate this concept with the Rac/Cdc42 effector p21-activated kinase (Pak1) and the Rho effector mammalian diaphanous-related formin (mDia1).

1 Introduction

1.1 Small-Molecule Inhibitors as Biological Probes

There are many approaches to experimentally eliminate the function of individual proteins. These include genetic knockouts, introduction of dominant negative protein forms, antisense or RNA interference-mediated knockdown of expression, and the use of specific chemical inhibitors. Drug-like small molecules offer a number of advantages over other methods, although thus far the number of proteins for which such inhibitors exist is relatively small.

Small-molecule inhibitors are particularly useful for studying highly dynamic biological systems, such as the cytoskeletal network in cells (Peterson and Mitchison 2002). Small-molecule inhibitors can take effect on a timescale of seconds, thereby offering exquisite temporal control over protein inhibition. This rapid means of protein inactivation can allow for more informative perturbation experiments, as biologically important events can occur on a sub-second timescale. By contrast, genetic knockouts, dominant negative protein forms, and RNA interference-mediated protein downregulation typically require substantially greater time to take effect, opening the possibility of functional compensation through, for example, upregulated expression of other functionally redundant proteins. Thus, chemical inhibitors can be used to reveal the unique consequences of acute loss of protein function.

An additional strength of small molecules as research tools is that they are frequently effective in multiple cell types and species. Chemical compounds developed using one experimental system can often be applied to others, whereas, for example, genetic knockouts are inherently species specific. Furthermore, genes required for organismal viability can require the cumbersome generation of conditional knockouts

for functional analysis. The use of temperature-sensitive alleles can mitigate this problem, but in some cases temperature shifts alone can independently introduce perturbations (Gasch et al. 2000). RNA interference reagents are also typically species specific due to nucleotide differences in genes from even closely related organisms. Thus, the "portability" of small molecules renders them more broadly useful to study diverse biological systems.

In addition, small-molecule inhibitors may allow for inhibition of a subset of a protein's functions, thus providing a finer scalpel for dissecting protein function. Whereas genetic knock-out of protein expression or knock-down by RNA interference eliminates all protein function, chemical inhibitors could, for example, inhibit catalytic activity while preserving target scaffolding functions (Knight and Shokat 2007). Similarly, inhibiting protein function through the use of so-called "dominant negative" forms of proteins, which often act by titrating interacting partners away from the corresponding endogenous, wild-type protein, can produce off-target effects that may be avoided by small molecules that bind and inhibit their targets directly.

For these reasons, small-molecule inhibitors offer a powerful and complementary approach for investigating protein function that is distinct from genetic, RNAi-dependent approaches and the use of dominant negative protein forms.

1.2 Chemical Genetics

Small-molecule inhibitors are typically identified through high-throughput screening of large libraries of chemical compounds. These screens can be "target-specific," for example, when utilizing purified preparations of the targeted protein, or "phenotypic," when compounds are tested in more complex biological systems. This latter approach is often referred to as "chemical genetics," based on an analogy to traditional genetics. Traditional forward genetics seeks to link biological phenotypes identified in screens with mutations in specific genes. In forward *chemical* genetic screens, compounds are screened for their ability to produce a phenotype of interest without regard to the specific target. Subsequent identification of the protein targeted by the small molecule thereby causally links the small-molecule target with the biological process under study. Thus, phenotypic *chemical* genetic screens, like *traditional* genetic screens, can be used to elucidate molecular mechanisms through the identification of proteins essential for a biological process. In addition to providing a means to discover proteins, however, this approach also identifies corresponding chemical inhibitors that can be used to elucidate protein function. For example, the compound secramine was identified in a phenotypic screen for compounds inhibiting trafficking of the vesicular stomatitis virus G protein from the Golgi to the plasma membrane (Pelish et al. 2001). Subsequent identification of the target of secramine as the Rho GTPase Cdc42 thereby linked Cdc42 to vesicular trafficking from the Golgi and provided a novel reagent for studying this function of Cdc42 (Pelish et al. 2006).

1.3 Chemical Genetics as a Tool to Dissect the Regulation of Rho GTPase Signaling

Small-molecule inhibitors can be used to dissect complex cellular signaling networks based on their ability to rapidly inhibit individual proteins participating within a network. For example, kinase inhibitors have been invaluable reagents for revealing epistatic relationships in signal transduction cascades (Knight and Shokat 2005). An example of a complex signaling network whose study could benefit from specific inhibitors is the signaling network centered around the Rho subfamily of the Ras superfamily of small GTP-binding proteins. The Rho GTPases are a family of signaling proteins originally linked closely to the regulation of the actin cytoskeleton (Ridley and Hall 1992; Ridley et al. 1992; Nobes and Hall 1995); however, ensuing studies have uncovered a myriad of cellular signaling pathways that involve the Rho GTPases (Jaffe and Hall 2005). As such, Rho GTPases have been implicated in a plethora of biological functions and disease states, including membrane trafficking, mitosis, transformation, and cancer (reviewed in Sahai and Marshall 2002).

The complexity of Rho GTPase signaling is due largely to the extensive number of both upstream and downstream binding partners. Rho proteins are molecular switches that cycle between inactive GDP-bound and active GTP-bound states through the actions of upstream regulatory proteins. GTPase-activating proteins promote the hydrolysis of bound GTP to GDP, and guanine nucleotide exchange factors catalyze the release of bound GDP, facilitating binding of GTP (Bos et al. 2007). Over 70 guanine nucleotide exchange factors have been identified for Rho GTPases (Meller et al. 2005; Rossman et al. 2005; Bos et al. 2007), thereby providing a mechanism for coupling a broad spectrum of upstream pathways to Rho GTPase activation. When GTP-bound, Rho GTPases can bind any of over 30 different effector proteins, including enzymes (e.g., kinases, oxidases, and lipases) as well as proteins that appear to serve predominantly scaffolding functions (Karnoub et al. 2004). However, the biological roles of many of these effectors are poorly understood, and a major emphasis in the field of Rho GTPase signaling has been the identification and functional characterization of upstream and downstream binding partners.

Adding to the complexity is the existence of 20 different Rho GTPases in humans (Boureux et al. 2007). Some binding partners are shared by multiple Rho GTPases, whereas others appear more GTPase-specific (Karnoub et al. 2004). Three of the best-studied Rho GTPases are Rho, Rac, and Cdc42. Rac and Cdc42 are the most closely related and share a number of effector proteins (Karnoub et al. 2004). The Rho family, by contrast, signals via a largely non-overlapping series of effectors (Karnoub et al. 2004).

The development of small-molecule inhibitors of components of Rho GTPase-mediated signaling could facilitate the elucidation of the functional roles of the proteins within these networks and may substantially further our ability to define the nature of the complex signaling networks themselves. In the following section,

we will describe a forward chemical genetic screen to identify small molecules that may target proteins both upstream and downstream of Cdc42, a Rho GTPase identified as a key signaling module in the regulation of actin filament assembly.

2 A Forward Chemical Genetic Screen Identifies Wiskostatin, a Small-Molecule Inhibitor of N-WASP

In order to identify small molecules that perturb Rho GTPase-dependent signaling, we conducted a high-throughput screen for compounds that inhibit Cdc42-dependent actin filament assembly in cytoplasmic extracts of *Xenopus laevis* eggs. These extracts are prepared by simple mechanical disruption of intact eggs by gentle centrifugation rather than through detergent extraction, resulting in essentially undiluted, detergent-free cytoplasm containing proteins at physiological concentrations (Lebensohn et al. 2006). Consequently, these extracts are widely used as a model system to study diverse cellular signaling and regulatory networks (Chen and Murray 1997; de la Barre et al. 1999; Salic et al. 2000; Castro et al. 2006; Chan and Forbes 2006; Deming and Kornbluth 2006; Lebensohn et al. 2006; Moon et al. 2006; Shennan 2006; Tutter and Walter 2006). High-speed centrifugation depletes the extract of endogenous lipid membranes, resulting in a quiescent extract termed HSS (high-speed supernatant) in which actin monomers do not spontaneously polymerize. Subsequent stimulation of the extract by the addition of lipid vesicles (liposomes) containing small amounts of phosphatidylinositol 4,5-bisphosphate (PIP2) results in the polymerization of endogenous actin onto the surface of the liposomes (Ma et al. 1998a; Peterson et al. 2001). Although this activity may recapitulate the localized polymerization of actin at the plasma membrane (Ma et al. 1998a), recent studies suggest that this pathway may also be used physiologically to mediate endocytosis and the intracellular motility of endosomally derived vesicles (Taunton et al. 2000; Sokac et al. 2003). As described below, actin assembly by PIP2-stimulated HSS has proven a robust assay for identifying components of the actin regulatory machinery that are critical for cell motility and morphology in many systems.

2.1 Actin Polymerization Induced by PIP2 and Cdc42

PIP2 promotes the nucleation of actin filaments in *Xenopus* egg extract via a Cdc42-dependent pathway (Ma et al. 1998a; Pelish et al. 2006; Peterson et al. 2006). PIP2 has also been linked to activation of Cdc42 in mammalian cells (Martin-Belmonte et al. 2007). PIP2 activation of Cdc42 is accomplished in part by promoting the membrane recruitment of Cdc42 through a direct interaction with a poly-basic stretch of amino acids in the C-terminal region of Cdc42 (Heo

et al. 2006) or, alternatively, through indirect recruitment by the PIP2-binding protein annexin 2 (Martin-Belmonte et al. 2007). In addition, PIP2 may promote guanine nucleotide exchange on Cdc42 either directly (Zheng et al. 1996) or indirectly through the regulation of guanine nucleotide exchange factors (Baumeister et al. 2006). PIP2 can also contribute to actin assembly by binding and promoting the activation of a ubiquitously expressed Cdc42 effector called N-WASP (neural Wiskott-Aldrich syndrome protein) (Miki et al. 1996; Prehoda et al. 2000; Rohatgi et al. 2000, 2001; Papayannopoulos et al. 2005) or by preventing capping protein from capping actin filament barbed ends (Schafer et al. 1996). It should be noted, however, that these latter two roles for PIP2 appear to be minor in *Xenopus* HSS compared to its role in activating Cdc42 (Rohatgi et al. 2000; Ho et al. 2004).

Cdc42 has been shown in many systems to direct the recruitment and activation of proteins that mediate the nucleation and polymerization of actin filaments (Rohatgi et al. 1999; Taunton et al. 2000; Lechler et al. 2001; Sokac et al. 2003). Studies concurrent with our work used an activity-based biochemical purification to identify proteins in *Xenopus* egg extract required for mediating actin polymerization downstream of Cdc42 (Ma et al. 1998b; Rohatgi et al. 1999; Ho et al. 2004). These studies identified three protein factors required for actin nucleation by Cdc42: the Cdc42-binding scaffolding protein N-WASP, a second Cdc42-binding protein known as Toca-1 (transducer of Cdc42-mediated actin assembly), and the ubiquitous actin filament nucleating protein complex, the Arp2/3 complex. Cdc42, together with Toca-1, binds N-WASP and promotes N-WASP activation (Rohatgi et al. 2000; Ho et al. 2006). Active N-WASP, in turn, binds and stimulates the actin filament nucleation activity of the Arp2/3 complex (Rohatgi et al. 1999). Both N-WASP and Arp2/3 complex are now appreciated as important for actin filament nucleation in many contexts (Millard et al. 2004; Vartiainen and Machesky 2004; Stradal and Scita 2006; Takenawa and Suetsugu 2007).

To identify proteins linking PIP2 signaling to actin filament assembly as well as to develop reagents for studying this signaling pathway in other systems, we screened unbiased libraries of chemical compounds for their ability to inhibit actin assembly in PIP2-stimulated HSS. These compounds included both structurally diverse, drug-like small molecules and a library of cyclic peptides. Over 26,000 compounds were tested for their ability to inhibit actin assembly induced by 10 μM liposomes containing PIP2 (PIP2: phosphatidylcholine:phosphatidylinositol, 4:48:48 molar ratio) in HSS. Actin assembly was measured in HSS by supplementing the HSS with trace amounts of rabbit muscle actin monomers covalently labeled with the flourophore pyrene. Incorporation of pyrene-actin into actin filaments results in a dramatic increase in pyrene fluorescence that can be measured in the intact HSS (Kouyama and Mihashi 1981; Ma et al. 1998a; Peterson et al. 2001; Lebensohn et al. 2006). Compounds were tested in a high-throughput manner utilizing 384-well plates. Active compounds identified by the screen were ranked based on their potency determined in dose-response experiments using the primary screening assay. Among the most potent compounds identified were a 14-amino-acid cyclic peptide called 187-1 (Peterson et al. 2001), a tetracyclic indole called

pirl1 (Peterson et al. 2006), and an N-alkylated carbazole derivative that we subsequently termed wiskostatin (Peterson et al. 2004; and see below).

Wiskostatin is a 3,6-dibromocarbazole derivative, N-alkylated with a dimethylaminopropan-2-ol group. In addition to wiskostatin, an otherwise similar 3,6-dichlorocarbazole variant of wiskostatin was identified by the primary screen, although it was slightly less potent. Wiskostatin inhibited actin polymerization in PIP2-stimulated *Xenopus* egg extract with an EC_{50} of ~4 μM. Thus, the maximal rate of actin polymerization was slowed by 50% relative to solvent-alone controls in the presence of this dose of wiskostatin. Wiskostatin concentrations >10 μM completely suppressed actin assembly stimulated by PIP2-containing liposomes.

2.2 The Target of Wiskostatin is N-WASP

A central challenge of forward chemical genetic screens is the identification of protein targets of small-molecule inhibitors (Stockwell 2000; Burdine and Kodadek 2004; Tochtrop and King 2004). Although a number of conceptually distinct approaches have been used successfully for inhibitor target identification (Burdine and Kodadek 2004; Tochtrop and King 2004), no single method has proven universally applicable. To identify the target of wiskostatin, we therefore began with a candidate-testing approach. As described above, several proteins that mediate actin assembly downstream of Cdc42 have been discovered, and *in vitro* assays using purified preparations of these proteins have been developed that successfully measure their biochemical activities (Ma et al. 1998b; Rohatgi et al. 1999; Ho et al. 2004, 2006). We therefore used these assays to determine if wiskostatin inhibited any of the known proteins that mediate actin assembly downstream of Cdc42.

Initial experiments tested whether wiskostatin might inhibit PIP2-stimulated actin assembly by binding actin monomers directly and preventing their incorporation into growing filaments. The polymerization kinetics of purified native rabbit muscle actin was monitored by the fluorescence increase of added pyrene-labeled actin monomers. Whereas wiskostatin inhibited actin assembly in PIP2-stimulated extract with an EC_{50} of 4 μM, up to 20 μM wiskostatin did not affect the polymerization of purified actin. Higher wiskostatin doses, however, modestly inhibited the polymerization of purified actin directly. The apparent inability of wiskostatin to inhibit actin polymerization directly suggests that wiskostatin must target a signaling component upstream of actin itself.

We next tested if wiskostatin inhibited the actin filament nucleating activity of the Arp2/3 complex, an essential mediator of Cdc42-dependent actin assembly in HSS (Ma et al. 1998b). Purified bovine Arp2/3 complex is inactive, but can be activated experimentally by the addition of a recombinant polypeptide derived from the C-terminus of N-WASP termed the VCA segment (named for verprolin-homologous, cofilin-homologous, and acidic amino acid sequences) (Rohatgi et al. 1999). In the presence of VCA, the actin-nucleating activity of Arp2/3 complex is potently activated and can be monitored using the pyrene-actin fluorescence assay.

VCA-activated Arp2/3 complex was not inhibited by 10 μM wiskostatin, indicating that the inhibitor must target some other component besides the Arp2/3 complex or the VCA segment of N-WASP.

Arp2/3 complex is activated in PIP2-stimulated HSS by its binding to the VCA region of endogenous N-WASP. Whereas the VCA segment is a potent activator of Arp2/3 complex, the activity of full-length N-WASP is sharply reduced due to autoinhibitory interactions between the VCA segment and an N-terminal domain called the GTPase-binding domain (GBD) (Kim et al. 2000; Prehoda et al. 2000; Rohatgi et al. 2000). Autoinhibition is relieved by the binding of active Cdc42 to the GBD, which releases the VCA segment, allowing VCA to activate Arp2/3 complex (Fig. 1a) (Prehoda et al. 2000; Rohatgi et al. 2000). We therefore tested whether wiskostatin inhibited N-WASP activation using purified pyrene-actin assays in the presence of purified Arp2/3 complex and recombinant N-WASP. Both the modest basal activity of autoinhibited N-WASP and the robust activity of N-WASP in the presence of PIP2 and Cdc42 were inhibited by wiskostatin. Thus, although wiskostatin did not inhibit Arp2/3 complex activation by the isolated VCA segment of N-WASP, it did inhibit Arp2/3 complex activation by full-length N-WASP. Importantly, wiskostatin inhibited N-WASP activity at doses consistent with its potency in HSS. Furthermore, several analogues of wiskostatin that were inactive at inhibiting PIP2-induced actin assembly in HSS also did not inhibit N-WASP activity *in vitro*, indicating that inhibition of N-WASP can explain wiskostatin inhibition of actin assembly in PIP2-stimulated HSS. The name "wiskostatin" was chosen for this compound based on its ability to inhibit a member of the Wiskott-Aldrich syndrome protein family.

2.3 Mechanism of N-WASP Inhibition by Wiskostatin

Since wiskostatin inhibits the activity of full-length N-WASP, but not that of the isolated VCA domain, this suggested that wiskostatin might perturb the conformational changes in N-WASP that accompany activation. How it might do so was of particular interest because N-WASP is a scaffolding protein not previously known to bind a small molecule. Available three-dimensional structural data for N-WASP that could facilitate prediction of binding sites for wiskostatin are limited to crystal structures of the N-terminal EVH1 domain of N-WASP (Volkman et al. 2002; Peterson et al. 2007). This domain does not, however, appear to play a role in either Cdc42 binding or Arp2/3 complex activation (Prehoda et al. 2000; Rohatgi et al. 2000). N-WASP is highly conserved in sequence and regulation with its hematopoetic-cell-specific paralogue, WASP, for which additional structures are available. For example, an NMR structure has been reported of a truncated WASP fragment comprising the GBD linked to a C-terminal element involved in Arp2/3 complex activation (Kim et al. 2000). This structure and accompanying biochemical experiments revealed the core interactions responsible for maintaining the autoinhibited conformation of WASP (and presumably N-WASP). Given the availability of

chemical shift assignments for the autoinhibited core of WASP and the 68% sequence identity with the corresponding domains of N-WASP, we chose to test whether binding of wiskostatin to WASP could be detected by NMR. Michael Rosen's group (UT Southwestern) conducted NMR titration experiments with wiskostatin and the autoinhibited core domains of WASP. Titrations of this WASP construct with wiskostatin resulted in dose-dependent and saturable amide proton chemical shift changes for a limited number of residues in the GBD of WASP. These initial experiments demonstrated the existence of a discrete wiskostatin-binding site in WASP.

Rosen's group then determined the structure of wiskostatin bound to the autoinhibited core of WASP by NMR (Fig. 1b). Remarkably, the structure of this region of WASP was largely unchanged by wiskostatin binding. The average backbone root mean square deviation between the liganded and free forms of WASP was 1.51 Å within the structured domains. The compound occupied a shallow pocket within the hydrophobic core of the WASP GBD that was well defined by 30 intermolecular nuclear Overhauser effects between the wiskostatin carbazole ring and the GBD. Importantly, 14 of the 15 amino acid residues most proximal to wiskostatin in WASP are conserved in N-WASP, suggesting that these paralogues likely both interact with the compound similarly.

Previous studies have demonstrated that the GBD of WASP is conformationally plastic, alternately adopting one conformation in autoinhibited WASP and another, mutually exclusive conformation when bound to Cdc42 (Abdul-Manan et al. 1999; Kim et al. 2000). This conformational change underlies the transition between autoinhibited WASP and active WASP in which Cdc42 binding to the GBD releases the VCA element, allowing Arp2/3 complex activation (Fig. 1a). A recombinant polypeptide comprising the isolated GBD is unstructured in aqueous buffer, indicating that folding of this segment is driven by these intra- or intermolecular interactions, respectively (Kim et al. 2000). The lack of significant changes in the conformation of the GBD of autoinhibited WASP on wiskostatin binding suggested that, instead of perturbing the conformation of WASP, the compound might act by stabilizing this inherently inactive conformation.

To test whether wiskostatin could stabilize the autoinhibited conformation of the GBD, the otherwise unstructured, isolated GBD polypeptide was titrated with wiskostatin and conformational changes in the GBD were monitored by NMR. Wiskostatin, but not an inactive derivative, induced folding of the GBD as evidenced by greater chemical shift dispersion in ^{1}H-^{15}N- and ^{1}H-^{13}C HSQC experiments. At saturating doses of wiskostatin, the resulting spectrum evidenced many characteristics of the spectrum of GBD when present in the autoinhibited conformation, strongly suggesting that wiskostatin alone could drive folding of the GBD into the autoinhibited conformation. This observation suggests that wiskostatin inhibits activation of N-WASP by binding to its autoinhibited conformation and antagonizing the conformational change mediated by Cdc42 that is required for N-WASP activation of Arp2/3 complex.

Activation of N-WASP by Cdc42 can be seen as the modulation of a conformational equilibrium in the GBD from a predominantly "closed," inactive form to an

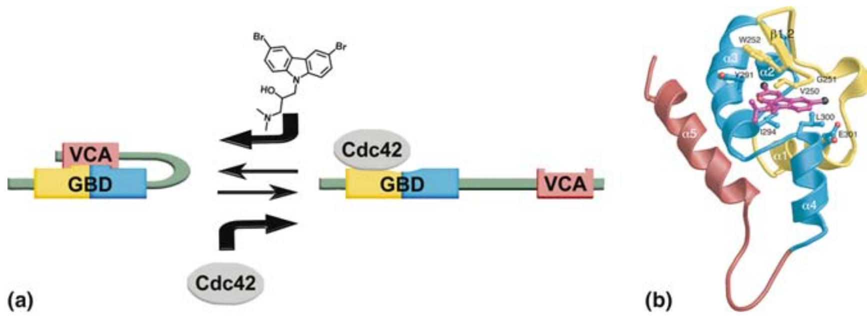

Fig. 1 The small-molecule N-WASP inhibitor wiskostatin antagonizes activation of N-WASP by Cdc42. **(a)** Intramolecular interactions between the GTPase-binding domain (*GBD*) and the verprolin homologous, cofilin homologous, acidic amino acid sequences (*VCA*) domain maintains N-WASP in an autoinhibited conformation (*left*). Binding of activated Cdc42 causes a restructuring of the GBD that relieves these interactions, leading to N-WASP activation (*right*). The small-molecule inhibitor wiskostatin binds the GBD and pulls the conformational equilibrium of N-WASP toward the autoinhibited state. **(b)** Structure of wiskostatin bound to the autoinhibited conformation of WASP (*left side* of **a**). A representative conformer from the NMR structure of the relevant domains of WASP bound to wiskostatin. Wiskostatin and side chains exhibiting nuclear Overhauser effects to wiskostatin are shown in *ball-and-stick representation*. Corresponding sequence elements are colored as in **(a)**. Panel **(b)** is reproduced from Peterson et al. 2004

active conformation. Our results suggest that wiskostatin and Cdc42 can influence this structural change by binding to the opposing GBD conformational states and titrating the equilibrium toward each state (Fig. 1). Consistent with such an underlying equilibrium, we found that inhibition by wiskostatin can be overcome by increasing the concentration of activators Cdc42 and PIP2 in *in vitro* pyrene-actin assays. Thus, wiskostatin exploits an inherent regulatory equilibrium in N-WASP by binding and stabilizing a native, autoinhibited conformation. Because the structure of the GBD when bound to VCA is incompatible with the GBD-Cdc42 binding (Kim et al. 2000), we infer that wiskostatin prevents the interaction of Cdc42 with N-WASP. Wiskostatin therefore mediates its inhibitory activity, in part, by preventing Cdc42-N-WASP binding.

2.4 Autoinhibition of Rho GTPase Effectors as a Target for Small-Molecule Inhibitors

The regulation of N-WASP activity involves the binding of an activator (Cdc42) to the autoinhibitory element (GBD), which displaces this element from the functional domain (VCA). Strikingly, a similar paradigm can be found in the regulation of many other proteins including other Rho family GTPase effectors, such as members of the group I p21-activated kinases (Paks) and the diaphanous-related formins (DRF). The shared regulatory strategy of autoinhibition observed in each of these

proteins along with our understanding of N-WASP inhibition by wiskostatin suggests that the conformational changes of group I Paks and DRFs could be targets for small-molecule inhibition. Indeed, we have proposed that, in general, proteins that adopt autoinhibited conformations could be susceptible to small molecules that stabilize the inhibited conformation (Peterson and Golemis 2004). Importantly, as illustrated by N-WASP, proteins targeted by this strategy need not be enzymes or possess already known small-molecule binding sites.

Small-molecule inhibitors that target Paks and DRFs could be used to dissect the specific role of these effectors downstream of Rho GTPases. In addition, chemical inhibition of Pak1 may serve as the basis for the development of novel therapeutics, as there is increasing evidence for the involvement of this protein in human disease (discussed below). In the following sections, we will discuss in detail the regulation of Pak1 and the DRF mDia1 as examples of other Rho GTPase effectors that adopt autoinhibited conformations and propose screening strategies that could lead to the identification of allosteric inhibitors of these effectors.

3 p21-Activated Kinases as Targets for Allosteric Inhibition

3.1 The Pak Family of Rho GTPase Effectors

The human Paks are a family of six serine/threonine-directed protein kinases identified as effectors of the small GTPases Rac (p21) and Cdc42 (Manser et al. 1994). Pak kinases are divided into two subgroups based on sequence similarity. Group I Paks (Paks 1, 2, and 3) contain an N-terminal autoregulatory domain that is involved in the formation of autoinhibited heterodimers (see below). In contrast, the group II Paks (Paks 4, 5, and 6) do not contain the autoregulatory sequences observed in group I Paks required for autoinhibition, and as such are constitutively active (for a review of group II Paks, see Jaffer and Chernoff 2002). The group I Paks contain a GTPase-binding domain (GBD) that is homologous in both sequence and three-dimensional structure to the GBD of WASP (Kim et al. 2000; Lei et al. 2000). Binding of activated GTPases to group I Paks stimulates Pak autophosphorylation as well as phosphorylation of downstream substrates. Group II Paks also bind Rac and Cdc42, but this interaction does not appear to regulate their catalytic activity (Jaffer and Chernoff 2002; Cotteret et al. 2003). Instead, evidence suggests that GTPase binding mediates recruitment of group II Paks to specific subcellular locations (Wu and Frost 2006). The best-characterized member of the Pak family is Pak1, which has been shown to regulate actin dynamics downstream of Rac and Cdc42, thereby affecting cell morphology, polarity, and cell migration. In addition, Pak1 activity has been linked to the signaling pathways that control cell growth, survival, and cell cycle progression. For an excellent review of group I Pak biology and function, see (Bokoch 2003).

3.2 Autoinhibition of Pak1

Like N-WASP, Pak1 catalytic activity is regulated by an autoinhibitory domain whose binding to the catalytic domain is released on binding-activated Rho GTPases (Lei et al. 2000). Unlike in N-WASP, however, the interactions between the Pak1 autoinhibitory domain and the catalytic domain occur between two Pak1 monomers *in trans,* resulting in the formation of an autoinhibited "head-to-tail" Pak1 dimer (Fig. 2 and Parrini et al. 2002). The autoregulatory region of Pak1 is made up of distinct sequence elements: the GBD element overlaps partially in sequence with an element termed the inhibitory switch (IS) domain, which anchors the autoregulatory domain of one Pak1 monomer to the kinase domain of the partner Pak1 monomer. C-terminal to this element is the kinase-inhibitory (KI) segment, which occupies the partner's active site, obstructing ATP and substrate binding, and disrupting the active site architecture (Zhao et al. 1998; Tu and Wigler 1999; Lei et al. 2000).

Similar to N-WASP regulation, release of Pak1 from its autoinhibited conformation is initiated by the binding of activated Rac or Cdc42 to the GBD. Binding of GTPase leads to an unfolding of the IS (Lei et al. 2000). This conformational change displaces the KI region from the Pak1 catalytic cleft, exposing the activation loop and allowing for phosphorylation of threonine 423, which is required to achieve full kinase activity (Manser et al. 1997; Fig. 2b). Additional phosphorylations occur at several sites in the Pak1 monomer to further facilitate kinase activation (Buchwald et al. 2001; Chong et al. 2001). Once phosphorylated, Pak1 monomers cannot reform the autoinhibited dimer until acted upon by protein phosphatases, such as POPX (Koh et al. 2002; Zhan et al. 2003).

The similarities between Pak1 and N-WASP regulation are also reflected in structural homology in their autoregulatory domains. Comparison of the structure

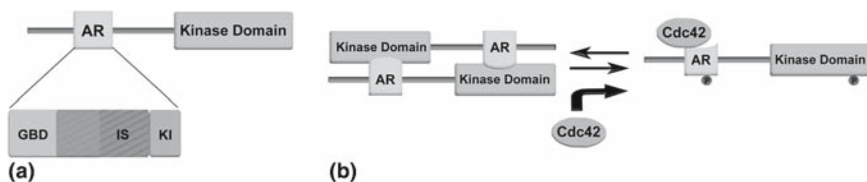

Fig. 2 Autoinhibition of Pak1. **(a)** The Pak1 autoregulatory region (*AR*) consists of a number of sequence elements. The GTPase-binding domain (*GBD*) overlaps with the sequence of the inhibitory switch domain (*IS*), which anchors the autoregulatory domain to the kinase domain. The binding of the AR of one Pak1 monomer to the kinase domain of another Pak1 monomer leads to the formation of the autoinhibited Pak1 dimer (*left side* of **b**). The kinase inhibitory (*KI*) segment intercalates within and disrupts the architecture of the partner's catalytic site. (Adapted from Lei et al. 2000). **(b)** Binding of activated Rac or Cdc42 to the GBD relieves the autoinhibitory interaction between the AR and the kinase domain, facilitating dissociation of Pak1 monomers. Autophosphorylation of GTPase-bound Pak1 completes the activation process and prevents reformation of the autoinhibited dimer

of the IS domain within the autoinhibited Pak1 dimer revealed a remarkable similarity to the WASP autoregulatory domain (Kim et al. 2000; Lei et al. 2000). In both the Pak1 and WASP structures, this autoregulatory element binds a structurally analogous alpha helix in either the kinase domain or VCA segment, respectively. Thus, a structurally conserved regulatory module is utilized by both proteins to regulate distinct signaling activities. Given the structural and mechanistic similarities between these two proteins, it is possible that, like N-WASP, Pak1 could be susceptible to inhibition by a small molecule that stabilizes its autoinhibited conformation.

3.3 Relevance of an Allosteric Pak1 Inhibitor

Current methods used to eliminate Pak1 function include classical genetic knockout (Hofmann et al. 2004) as well as transfection of interfering reagents, such as siRNAs (Tang et al. 2005), constructs encoding the IS and KI sequence elements (Beeser and Chernoff 2005), or catalytically inactive, full-length versions of the kinase (Adam et al. 2000). These approaches, while useful in abrogating Pak1 activity, are limited in their degree of control of Pak1 activity, as well as by a lack of control over the time required to achieve Pak1 inhibition. In addition, overexpression of kinase-dead or truncated Pak1 constructs may titrate away relevant Pak1 binding partners, such as the guanine nucleotide exchange factor Pak interacting exchange factor (Pix) or Nck (Bokoch et al. 1996; Galisteo et al. 1996; Bagrodia et al. 1998; Manser et al. 1998), leading to complications in the interpretation of observed phenotypes. Small-molecule inhibitors can address some of the limitations associated with these currently used methods of inhibiting Pak1 activity. Indeed, kinase inhibitors in general have had a tremendous impact on our understanding of the functions of specific kinases (Knight and Shokat 2005). The advantages of using chemical inhibitors make small-molecule inhibitors of Pak1 a potentially important and complementary tool in the study of Pak1 function in cells.

The vast majority of existing kinase inhibitors binds to the kinase active site in a manner competitive with ATP. Since the ATP-binding pocket of kinases is highly conserved in sequence and structure, most inhibitors target multiple related kinases in addition to their intended target (Davies et al. 2000; Fabian et al. 2005). Indeed, a chemical inhibitor of Pak1 that is based on the ATP-competitive inhibitor CEP-1347 (Nheu et al. 2002) is also a potent inhibitor of mixed-lineage kinases 1–3 (Maroney et al. 2001; Nheu et al. 2002). In contrast, an allosteric inhibitor that stabilizes the autoinhibited Pak conformation might exhibit significantly greater kinase specificity due to the fact that the autoregulatory domain of group I Paks is not conserved in other protein kinases. Thus, allosteric inhibitors that target this domain to stabilize the inactive Pak1 dimer would not be expected to inhibit other kinases. Furthermore, since autoinhibition is not conserved in group II Pak family members, one would expect such inhibitors to target only the group I Paks, despite

significant sequence conservation in the catalytic domains of these two groups. As such, allosteric inhibitors of Pak1 could serve as highly specific tools to elucidate group I Pak functions.

Small-molecule Pak1 inhibitors may also have clinical relevance. Pak1 expression and activity have been linked to tumor development in a number of different cancers including colorectal, ovarian, and breast (Kumar and Vadlamudi 2002; Schraml et al. 2003; Carter et al. 2004), making identification of clinically relevant Pak1 inhibitors of great interest (Kumar et al. 2006). Paks involvement in tumorigenesis is likely due in part to its ability to promote activation of the mitogen-activated protein kinase (MAPK) pathway. Group I Paks directly phosphorylate two kinases in this pathway, c-Raf and MEK1, and Pak1 phosphorylation of these kinases is thought to be required for their full activation by the Ras oncogene (Frost et al. 1997; King et al. 1998; Chaudhary et al. 2000; Eblen et al. 2002; Zang et al. 2002; Slack-Davis et al. 2003; Tran and Frost 2003). Indeed, expression of dominant negative Pak1 constructs inhibits Ras-mediated transformation in Rat1 fibroblasts, rat Schwann cells, and in a neurofibrosarcoma cell line (Tang et al. 1997, 1998). Conversely, expression of an activated form of Pak1 stimulates the MAPK pathway in HEK 293 cells (Lu et al. 1997) and is tumorigenic in the mammary glands of transgenic mice (Wang et al. 2006), again underscoring the importance of this kinase in cell proliferation signaling.

A role for Pak1 in tumorigenesis is also supported by the interaction of Pak with a number of proteins involved in cell cycle progression. Pak1 phosphorylates the DNA scaffolding protein histone H3 on serine 10 both *in vitro* and in human breast cancer cells, and phosphorylation at this site has been linked to mitotic chromosome assembly, segregation, and movement to the metaphase plate (Cheung et al. 2000; Li et al. 2002). Additionally, Pak1 is localized to centrosomes (Banerjee et al. 2002) and was recently shown to phosphorylate and activate the mitotic kinase Aurora A. Inhibition of Pak1 in this study led to a delay in both recruitment of Aurora-A to centrosomes as well as centrosome duplication (Zhao et al. 2005). Finally, Pak1 is known to induce the expression of cyclin D1 (Balasenthil et al. 2004), a key regulator of G1 cell cycle progression.

Elevated Pak activity has been observed in a number of highly invasive cancer cells, suggesting that Pak1 may also play a role in the cell motility underlying metastasis. Expression of constitutively active Pak1 increases the motility of breast cancer-derived MCF7 cells (Vadlamudi et al. 2000). Conversely, expression of a kinase-dead Pak1 mutant resulted in a three-fold decrease in migration of MDA-MB-435 melanoma cells (Adam et al. 2000). The role of Pak1 in mediating cell migration may involve Pak1 phosphorylation of the cytoskeletal signaling protein LIM kinase (LIMK), which increases LIMK activity ten-fold (Edwards et al. 1999). Activated LIMK phosphorylates and deactivates the F-actin severing protein cofilin, leading to changes in actin cytoskeletal dynamics (Arber et al. 1998; Yang et al. 1998). Indeed, LIMK activity has been shown to be required for migration in breast cancer-derived cells (Yoshioka et al. 2003), highlighting the role of this Pak1 substrate in tumor cell motility.

3.4 Endogenous Protein Inhibitors of Pak1 Bind the Autoregulatory Domain

In addition to the intramolecular interactions that regulate Pak1 activity, there are a number of other proteins that interact with and modulate Pak1 activity. Interestingly, many of these proteins bind within the Pak1 autoregulatory region. For example, human Pak1-interacting protein (hPIP1) is a WD repeat containing protein that was shown to inhibit Pak1 kinase activity as well as the signaling of Pak1 effectors JNK and NFκB in human cells. hPIP1 binds within the autoregulatory region of Pak1, suggesting that it could inhibit Pak1 activity by stabilizing the autoinhibited conformation (Xia et al. 2001).

The product of the tumor suppressor gene NF2, termed merlin, is another cellular protein with the ability to inhibit Pak1 activity. While the full spectrum of merlin functions are still under investigation, it has been demonstrated that overexpression of merlin inhibited Pak1 activation as well as Pak1 interaction with the focal adhesion protein paxillin (Kissil et al. 2003; Xiao et al. 2005). Conversely, loss of merlin expression resulted in an increase in Pak1 activation (Kissil et al. 2003). Merlin binds Pak1 within the autoregulatory region and inhibits the interaction between Pak1 and Rac (Kissil et al. 2003), indicating that this protein functions by disrupting a protein interaction required for Pak1 activation. In addition to acting as an inhibitor of Pak activity, merlin is also a Pak substrate, as it was shown that Pak phosphorylates merlin on serine 518 (Kissil et al. 2002; Xiao et al. 2002). Phosphorylation on this residue alters merlin localization and anti-proliferation activity (Shaw et al. 2001; Kissil et al. 2002; Xiao et al. 2002), suggesting a feedback signaling relationship between these two proteins.

The recently identified protein Cysteine Rich-Inhibitor of Pak1 (CRIPak) was shown to inhibit Pak1 activity both *in vitro* and in cells, and blocked the activation of the Pak1 effector LIM kinase (Talukder et al. 2006). CRIPak also binds within the autoregulatory region of Pak1, and therefore could also act to by stabilizing autoinhibited Pak1 dimers. However, this region of interaction overlaps with the Pix-binding region of Pak1, raising the possibility that CRIPak could antagonize Pak1 activity by inhibiting the binding of Pix (Talukder et al. 2006).

Interestingly, CIB1, an EF-hand domain-containing protein, also binds to the Pak1 autoregulatory domain of Pak1; however, this binding results in *activation* of Pak1 catalytic activity (Leisner et al. 2005). Thus, activation and inhibition of Pak1 activity through the binding of proteins to the Pak1 autoregulatory domain are a biologically relevant regulatory mechanism.

The studies discussed above suggest that some endogenous Pak1 protein inhibitors may act by stabilizing the autoinhibited conformation of Pak1. Thus, the binding of proteins other than Rac or Cdc42 to the autoregulatory domain may be a physiological mechanism for negatively regulating Pak1 kinase activity. It may therefore be possible for a small molecule to exploit this inherent regulatory mechanism in a similar way. In the following section, we will discuss screening strategies that could be employed to specifically identify such an inhibitor for members of the group I Pak family.

3.5 Screening Approaches to Identify Allosteric Group I Pak Inhibitors

Many high-throughput assays have been developed for the identification of kinase inhibitors. The majority of these assays simply report on the phosphorylation of model substrates and therefore do not discriminate between compounds acting via a particular mechanism. The identification of compounds that target group I Paks via the allosteric mechanism of stabilization of the native autoinhibited conformation requires special considerations in assay design. For example, kinase inhibitor screens frequently utilize constitutively active, truncated fragments comprising the isolated kinase domain. These constructs may lack important domains that regulate catalytic activity that could be targeted by an allosteric inhibitor. Therefore, full-length Pak1 should be used to enable the detection of allosteric inhibitors. In addition, kinase activity screens are frequently performed in the presence of low (μM) ATP concentrations, thus rendering these assays highly sensitive to ATP-competitive inhibitors. One simple change that could bias such a screen towards non-ATP competitive inhibitors would be to monitor Pak1 kinase activity at high (mM) ATP concentrations. Subsequent mechanistic or enzymological follow-up studies could then be conducted to determine if inhibitory compounds are indeed non-competitive with ATP.

Another approach to address a Pak1 inhibitor's mechanism of action takes advantage of the ability to control the activation state of Pak1 *in vitro*. The interaction between purified autoregulatory and kinase domains of Pak1 is abolished when these fragments are autophosphorylated by Pak1 (Zenke et al. 1999; Buchwald et al. 2001), suggesting that once activated (and autophosphorylated), full length Pak1 does not reform the autoinhibited conformation until acted upon by protein phosphatases. Therefore, in an *in vitro* assay with no phosphatases present, it is possible to drive the regulatory equilibrium of Pak1 irreversibly towards the activated state by pre-incubating the kinase with activated Rac or Cdc42 and ATP. A small molecule that inhibits Pak1 activity by stabilizing the autoinhibited Pak1 complex should be ineffective at inhibiting this pre-active Pak1. This suggests that a simple "order of addition" experiment could be used to discriminate between inhibitors targeting the active site and those that target the Pak1 activation mechanism. In this approach, two separate kinase assays would be conducted in parallel in which the kinase reaction components are the same, but the order of their addition to the reaction is different. In the first assay, potential inhibitors would be added to autoinhibited Pak1 dimers prior to the addition of activator (activated Rac or Cdc42) and ATP. In the second assay, small molecules would be added to Pak1 after activation of the kinase via incubation with activator and ATP. Compounds that inhibit substrate phosphorylation by Pak1 in both assays likely do not depend on the formation of inactive Pak1 dimers. However, inhibition of Pak1 catalytic activity by a compound only when added prior to Pak1 activation would be consistent with a compound antagonizing the activation process. In this manner it may be possible to initially identify allosteric Pak1 inhibitors.

Alternatively, an assay that directly monitors the interaction between Pak1 dimers could be performed so as to select for compounds that stabilize the Pak1 autoinhibited conformation. One such assay could involve a fluorescence resonance energy transfer (FRET)-based approach utilizing recombinant Pak1 fragments comprising the catalytic domain and the autoregulatory domain individually tagged with either FRET donor or acceptor molecules. In the autoinhibited state, these tagged Pak1 fragments would dimerize and undergo FRET. Upon addition of Cdc42, FRET would be abolished due to the dissociation of the autoregulatory domain from the kinase domain. In this assay, individual compounds would be added to inactive Pak1 dimers and the maintenance of FRET monitored upon addition of Cdc42. Those reactions that maintain FRET in the presence of Cdc42 could act by stabilizing Pak1 dimers. Subsequent assays could then be performed to confirm that compounds identified by this approach do indeed inhibit Pak1 catalytic activity.

4 DRFs as Targets for Allosteric Inhibition

4.1 Formins Are a Family of Actin-Nucleating Proteins

The nucleation of actin filaments is an important rate-limiting step in de novo actin filament polymerization. Currently there are three known classes of proteins with the ability to nucleate actin filaments: the Arp2/3 complex, Spire, and the formin family of proteins (Machesky et al. 1994; Quinlan et al. 2005). The best-characterized actin nucleator is the Arp2/3 complex, which binds to the side of existing actin filaments to nucleate new filaments that grow at the barbed end, resulting in the formation of a branched actin network. Much less is known about Spire, which nucleates linear actin filaments and binds to the pointed end of the new filament (Quinlan et al. 2005). The formins comprise seven mammalian subfamilies of proteins characterized by conserved Formin Homology 1 (FH1) and FH2 domains, which convey the ability to nucleate actin filaments (Higgs 2005; Higgs and Peterson 2005). Unlike the Arp2/3 complex and Spire, formins nucleate actin filaments from the barbed end and remain bound to the elongating barbed end (Pruyne et al. 2002). The actin-nucleating activity of the formins is linked to such cellular processes as cytokinesis, cell migration, endocytosis, and the establishment of cell polarity (for a review of formin structure and function, see Faix and Grosse 2006).

4.2 Diaphanous-Related Formins Are Rho GTPase Effectors That Undergo Autoinhibition

The best-studied subfamily within the formin family of actin nucleators is the diaphanous-related formins (DRFs). In addition to the conserved FH1 and FH2

domains, DRFs contain a GTPase-binding domain (GBD) responsible for the interaction of these proteins with members of the Rho GTPases (Watanabe et al. 1997). Binding of Rho GTPases to the GBD is thought to activate DRFs through relief of autoinhibitory interactions involving additional domains (see below). The majority of our understanding of DRF autoregulation comes from studies of the mammalian homologue of diaphanous (mDia) DRF subgroup, of which there are three isoforms (mDia 1–3). mDia isoforms display overlapping functions in a number of cases (Tominaga et al. 2000; Copeland and Treisman 2002; Peng et al. 2003), and the domains responsible for mDia autoregulation are highly conserved among mDia1, mDia2, and mDia3 (Watanabe et al. 1997; Petersen et al. 1998; Alberts 2001). Therefore, for the purpose of clarity the following sections will focus on the best-characterized member of this group, mDia1.

4.3 Autoinhibition of mDia1

A number of studies have begun to unravel the mechanism of mDia1 autoinhibition. Early cellular studies utilizing truncated mDia1 constructs demonstrated the involvement of both N-terminal and C-terminal domains in the regulation of mDia-mediated actin filament assembly (Watanabe et al. 1997; Tominaga et al. 2000; Alberts 2001). Subsequently, these regulatory activities were discovered to be the result of an interaction between a domain in the amino terminus of mDia1 termed the diaphanous inhibitor domain (DID) and a domain adjacent to the C-terminal FH2 domain termed the diaphanous autoregulatory domain (DAD) (Fig. 3). Biophysical studies demonstrated binding of the DID to the DAD with sub-micromolar affinity (Lammers et al. 2005; Li and Higgs 2005), and introduction of a recombinant peptide comprising the DID is sufficient to inhibit FH2-DAD-induced

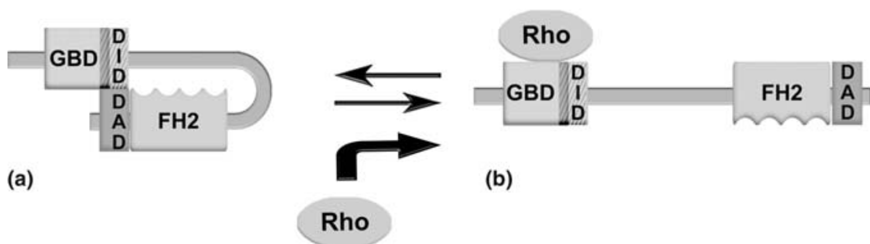

Fig. 3 Schematic representation of autoinhibition of mDia1 (mDia1 is shown as a monomer for clarity). Interaction of the diaphanous inhibitor domain (*DID*) with the diaphanous autoregulatory domain (*DAD*) maintains mDia1 in a conformation that renders the protein unable to nucleate actin filaments via the FH2 domain (**a**). Binding of activated Rho to the GTPase binding domain (*GBD*), which partially overlaps with DID, displaces the DAD, leading to the relief of autoinhibition (**b**)

actin polymerization (Li and Higgs 2005). Structural studies have revealed extensive hydrophobic contacts between a helix within the core DAD and a number of the armadillo-repeats contained within the DID (Lammers et al. 2005). Although the autoinhibitory domains of mDia1 are distinct in both amino acid sequence and three-dimensional structure from those found in N-WASP and Pak1, the general strategy of autoinhibition involving an interaction between autoregulatory domains and domains within the catalytic portion of the protein is shared among all three proteins (compare Figs. 1a, 2b, and 3).

Also akin to N-WASP and Pak1, DRFs contain an amino-terminal region that mediates the interaction between these proteins and Rho GTPases. The GTPase-binding sites of DRFs are less well defined, in general, than for N-WASP and Pak1 (Rivero et al. 2005). However, conserved CRIB-type GTPase binding domains are observed in mDia family members (Peng et al. 2003). mDia1 has been shown to interact with Rho A, B, and C, but not Rac or Cdc42 (Watanabe et al. 1997, 1999). Additionally, other mDia isoforms have been shown to interact with other Rho GTPases (Alberts et al. 1998; Gasman et al. 2003; Peng et al. 2003; Yasuda et al. 2004). The mDia1 GBD partially overlaps with the autoregulatory region of the protein (Fig. 3), and a number of studies demonstrate that binding of activated Rho to mDia1 fragments containing the GBD antagonizes the DID/DAD interaction by actively displacing the DAD from the DID (Watanabe et al. 1997; Lammers et al. 2005; Li and Higgs 2005; Rose et al. 2005). The functional relevance of this disruption is highlighted by data demonstrating that Rho partially relieves the inhibitory effects of the DID on mDia-mediated actin polymerization (Li and Higgs 2003).

Although much has been learned concerning the autoregulatory cycle of mDia1, the molecular details of Rho-mediated relief of autoinhibition are not entirely clear. For example, binding of activated Rho or the DAD to DID-containing fragments is mutually exclusive, yet they do not bind to completely overlapping DID sequences (Lammers et al. 2005; Rose et al. 2005). Unlike what is observed in N-WASP and Pak1, release of mDia1 from the autoinhibited state does not appear to involve conformational changes within the autoregulatory domain, as the structure of DID fragments are not significantly different when examined alone (Otomo et al. 2005), in a complex with active Rho (Rose et al. 2005), or when bound to the DAD (Lammers et al. 2005). Currently there is no crystal structure available for the DAD alone, precluding any comparisons between the conformation of the DAD in the unbound state and the conformation of the DAD complexed with the DID. Comparison of the structures of DID-containing fragments complexed with either the DAD or RhoC led Lammers et al. to suggest a theoretical ternary Rho/DID/DAD complex in which residues within the DAD (1179–1187) and RhoC (64–67) sterically and electrostatically repel each other, mediating the release of the DAD from the DID (Lammers et al. 2005). However, further study is required to demonstrate the true role of these residues in the proposed interactions.

Despite using structurally distinct domains, the regulatory mechanism of mDia1 is remarkably similar to that observed for N-WASP and Pak1. It is therefore conceivable that, much like N-WASP, mDia1 may be susceptible to inhibition by allosteric inhibition. In the following sections, we will discuss the relevance of such

an inhibitor and propose screening strategies that could be used to identify small-molecule mDia1 inhibitors that target this protein by an allosteric mechanism.

4.4 Relevance of an Allosteric mDia1 Inhibitor

A major challenge in the actin field is the difficulty of determining the contribution of different classes of actin filament nucleators to the generation of new actin filaments because of the presence of other abundant actin filament nucleating activities. A small-molecule inhibitor that is specific for the formin family would therefore be useful in discriminating the relative contribution of the formins in the context of the three classes of actin nucleators (Arp2/3 complex, Spire, and formins). However, sequence differences within the formin family suggest that a greater degree of inhibitor specificity may be possible. In contrast to the catalytic FH2 domain found in all members of the formin family, clear DID and DAD-like sequences are found only in three formin subfamilies: mammalian diaphanous (mDia) subfamily, formin-related gene in leukocytes (FRL) subfamily, and disheveled-associated activator of morphogenesis (DAAM) subfamily (Higgs and Peterson 2005). This suggests that small molecules that target the DID/DAD regulatory domains might exhibit formin subfamily specificity, which would aid in characterizing the role of these functionally related proteins. Furthermore, while core residues are conserved between the DID and DAD sequences across these three subfamilies, they differ significantly in overall DID and DAD sequence (Higgs and Peterson 2005), suggesting the possibility of identifying mDia-specific, FRL-specific, and DAAM-specific inhibitors. Finally, even within the mDia subfamily, limited differences in the sequence of the paralogues mDia1, mDia2, and mDia3 allow for isoform-specific small-molecule inhibitors that could be used to discriminate the distinct roles of mDia family members (Higgs and Peterson 2005).

A small-molecule inhibitor of mDia1 would offer additional advantages over conventional research tools used to study this protein. For example, it has been observed that deletion of the gene encoding mDia1 results in the up-regulation of mDia2 expression (Peng et al. 2003). A rapid-acting chemical inhibitor of mDia1 might allow for the study of loss of mDia1 function while avoiding the compensatory up-regulation of mDia2 expression, thereby facilitating the identification of isoform-specific mDia functions. The temporal control afforded by the use of chemical inhibitors allows for inhibitor wash-out experiments, which may shed light on the role of mDia1 in the formation of such dynamic structures as filopodia (Peng et al. 2003; Higashida et al. 2004), the cytokinetic ring (Watanabe et al. 1997; Tominaga et al. 2000), and the mitotic spindle (Kato et al. 2001). Finally, chemical inhibition offers the advantage of knocking down protein function without the expression of dominant negative peptide fragments that may interact with endogenous binding partners (Watanabe et al. 1999). This advantage is especially relevant in the context of Rho GTPase effectors, where dominant negative

constructs may bind to and titrate away the pool of activated Rho GTPases, clouding the interpretation of resultant phenotypes.

An allosteric mDia1 inhibitor may also serve as a useful tool in furthering our understanding of the molecular details of mDia regulation. For example, binding of Rho only partially relieves the autoinhibition of mDia1 actin nucleation *in vitro* (Li and Higgs 2003, 2005), which raises the possibility that additional, unidentified regulatory proteins are involved in mDia activation. A small-molecule inhibitor that stabilizes the cellular pool of mDia1 into an autoinhibited conformation may allow for the purification and identification of "dead end" mDia1/activator complexes for subsequent analysis and identification of interacting proteins.

Finally, while there is as yet no direct link between mDia activity and the development of disease states, DRFs have been proposed as novel drug targets in cancer due to their role in regulating both the actin and microtubule cytoskeleton (Faix and Grosse 2006) and the importance of these cytoskeletal elements for mitosis and cytokinesis. mDia1 interacts with the tumor suppressor adenomatous polyposis coli (APC), and may serve as a scaffold for APC-mediated microtubule stabilization (Wen et al. 2004). In addition, mDia1 binds polycystin 2 (PKD2), a protein linked to the development of polycystic kidney disease (Rundle et al. 2004). Additional evidence for the involvement of these cytoskeletal regulators in the development of disease comes from the human DRFs, where mutations in the genes encoding hDia1 and hDia2 have been associated with non-syndromic deafness (Lynch et al. 1997) and premature ovarian failure (Bione et al. 1998). Given the role of DRFs in a number of important cellular processes, it is likely that aberrant DRF function will be implicated in additional human disease states. As such, small-molecule inhibitors of mDia1 could help define the role of these proteins in disease development.

4.5 Screening Approaches to Identify Allosteric mDia1 Inhibitors

Similar to the approaches described for Pak1, chemical screens to identify allosteric inhibitors of mDia should be designed such that the chosen assays address both the inhibitory activity of a compound as well as the mechanism of action. One such approach would involve performing parallel *in vitro* actin polymerization assays (Cooper et al. 1983): one with full-length mDia1 proteins, the other with a truncated mDia1 protein containing only the FH2-DAD domains. In the first screen, the ability of full-length mDia1 to stimulate actin polymerization in the presence of activator (GTP-bound Rho) and individual compounds would be assayed. A second screen would then be performed that would test the ability of the same compounds to inhibit the actin-nucleating activity of a truncated mDia1 protein containing only the catalytic FH2-DAD domains. This FH2-DAD fragment lacks the autoregulatory domains required for autoinhibition, and as such displays high intrinsic catalytic activity (Li and Higgs 2003). Compounds that inhibit only the full-length protein, and not the isolated catalytic fragment, may target the autoregulatory

domains of mDia1. In contrast, compounds that inhibit both the full-length and truncated mDia1 proteins are likely to act either non-specifically or by targeting the catalytic domains of the protein. This type of assay was used in the characterization of wiskostatin, where it was demonstrated that wiskostatin was unable to inhibit the catalytic VCA fragment of N-WASP, but significantly inhibited the catalytic activity of the full length N-WASP construct (see above and Peterson et al. 2004).

Another approach to assay compounds for their mechanism of action could be to directly monitor the interaction between recombinant protein fragments containing the autoregulatory DID and DAD domains *in vitro* using fluorescence polarization assays. These assays are commonly used in high throughput screens to identify compounds that disrupt protein–protein interactions (Owicki 2000; Burke et al. 2003); however, in this case the technique could be used to identify compounds that stabilize the autoinhibitory interactions of the DID and DAD. Fluorescence polarization assays have been used to investigate DID/DAD interactions (Lammers et al. 2005; Li and Higgs 2005; Rose et al. 2005; Wallar et al. 2006), and this assay has been used to demonstrate the mutually exclusive binding of Rho-GTP and a mDia1 construct containing the DID (Rose et al. 2005). In this report, an mDia fragment containing the DID was added to a DAD-containing fragment labeled with the fluorophore 7-amino-4-methylcoumarin-3-acetic acid (AMCA). The interaction between these two fragments resulted in a dramatic increase in the fluorescence polarization of the DAD construct, indicative of a binding interaction. Addition of activated RhoA, but not RhoA-GDP, reduced the fluorescence polarization back to basal levels, indicating a disruption of the DID/DAD interaction (Rose et al. 2005). This assay could be adapted to test the ability of individual compounds to stabilize the interaction between the DID and the DAD in the presence of active RhoA. Compounds that stabilize the interactions between the DID and fluorescently labeled DAD would result in increased fluorescence polarization of DID/DAD complexes that persist despite the addition of active RhoA. To further characterize those compounds that appear to stabilize the DID/DAD complex, a follow-up fluorescence polarization assay could be performed using a DID-containing fragment mutated from Ala to Asp at residue 256. This DID mutant is unable to interact with DAD-containing protein fragments (Otomo et al. 2005; Rose et al. 2005). Therefore, compounds that stabilize the interaction of mutated-DID/DAD interaction in this assay may be acting through a non-specific mechanism.

5 Concluding Remarks/Perspective

Autoinhibition is a regulatory strategy employed by many Rho GTPase effectors including enzymes and non-catalytic, scaffolding proteins. The conformational changes and binding events that activate and relieve autoinhibition can provide opportunities for small-molecule binding and intervention as illustrated by N-WASP and wiskostatin. In particular, the trapping of native, inactive conformations may be a broadly applicable approach to inhibiting proteins regulated in this way. In principle,

this mechanism provides two key advantages over active site-directed inhibitors. First, inhibition by stabilization of autoinhibited states allows for the targeting of proteins, such as scaffolding proteins, which lack classical small molecule-binding active sites and have not been considered “druggable” targets. As such, the spectrum of proteins amenable to small-molecule inhibition may be significantly greater than previously appreciated, implying the existence of a larger number of potential targets for therapeutic intervention. Second, increased inhibitor specificity may be achieved since autoregulatory domains may not be as evolutionarily conserved in sequence as catalytic sites between closely related proteins. Indeed, the well-documented promiscuity of many ATP-competitive kinase inhibitors illustrates the challenge of achieving target specificity with active site-directed inhibitors.

Wiskostatin was identified in a phenotypic screen for inhibitors of a signaling cascade without preordination of target or mechanism of action. Nevertheless, inhibitors that act by a similar mechanism should, in principle, be identifiable by targeted screens using purified proteins that include the relevant autoinhibitory domains. Consequently, prior detailed biochemical and/or structural knowledge of the regulatory mechanism of the targeted protein facilitates the design of assays that specifically detect compounds that exploit that mechanism. Pak1 and mDia1 are two Rho GTPase effectors for which a wealth of biochemical and structural data are available regarding their mechanisms of autoinhibition and activation. We used these examples here to illustrate how this knowledge can be applied to specifically identify inhibitors that stabilize their autoinhibited states. Furthermore, specific chemical inhibitors of these proteins have not been reported and would be novel and complementary tools to elucidate their biological functions.

Stabilization of autoinhibited conformations need not be limited to effectors of the Rho GTPases, however. Many other proteins are regulated by defined autoinhibitory domains and could be amenable to the screening strategies and allosteric small-molecule inhibition as described here. The example of wiskostatin hints that autoinhibited proteins, in general, could represent a hitherto largely unexploited class of small-molecule targets.

Acknowledgements The authors gratefully acknowledge Drs. Jonathan Chernoff and Harry Higgs for critical reading of the manuscript.

References

Abdul-Manan N, Aghazadeh B, Liu GA, Majumdar A, Ouerfelli O, Siminovitch KA, Rosen MK (1999) Structure of Cdc42 in complex with the GTPase-binding domain of the ‘Wiskott-Aldrich syndrome’ protein. Nature 399:379–383

Adam L, Vadlamudi R, Mandal M, Chernoff J, Kumar R (2000) Regulation of microfilament reorganization and invasiveness of breast cancer cells by kinase dead p21-activated kinase-1. J Biol Chem 275:12041–12050

Alberts AS (2001) Identification of a carboxyl-terminal diaphanous-related formin homology protein autoregulatory domain. J Biol Chem 276:2824–2830

Alberts AS, Bouquin N, Johnston LH, Treisman R (1998) Analysis of RhoA-binding proteins reveals an interaction domain conserved in heterotrimeric G protein beta subunits and the yeast response regulator protein Skn7. J Biol Chem 273:8616–8622

Arber S, Barbayannis FA, Hanser H, Schneider C, Stanyon CA, Bernard O, Caroni P (1998) Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature 393:805–809

Bagrodia S, Taylor SJ, Jordon KA, Van Aelst L, Cerione RA (1998) A novel regulator of p21-activated kinases. J Biol Chem 273:23633–23636

Balasenthil S, Sahin AA, Barnes CJ, Wang RA, Pestell RG, Vadlamudi RK, Kumar R (2004) p21-activated kinase-1 signaling mediates cyclin D1 expression in mammary epithelial and cancer cells. J Biol Chem 279:1422–1428

Banerjee M, Worth D, Prowse DM, Nikolic M (2002) Pak1 phosphorylation on t212 affects microtubules in cells undergoing mitosis. Curr Biol 12:1233–1239

Baumeister MA, Rossman KL, Sondek J, Lemmon MA (2006) The Dbs PH domain contributes independently to membrane targeting and regulation of guanine nucleotide-exchange activity. Biochem J 400:563–572

Beeser A, Chernoff J (2005) Production and use of a cell permeable inhibitor of group A Paks (TAT-PID) to analyze signal transduction. Methods 37:203–207

Bione S, Sala C, Manzini C, Arrigo G, Zuffardi O, Banfi S, Borsani G, Jonveaux P, Philippe C, Zuccotti M, Ballabio A, Toniolo D (1998) A human homologue of the Drosophila melanogaster diaphanous gene is disrupted in a patient with premature ovarian failure: evidence for conserved function in oogenesis and implications for human sterility. Am J Hum Genet 62:533–541

Bokoch GM (2003) Biology of the p21-activated kinases. Annu Rev Biochem 72:743–781

Bokoch GM, Wang Y, Bohl BP, Sells MA, Quilliam LA, Knaus UG (1996) Interaction of the Nck adapter protein with p21-activated kinase (PAK1). J Biol Chem 271:25746–25749

Bos JL, Rehmann H, Wittinghofer A (2007) GEFs and GAPs: critical elements in the control of small G proteins. Cell 129:865–877

Boureux A, Vignal E, Faure S, Fort P (2007) Evolution of the Rho family of ras-like GTPases in eukaryotes. Mol Biol Evol 24:203–216

Buchwald G, Hostinova E, Rudolph MG, Kraemer A, Sickmann A, Meyer HE, Scheffzek K, Wittinghofer A (2001) Conformational switch and role of phosphorylation in PAK activation. Mol Cell Biol 21:5179–5189

Burdine L, Kodadek T (2004) Target identification in chemical genetics: the (often) missing link. Chem Biol 11:593–597

Burke TJ, Loniello KR, Beebe JA, Ervin KM (2003) Development and application of fluorescence polarization assays in drug discovery. Comb Chem High Throughput Screen 6:183–194

Carter JH, Douglass LE, Deddens JA, Colligan BM, Bhatt TR, Pemberton JO, Konicek S, Hom J, Marshall M, Graff JR (2004) Pak-1 expression increases with progression of colorectal carcinomas to metastasis. Clin Cancer Res 10:3448–3456

Castro A, Vigneron S, Bernis C, Labbe JC, Lorca T (2006) Ubiquitin-mediated protein degradation in Xenopus egg extracts. Methods Mol Biol 322:223–234

Chan RC, Forbes DI (2006) *In vitro* study of nuclear assembly and nuclear import using Xenopus egg extracts. Methods Mol Biol 322:289–300

Chaudhary A, King WG, Mattaliano MD, Frost JA, Diaz B, Morrison DK, Cobb MH, Marshall MS, Brugge JS (2000) Phosphatidylinositol 3-kinase regulates Raf1 through Pak phosphorylation of serine 338. Curr Biol 10:551–554

Chen RH, Murray A (1997) Characterization of spindle assembly checkpoint in Xenopus egg extracts. Methods Enzymol 283:572–584

Cheung P, Allis CD, Sassone-Corsi P (2000) Signaling to chromatin through histone modifications. Cell 103:263–271

Chong C, Tan L, Lim L, Manser E (2001) The mechanism of PAK activation. Autophosphorylation events in both regulatory and kinase domains control activity. J Biol Chem 276:17347–17353

Cooper JA, Walker SB, Pollard TD (1983) Pyrene actin: documentation of the validity of a sensitive assay for actin polymerization. J Muscle Res Cell Motil 4:253–262
Copeland JW, Treisman R (2002) The diaphanous-related formin mDia1 controls serum response factor activity through its effects on actin polymerization. Mol Biol Cell 13:4088–4099
Cotteret S, Jaffer ZM, Beeser A, Chernoff J (2003) p21-Activated kinase 5 (Pak5) localizes to mitochondria and inhibits apoptosis by phosphorylating BAD. Mol Cell Biol 23:5526–5539
Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95–105
de la Barre AE, Robert-Nicoud M, Dimitrov S (1999) Assembly of mitotic chromosomes in Xenopus egg extract. Methods Mol Biol 119:219–229
Deming P, Kornbluth S (2006) Study of apoptosis *in vitro* using the Xenopus egg extract reconstitution system. Methods Mol Biol 322:379–393
Eblen ST, Slack JK, Weber MJ, Catling AD (2002) Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. Mol Cell Biol 22:6023–6033
Edwards DC, Sanders LC, Bokoch GM, Gill GN (1999) Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat Cell Biol 1:253–259
Fabian MA, Biggs WH 3rd, Treiber DK, Atteridge CE, Azimioara MD, Benedetti MG, Carter TA, Ciceri P, Edeen PT, Floyd M, Ford JM, Galvin M, Gerlach JL, Grotzfeld RM, Herrgard S, Insko DE, Insko MA, Lai AG, Lelias JM, Mehta SA, Milanov ZV, Velasco AM, Wodicka LM, Patel HK, Zarrinkar PP, Lockhart DJ (2005) A small molecule-kinase interaction map for clinical kinase inhibitors. Nat Biotechnol 23:329–336
Faix J, Grosse R (2006) Staying in shape with formins. Dev Cell 10:693–706
Frost JA, Steen H, Shapiro P, Lewis T, Ahn N, Shaw PE, Cobb MH (1997) Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. Embo J 16:6426–6438
Galisteo ML, Chernoff J, Su YC, Skolnik EY, Schlessinger J (1996) The adaptor protein Nck links receptor tyrosine kinases with the serine-threonine kinase Pak1. J Biol Chem 271:20997–21000
Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11:4241–4257
Gasman S, Kalaidzidis Y, Zerial M (2003) RhoD regulates endosome dynamics through Diaphanous-related Formin and Src tyrosine kinase. Nat Cell Biol 5:195–204
Heo WD, Inoue T, Park WS, Kim ML, Park BO, Wandless TJ, Meyer T (2006) PI(3,4,5)P3 and PI(4,5)P2 lipids target proteins with polybasic clusters to the plasma membrane. Science 314:1458–1461
Higashida C, Miyoshi T, Fujita A, Oceguera-Yanez F, Monypenny J, Andou Y, Narumiya S, Watanabe N (2004) Actin polymerization-driven molecular movement of mDia1 in living cells. Science 303:2007–2010
Higgs HN (2005) Formin proteins: a domain-based approach. Trends Biochem Sci 30:342–353
Higgs HN, Peterson KJ (2005) Phylogenetic analysis of the formin homology 2 domain. Mol Biol Cell 16:1–13
Ho HY, Rohatgi R, Lebensohn AM, Le M, Li J, Gygi SP, Kirschner MW (2004) Toca-1 mediates Cdc42-dependent actin nucleation by activating the N-WASP-WIP complex. Cell 118:203–216
Ho HY, Rohatgi R, Lebensohn AM, Kirschner MW (2006) *In vitro* reconstitution of cdc42-mediated actin assembly using purified components. Methods Enzymol 406:174–190
Hofmann C, Shepelev M, Chernoff J (2004) The genetics of Pak. J Cell Sci 117:4343–4354
Jaffe AB, Hall A (2005) Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol 21:247–269
Jaffer ZM, Chernoff J (2002) p21-activated kinases: three more join the Pak. Int J Biochem Cell Biol 34:713–717
Karnoub AE, Symons M, Campbell SL, Der CJ (2004) Molecular basis for Rho GTPase signaling specificity. Breast Cancer Res Treat 84:61–71

Kato T, Watanabe N, Morishima Y, Fujita A, Ishizaki T, Narumiya S (2001) Localization of a mammalian homolog of diaphanous, mDia1, to the mitotic spindle in HeLa cells. J Cell Sci 114:775–784
Kim AS, Kakalis LT, Abdul-Manan N, Liu GA, Rosen MK (2000) Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. Nature 404:151–158
King AJ, Sun H, Diaz B, Barnard D, Miao W, Bagrodia S, Marshall MS (1998) The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. Nature 396:180–183
Kissil JL, Johnson KC, Eckman MS, Jacks T (2002) Merlin phosphorylation by p21-activated kinase 2 and effects of phosphorylation on merlin localization. J Biol Chem 277:10394–10399
Kissil JL, Wilker EW, Johnson KC, Eckman MS, Yaffe MB, Jacks T (2003) Merlin, the product of the Nf2 tumor suppressor gene, is an inhibitor of the p21-activated kinase, Pak1. Mol Cell 12:841–849
Knight ZA, Shokat KM (2005) Features of selective kinase inhibitors. Chem Biol 12:621–637
Knight ZA, Shokat KM (2007) Chemical genetics: where genetics and pharmacology meet. Cell 128:425–430
Koh CG, Tan EJ, Manser E, Lim L (2002) The p21-activated kinase PAK is negatively regulated by POPX1 and POPX2, a pair of serine/threonine phosphatases of the PP2C family. Curr Biol 12:317–321
Kouyama T, Mihashi K (1981) Fluorimetry study of N-(1-pyrenyl)iodoacetamide-labelled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromyosin. Eur J Biochem 114:33–38
Kumar R, Vadlamudi RK (2002) Emerging functions of p21-activated kinases in human cancer cells. J Cell Physiol 193:133–144
Kumar R, Gururaj AE, Barnes CJ (2006) p21-activated kinases in cancer. Nat Rev Cancer 6:459–471
Lammers M, Rose R, Scrima A, Wittinghofer A (2005) The regulation of mDia1 by autoinhibition and its release by Rho*GTP. Embo J 24:4176–4187
Lebensohn AM, Ma L, Ho HY, Kirschner MW (2006) Cdc42 and PI(4,5)P2-induced actin assembly in Xenopus egg extracts. Methods Enzymol 406:156–173
Lechler T, Jonsdottir GA, Klee SK, Pellman D, Li R (2001) A two-tiered mechanism by which Cdc42 controls the localization and activation of an Arp2/3-activating motor complex in yeast. J Cell Biol 155:261–270
Lei M, Lu W, Meng W, Parrini MC, Eck MJ, Mayer BJ, Harrison SC (2000) Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. Cell 102:387–397
Leisner TM, Liu M, Jaffer ZM, Chernoff J, Parise LV (2005) Essential role of CIB1 in regulating PAK1 activation and cell migration. J Cell Biol 170:465–476
Li F, Higgs HN (2003) The mouse Formin mDia1 is a potent actin nucleation factor regulated by autoinhibition. Curr Biol 13:1335–1340
Li F, Higgs HN (2005) Dissecting requirements for auto-inhibition of actin nucleation by the formin, mDia1. J Biol Chem 280:6986–6992
Li F, Adam L, Vadlamudi RK, Zhou H, Sen S, Chernoff J, Mandal M, Kumar R (2002) p21-activated kinase 1 interacts with and phosphorylates histone H3 in breast cancer cells. EMBO Rep 3:767–773
Lu W, Katz S, Gupta R, Mayer BJ (1997) Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. Curr Biol 7:85–94
Lynch ED, Lee MK, Morrow JE, Welcsh PL, Leon PE, King MC (1997) Nonsyndromic deafness DFNA1 associated with mutation of a human homolog of the Drosophila gene diaphanous. Science 278:1315–1318
Ma L, Cantley LC, Janmey PA, Kirschner MW (1998a) Corequirement of specific phosphoinositides and small GTP-binding protein Cdc42 in inducing actin assembly in Xenopus egg extracts. J Cell Biol 140:1125–1136

Ma L, Rohatgi R, Kirschner MW (1998b) The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42. Proc Natl Acad Sci USA 95:15362–15367
Machesky LM, Atkinson SJ, Ampe C, Vandekerckhove J, Pollard TD (1994) Purification of a cortical complex containing two unconventional actins from Acanthamoeba by affinity chromatography on profilin-agarose. J Cell Biol 127:107–115
Manser E, Leung T, Salihuddin H, Zhao ZS, Lim L (1994) A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature 367:40–46
Manser E, Huang HY, Loo TH, Chen XQ, Dong JM, Leung T, Lim L (1997) Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. Mol Cell Biol 17:1129–1143
Manser E, Loo TH, Koh CG, Zhao ZS, Chen XQ, Tan L, Tan I, Leung T, Lim L (1998) PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol Cell 1:183–192
Maroney AC, Finn JP, Connors TJ, Durkin JT, Angeles T, Gessner G, Xu Z, Meyer SL, Savage MJ, Greene LA, Scott RW, Vaught JL (2001) Cep-1347 (KT7515), a semisynthetic inhibitor of the mixed lineage kinase family. J Biol Chem 276:25302–25308
Martin-Belmonte F, Gassama A, Datta A, Yu W, Rescher U, Gerke V, Mostov K (2007) PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. Cell 128:383–397
Meller N, Merlot S, Guda C (2005) CZH proteins: a new family of Rho-GEFs. J Cell Sci 118:4937–4946
Miki H, Miura K, Takenawa T (1996) N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. EMBO J 15:5326–5335
Millard TH, Sharp SJ, Machesky LM (2004) Signalling to actin assembly via the WASP (Wiskott-Aldrich syndrome protein)-family proteins and the Arp2/3 complex. Biochem J 380:1–17
Moon KH, Zhao X, Yu YT (2006) Pre-mRNA splicing in the nuclei of Xenopus oocytes. Methods Mol Biol 322:149–163
Nheu TV, He H, Hirokawa Y, Tamaki K, Florin L, Schmitz ML, Suzuki-Takahashi I, Jorissen RN, Burgess AW, Nishimura S, Wood J, Maruta H (2002) The K252a derivatives, inhibitors for the PAK/MLK kinase family selectively block the growth of RAS transformants. Cancer J 8:328–336
Nobes CD, Hall A (1995) Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 81:53–62
Otomo T, Otomo C, Tomchick DR, Machius M, Rosen MK (2005) Structural basis of Rho GTPase-mediated activation of the formin mDia1. Mol Cell 18:273–281
Owicki JC (2000) Fluorescence polarization and anisotropy in high throughput screening: perspectives and primer. J Biomol Screen 5:297–306
Papayannopoulos V, Co C, Prehoda KE, Snapper S, Taunton J, Lim WA (2005) A polybasic motif allows N-WASP to act as a sensor of PIP(2) density. Mol Cell 17:181–191
Parrini MC, Lei M, Harrison SC, Mayer BJ (2002) Pak1 kinase homodimers are autoinhibited in trans and dissociated upon activation by Cdc42 and Rac1. Mol Cell 9:73–83
Pelish HE, Westwood NJ, Feng Y, Kirchhausen T, Shair MD (2001) Use of biomimetic diversity-oriented synthesis to discover galanthamine-like molecules with biological properties beyond those of the natural product. J Am Chem Soc 123:6740–6741
Pelish HE, Peterson JR, Salvarezza SB, Rodriguez-Boulan E, Chen JL, Stamnes M, Macia E, Feng Y, Shair MD, Kirchhausen T (2006) Secramine inhibits Cdc42-dependent functions in cells and Cdc42 activation *in vitro*. Nat Chem Biol 2:39–46
Peng J, Wallar BJ, Flanders A, Swiatek PJ, Alberts AS (2003) Disruption of the Diaphanous-related formin Drf1 gene encoding mDia1 reveals a role for Drf3 as an effector for Cdc42. Curr Biol 13:534–545

Petersen J, Nielsen O, Egel R, Hagan IM (1998) FH3, a domain found in formins, targets the fission yeast formin Fus1 to the projection tip during conjugation. J Cell Biol 141:1217–1228
Peterson FC, Deng Q, Zettl M, Prehoda KE, Lim WA, Way M, Volkman BF (2007) Multiple WASP-interacting protein recognition motifs are required for a functional interaction with N-WASP. J Biol Chem 282:8446–8453
Peterson JR, Golemis EA (2004) Autoinhibited proteins as promising drug targets. J Cell Biochem 93:68–73
Peterson JR, Mitchison TJ (2002) Small molecules, big impact: a history of chemical inhibitors and the cytoskeleton. Chem Biol 9:1275–1285
Peterson JR, Lokey RS, Mitchison TJ, Kirschner MW (2001) A chemical inhibitor of N-WASP reveals a new mechanism for targeting protein interactions. Proc Natl Acad Sci USA 98:10624–10629
Peterson JR, Bickford LC, Morgan D, Kim AS, Ouerfelli O, Kirschner MW, Rosen MK (2004) Chemical inhibition of N-WASP by stabilization of a native autoinhibited conformation. Nat Struct Mol Biol 11:747–755
Peterson JR, Lebensohn AM, Pelish HE, Kirschner MW (2006) Biochemical suppression of small-molecule inhibitors: a strategy to identify inhibitor targets and signaling pathway components. Chem Biol 13:443–452
Prehoda KE, Scott JA, Mullins RD, Lim WA (2000) Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. Science 290:801–806
Pruyne D, Evangelista M, Yang C, Bi E, Zigmond S, Bretscher A, Boone C (2002) Role of formins in actin assembly: nucleation and barbed-end association. Science 297:612–615
Quinlan ME, Heuser JE, Kerkhoff E, Mullins RD (2005) Drosophila Spire is an actin nucleation factor. Nature 433:382–388
Ridley AJ, Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389–399
Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70:401–410
Rivero F, Muramoto T, Meyer AK, Urushihara H, Uyeda TQ, Kitayama C (2005) A comparative sequence analysis reveals a common GBD/FH3-FH1-FH2-DAD architecture in formins from Dictyostelium, fungi and metazoa. BMC Genomics 6:28
Rohatgi R, Ma L, Miki H, Lopez M, Kirchhausen T, Takenawa T, Kirschner MW (1999) The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. Cell 97:221–231
Rohatgi R, Ho HY, Kirschner MW (2000) Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate. J Cell Biol 150:1299–1310
Rohatgi R, Nollau P, Ho HY, Kirschner MW, Mayer BJ (2001) Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. J Biol Chem 276:26448–26452
Rose R, Weyand M, Lammers M, Ishizaki T, Ahmadian MR, Wittinghofer A (2005) Structural and mechanistic insights into the interaction between Rho and mammalian Dia. Nature 435:513–518
Rossman KL, Der CJ, Sondek J (2005) GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nat Rev Mol Cell Biol 6:167–180
Rundle DR, Gorbsky G, Tsiokas L (2004) PKD2 interacts and co-localizes with mDia1 to mitotic spindles of dividing cells: role of mDia1 IN PKD2 localization to mitotic spindles. J Biol Chem 279:29728–29739
Sahai E, Marshall CJ (2002) RHO-GTPases and cancer. Nat Rev Cancer 2:133–142
Salic A, Lee E, Mayer L, Kirschner MW (2000) Control of beta-catenin stability: reconstitution of the cytoplasmic steps of the wnt pathway in Xenopus egg extracts. Mol Cell 5:523–532
Schafer DA, Jennings PB, Cooper JA (1996) Dynamics of capping protein and actin assembly *in vitro*: uncapping barbed ends by polyphosphoinositides. J Cell Biol 135:169–179
Schraml P, Schwerdtfeger G, Burkhalter F, Raggi A, Schmidt D, Ruffalo T, King W, Wilber K, Mihatsch MJ, Moch H (2003) Combined array comparative genomic hybridization and tissue

microarray analysis suggest PAK1 at 11q13.5-q14 as a critical oncogene target in ovarian carcinoma. Am J Pathol 163:985–992
Shaw RJ, Paez JG, Curto M, Yaktine A, Pruitt WM, Saotome I, O'Bryan JP, Gupta V, Ratner N, Der CJ, Jacks T, McClatchey AI (2001) The Nf2 tumor suppressor, merlin, functions in Rac-dependent signaling. Dev Cell 1:63–72
Shennan KI (2006) Xenopus egg extracts: a model system to study proprotein convertases. Methods Mol Biol 322:199–212
Slack-Davis JK, Eblen ST, Zecevic M, Boerner SA, Tarcsafalvi A, Diaz HB, Marshall MS, Weber MJ, Parsons JT, Catling AD (2003) PAK1 phosphorylation of MEK1 regulates fibronectin-stimulated MAPK activation. J Cell Biol 162:281–291
Sokac AM, Co C, Taunton J, Bement W (2003) Cdc42-dependent actin polymerization during compensatory endocytosis in Xenopus eggs. Nat Cell Biol 5:727–732
Stockwell BR (2000) Chemical genetics: ligand-based discovery of gene function. Nat Rev Genet 1:116–125
Stradal TE, Scita G (2006) Protein complexes regulating Arp2/3-mediated actin assembly. Curr Opin Cell Biol 18:4–10
Takenawa T, Suetsugu S (2007) The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. Nat Rev Mol Cell Biol 8:37–48
Talukder AH, Meng Q, Kumar R (2006) CRIPak, a novel endogenous Pak1 inhibitor. Oncogene 25:1311–1319
Tang Y, Chen Z, Ambrose D, Liu J, Gibbs JB, Chernoff J, Field J (1997) Kinase-deficient Pak1 mutants inhibit Ras transformation of Rat-1 fibroblasts. Mol Cell Biol 17:4454–4464
Tang Y, Marwaha S, Rutkowski JL, Tennekoon GI, Phillips PC, Field J (1998) A role for Pak protein kinases in Schwann cell transformation. Proc Natl Acad Sci USA 95:5139–5144
Tang DD, Bai Y, Gunst SJ (2005) Silencing of p21-activated kinase attenuates vimentin phosphorylation on Ser-56 and reorientation of the vimentin network during stimulation of smooth muscle cells by 5-hydroxytryptamine. Biochem J 388:773–783
Taunton J, Rowning BA, Coughlin ML, Wu M, Moon RT, Mitchison TJ, Larabell CA (2000) Actin-dependent propulsion of endosomes and lysosomes by recruitment of N-WASP. J Cell Biol 148:519–530
Tochtrop GP, King RW (2004) Target identification strategies in chemical genetics. Comb Chem High Throughput Screen 7:677–688
Tominaga T, Sahai E, Chardin P, McCormick F, Courtneidge SA, Alberts AS (2000) Diaphanous-related formins bridge Rho GTPase and Src tyrosine kinase signaling. Mol Cell 5:13–25
Tran NH, Frost JA (2003) Phosphorylation of Raf-1 by p21-activated kinase 1 and Src regulates Raf-1 autoinhibition. J Biol Chem 278:11221–11226
Tu H, Wigler M (1999) Genetic evidence for Pak1 autoinhibition and its release by Cdc42. Mol Cell Biol 19:602–611
Tutter AV, Walter JC (2006) Chromosomal DNA replication in a soluble cell-free system derived from Xenopus eggs. Methods Mol Biol 322:121–137
Vadlamudi RK, Adam L, Wang RA, Mandal M, Nguyen D, Sahin A, Chernoff J, Hung MC, Kumar R (2000) Regulatable expression of p21-activated kinase-1 promotes anchorage-independent growth and abnormal organization of mitotic spindles in human epithelial breast cancer cells. J Biol Chem 275:36238–36244
Vartiainen MK, Machesky LM (2004) The WASP-Arp2/3 pathway: genetic insights. Curr Opin Cell Biol 16:174–181
Volkman BF, Prehoda KE, Scott JA, Peterson FC, Lim WA (2002) Structure of the N-WASP EVH1 domain-WIP complex: insight into the molecular basis of Wiskott-Aldrich Syndrome. Cell 111:565–576
Wallar BJ, Stropich BN, Schoenherr JA, Holman HA, Kitchen SM, Alberts AS (2006) The basic region of the diaphanous-autoregulatory domain (DAD) is required for autoregulatory interactions with the diaphanous-related formin inhibitory domain. J Biol Chem 281:4300–4307
Wang RA, Zhang H, Balasenthil S, Medina D, Kumar R (2006) PAK1 hyperactivation is sufficient for mammary gland tumor formation. Oncogene 25:2931–2936

Watanabe N, Madaule P, Reid T, Ishizaki T, Watanabe G, Kakizuka A, Saito Y, Nakao K, Jockusch BM, Narumiya S (1997) p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. EMBO J 16:3044–3056

Watanabe N, Kato T, Fujita A, Ishizaki T, Narumiya S (1999) Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. Nat Cell Biol 1:136–143

Wen Y, Eng CH, Schmoranzer J, Cabrera-Poch N, Morris EJ, Chen M, Wallar BJ, Alberts AS, Gundersen GG (2004) EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. Nat Cell Biol 6:820–830

Wu X, Frost JA (2006) Multiple Rho proteins regulate the subcellular targeting of PAK5. Biochem Biophys Res Commun 351:328–335

Xia C, Ma W, Stafford LJ, Marcus S, Xiong WC, Liu M (2001) Regulation of the p21-activated kinase (PAK) by a human Gbeta-like WD-repeat protein, hPIP1. Proc Natl Acad Sci USA 98:6174–6179

Xiao GH, Beeser A, Chernoff J, Testa JR (2002) p21-activated kinase links Rac/Cdc42 signaling to merlin. J Biol Chem 277:883–886

Xiao GH, Gallagher R, Shetler J, Skele K, Altomare DA, Pestell RG, Jhanwar S, Testa JR (2005) The NF2 tumor suppressor gene product, merlin, inhibits cell proliferation and cell cycle progression by repressing cyclin D1 expression. Mol Cell Biol 25:2384–2394

Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, Nishida E, Mizuno K (1998) Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature 393:809–812

Yasuda S, Oceguera-Yanez F, Kato T, Okamoto M, Yonemura S, Terada Y, Ishizaki T, Narumiya S (2004) Cdc42 and mDia3 regulate microtubule attachment to kinetochores. Nature 428:767–771

Yoshioka K, Foletta V, Bernard O, Itoh K (2003) A role for LIM kinase in cancer invasion. Proc Natl Acad Sci USA 100:7247–7252

Zang M, Hayne C, Luo Z (2002) Interaction between active Pak1 and Raf-1 is necessary for phosphorylation and activation of Raf-1. J Biol Chem 277:4395–4405

Zenke FT, King CC, Bohl BP, Bokoch GM (1999) Identification of a central phosphorylation site in p21-activated kinase regulating autoinhibition and kinase activity. J Biol Chem 274:32565–32573

Zhan Q, Ge Q, Ohira T, Van Dyke T, Badwey JA (2003) p21-activated kinase 2 in neutrophils can be regulated by phosphorylation at multiple sites and by a variety of protein phosphatases. J Immunol 171:3785–3793

Zhao ZS, Manser E, Chen XQ, Chong C, Leung T, Lim L (1998) A conserved negative regulatory region in alphaPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1. Mol Cell Biol 18:2153–2163

Zhao ZS, Lim JP, Ng YW, Lim L, Manser E (2005) The GIT-associated kinase PAK targets to the centrosome and regulates Aurora-A. Mol Cell 20:237–249

Zheng Y, Glaven JA, Wu WJ, Cerione RA (1996) Phosphatidylinositol 4,5-bisphosphate provides an alternative to guanine nucleotide exchange factors by stimulating the dissociation of GDP from Cdc42Hs. J Biol Chem 271:23815–23819

Pharmacological Interference with Protein–Protein Interactions Mediated by Coiled-Coil Motifs

H.M. Strauss and S. Keller(✉)

Contents

1 Introduction: The GCN4 Leucine Zipper 462
1.1 Function 463
1.2 Structure 464
2 Coiled Coils in Scaffolding Proteins 467
2.1 The Ndc80 Complex 468
2.2 SNARE Proteins 469
3 Role of Coiled Coils in Viral Infection 471
3.1 Class I Fusion Proteins 472
3.2 Influenza Hemagglutinin 474
3.3 Gp41 of HIV-1 474
4 Therapeutic Interference with Coiled-Coil Interactions 475
4.1 Botulinum Toxins 475
4.2 Viral Fusion Inhibitors 476
5 Other Therapeutic Applications of Coiled Coils 477
References 478

Abstract Coiled coils are bundles of intertwined α-helices that provide protein–protein interaction sites for the dynamic assembly and disassembly of protein complexes. The coiled-coil motif combines structural versatility and adaptability with mechanical strength and specificity. Multimeric proteins that rely on coiled-coil interactions are structurally and functionally very diverse, ranging from simple homodimeric transcription factors to elaborate heteromultimeric scaffolding clusters. Several coiled-coil-bearing proteins are of outstanding pharmacological importance, most notably SNARE proteins involved in vesicular trafficking of neurotransmitters and viral fusion proteins. Together with their crucial roles in many physiological and pathological processes, the structural simplicity and reversible nature of coiled-coil associations render them a promising target for

S. Keller
Leibniz Institute of Molecular Pharmacology FMP, Robert-Rössle-Str. 10, 13125 Berlin, Germany
Keller@fmp-berlin.de

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

pharmacological interference, as successfully exemplified by botulinum toxins and viral fusion inhibitors.

The α-helical coiled coil is a ubiquitous protein domain that mediates highly specific homo- and heteromeric protein–protein interactions among a wide range of proteins. The coiled-coil motif was first proposed by Crick on the basis of X-ray diffraction data on α-keratin more than 50 years ago (Crick 1952, 1953) and nowadays belongs to the best-characterized protein interaction modules. By definition, a coiled coil is an oligomeric protein assembly consisting of several right-handed amphipathic α-helices that wind around each other into a *superhelix* (or a *supercoil*) in which the hydrophobic surfaces of the constituent helices are in continuous contact, forming a hydrophobic core. Both homomeric and heteromeric coiled coils with different stoichiometries are possible, and the helices can be aligned in either a parallel or an antiparallel topology (Harbury et al. 1993, 1994). Stoichiometry and topology are governed by the primary structure, that is, the sequence of the polypeptide chains, and a given protein can participate in multiple assembly–disassembly equilibria among several coiled coils differing in stoichiometry and topology (Portwich et al. 2007).

Protein complexes whose oligomeric quaternary structures – and, hence, biological activities – depend on coiled-coil interactions include transcription factors, tRNA synthetases (Biou et al. 1994; Cusack et al. 1990), cytoskeletal and signal-transduction proteins, enzyme complexes, proteins involved in vesicular trafficking, viral coat proteins, and membrane proteins (Langosch and Heringa 1998). It is thus not surprising that coiled-coil motifs have gained great attention as potential targets for modulating protein–protein interactions implicated in a large number of diseases.

In this review, we will first discuss some fundamental functional and structural aspects of a simple and well-characterized representative of coiled-coil transcription factors (Sect. 1) before considering two more complex coiled coils found in scaffolding proteins involved in mitosis and meiosis and vesicular trafficking (Sect. 2). This will set the stage for addressing the role of coiled coils in viral infection (Sect. 3) as well as strategies of interfering with such protein–protein interactions therapeutically (Sect. 4 and 5).

1 Introduction: The GCN4 Leucine Zipper

The two-stranded transcription factor GCN4 (general control nondepressible) from *Saccharomyces cerevisiae* was the first coiled coil for which a high-resolution crystal structure became available (O'Shea et al. 1991) and until now has remained one of the best-studied coiled coils. Therefore, and owing to its structural and functional simplicity, GCN4 lends itself ideally as an example for introducing the basic features inherent to coiled-coil motifs.

1.1 Function

Transcription factors enable cells to quickly and adequately respond and adapt to environmental changes by reprogramming their transcription pattern, that is, by activating or repressing gene expression in a spatially and temporally controlled manner. The GCN4 protein was originally identified as a positive regulator of genes encoding enzymes required for amino acid biosynthesis (Hinnebusch 1992), but it has since been implicated in various other biosynthetic pathways as well (Hinnebusch and Natarajan 2002). Upon amino acid, purine, or nitrogen starvation, the GCN4 protein level is upregulated through several mechanisms, and GCN4 homodimers bind to a consensus sequence (TGACTC) found upstream of target genes, thus derepressing their transcription. These targets include more than 35 genes encoding enzymes involved in amino acid biosynthesis (Hinnebusch 1992); furthermore, transcription of at least 10% of all genes of the entire yeast genome is directly or indirectly stimulated by GCN4 (Hinnebusch and Natarajan 2002).

The GCN4 dimer belongs to the family of basic region leucine zipper (bZip) transcription factors, which are of paramount importance in all eukaryotic cells (Hurst 1994, 1995). The two vital functions of these proteins – that is, dimerization and ensuing DNA binding – are reflected in the structure of their bZip domain, which is about 60–80 amino acid residues long and consists of two distinct regions (Vinson et al. 1989): an N-terminal *basic region* responsible for binding to the major groove of double-stranded DNA and a C-terminal *leucine zipper* mediating dimerization (Ellenberger et al. 1992; Keller et al. 1995; König and Richmond 1993). The dimeric quaternary structure of GCN4 and other bZip transcription factors bears several advantages over transcription regulators acting as monomers. Trivially, although monomeric or multimeric proteins can serve the same purpose, binding to double-stranded DNA inherently reveals some bias in favor of dimeric protein complexes. More importantly, however, the dimeric nature of the active state brings about a more pronounced concentration dependence as compared with a biologically active protein monomer. This is due to the fact that the concentration of protein dimers is not a linear function of the concentration of monomeric subunits, but reveals a steeper dependence. Finally, the modular architecture of bZip transcription factors allows for a huge number of conceivable homo- and heterodimeric combinations resulting from a rather limited set of polypeptide chains. GCN4 natively self-associates into a homodimeric protein assembly, but other members of the bZip family are involved in simultaneous, competing homo- and heterodimeric association processes, giving rise to transcription factors differing in DNA-binding avidity and even target-gene specificity (O'Shea et al. 1989, 1992; Hurst 1994, 1995; Glover and Harrison 1995; Newman and Keating 2003).

1.2 Structure

The GCN4 protein is 281 amino acid residues long and contains a bZip domain at its C-terminus (O'Shea et al. 1991). The 31-residue coiled-coil leucine zipper spans residues 249–279 of the full-length protein; its sequence reads R $[\mathrm{M_2KQL_5EDK}]_{\mathrm{I}}$ $[\mathrm{V_9EEL_{12}LSK}]_{\mathrm{II}}$ $[\mathrm{N_{16}YHL_{19}ENE}]_{\mathrm{III}}$ $[\mathrm{V_{23}ARL_{26}KKL}]_{\mathrm{IV}}$ $\mathrm{V_{30}G}$ and reveals four so-called *heptad repeats* typical of all coiled coils. Each of these repeating heptads consists of seven amino acid residues, corresponding to two helical turns (see below), and is generically represented by $\mathbf{[abcdefg]}_i$, where *i* stands for the heptad number (heptads I–IV in the present case). This is conveniently illustrated in a *helical-wheel diagram* (Fig. 1), where all residues along the four heptads of the GCN4 coiled coil are projected onto a plane perpendicular to the supercoil axis.

Positions **a** and **d** (denoted by lower-case residue numbers in the above sequence) play a key role as they constitute the dimerization interface, that is, the hydrophobic core that is shielded from the aqueous environment upon coiled-coil assembly. These positions are usually occupied by hydrophobic, often branched amino acid residues, such as leucine, isoleucine, and valine (McLachlan and Stewart 1975; Conway and Parry 1990, 1991). In the case of the GCN 4 coiled coil, valine is the predominant residue found at the **a** positions, whereas all **d**

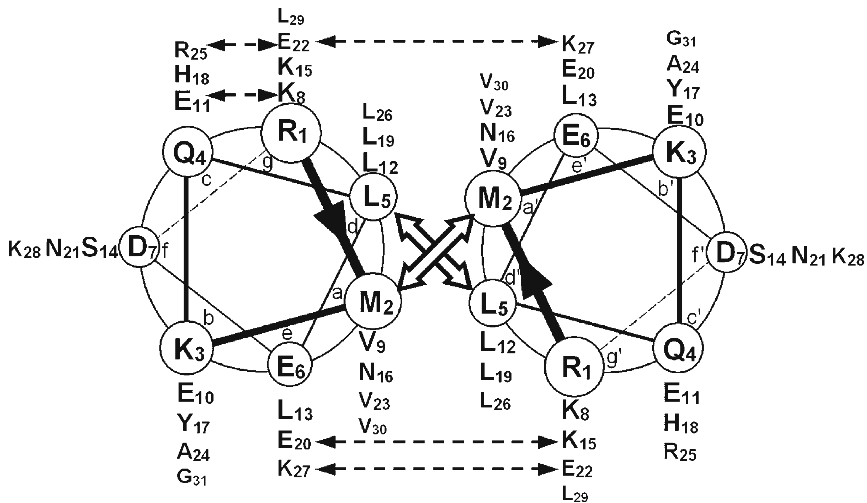

Fig. 1 Helical-wheel diagram of the homodimeric GCN4 leucine-zipper coiled coil, corresponding to residues 249–279 of the full-length GCN4 protein. View from N-terminus. Residues next to the viewer are surrounded by *circles*. *Single-letter code* for amino acids is used. *Crossed arrows* in the center denote interactions in the hydrophobic core, whereas *dashed arrows* represent inter- and intrahelical salt bridges (copyright Wiley-VCH Verlag GmbH & Co. KGaA, reproduced with permission from Portwich et al. 2007)

positions carry leucine residues. Like many similar dimeric transcription factors, GCN4 displays an asparagine residue (N_{16}) in the **a** position of the third heptad (Hurst 1994, 1995), which is crucial for dimerization. Substitution of this conserved asparagine by other amino acid residues, even glutamine, gives rise to a mixture of dimers and trimers (Harbury et al. 1993; Potekhin et al. 1994; Knappenberger et al. 2002) or destabilizes the dimeric coiled coil (Gonzalez et al. 1996). More generally, the residues in the hydrophobic core positions **a** and **d** act as a switch for the transition from the native dimeric to trimeric coiled-coil structures, and even very subtle modifications, such as supposedly conservative amino acid substitutions may exert a tremendous effect on coiled-coil stoichiometry (Portwich et al. 2007). Positions **b**, **c**, **e**, **f**, and **g** face the aqueous outside of the assembly and are mostly occupied by hydrophilic residues. In particular, the positions neighboring the hydrophobic core, that is, **e** and **g**, are rich in glutamic acid and lysine residues.

The X-ray structure of the parallel GCN4 homodimer (O'Shea et al. 1991; Fig. 2) confirmed the model of *knobs-into-holes packing* suggested by Crick (1953), in which each of the hydrophobic side chains at positions **a** and **d** of either helix protrudes into a cavity molded by the side chains of four residues of the opposing helix. Importantly, a slight distortion of both constituent helices as compared with a typical monomeric or globular α-helix causes the helical pitch to amount to 3.5 residues rather than the usual 3.6 residues. Therefore, each heptad corresponds to exactly two helical turns, allowing all **a** and **d** side chains along all four heptads to contact the hydrophobic helix–helix interface.

The glutamic acid and lysine residues at positions **e** and **g** stabilize the assembly in a twofold manner. On one hand, the methylene groups of their side chains shield the hydrophobic core from water (Alber 1992; Hodges et al. 1994). On the other hand, oppositely charged residues in these positions form inter- and intramolecular salt bridges (O'Shea et al. 1991). In the GCN4 leucine zipper, intrahelical salt bridges exist between residues K8 (**g**) and E11 (**c**) as well as E22 (**g**) and R25 (**c**), which most likely competes with an interhelical salt bridge between E22 (**g**) and K27 (**e′**). Two other interhelical salt bridges are established between E20 (**e**) and K15 (**g′**) as well as K27 (**e**) and E22 (**g′**). Since these intermolecular salt bridges are not entirely surrounded by water, but partially exposed to the hydrophobic core, ion-pair formation contributes considerably to coiled-coil stability (Krylov et al. 1994; Lavigne et al. 1996; Lumb and Kim 1995). Not unexpectedly, introduction of like charges into juxtaposing residues in positions **g** and **e′** or **g′** and **e** drastically destabilizes bZip coiled coils (O'Shea et al. 1992).

Close inspection of the crystal structure (Fig. 2) and, more obviously, of the pattern of salt bridges highlighted in the helical-wheel diagram (Fig. 1) reveals that the homodimeric GCN4 coiled coil is not strictly symmetric with respect to the supercoil axis. Also, the core-flanking positions **e** and **g** are not equivalent, as might naively be deduced from the helical-wheel diagram. This asymmetry manifests itself in the observation that position **g** is significantly more sensitive towards amino acid substitutions than position **e** (Portwich et al. 2007).

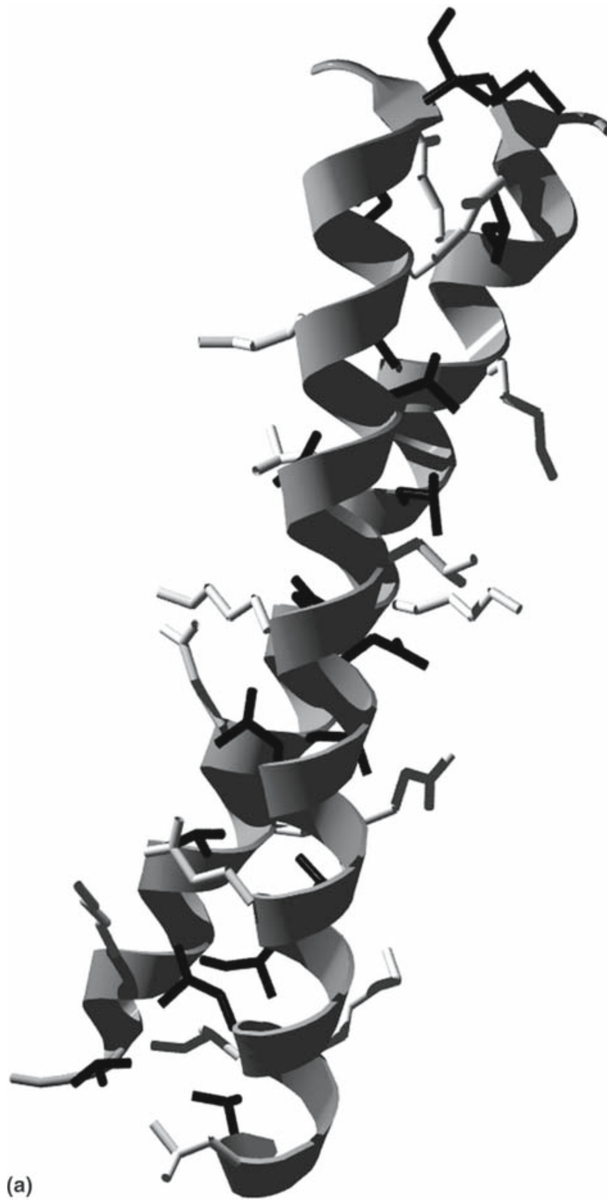

Fig. 2 The 1.8-Å X-ray structure of the homodimeric GCN4 leucine-zipper coiled coil, corresponding to residues 249–279 of the full-length GCN4 protein (PDB accession code 2ZTA; O'Shea et al. 1991). **(a)** *Side view*, N-terminus at top. **(b)** *Axial view* from N-terminus. Side chains of residues at the hydrophobic core positions **a** and **d** are shown in *black*, side chains of residues at the core-flanking positions **e** and **g** in *light gray*

Fig. 2 (continued)

2 Coiled Coils in Scaffolding Proteins

As pointed out above, dimeric transcription factors represent a particularly simple and well-studied example of α-helical leucine zippers. However, coiled coils can assume more complex quaternary structures and are involved in a plethora of other molecular interactions and key cellular processes. On the basis of 29 genomes sequenced as of 2001, ten percent of all eukaryotic proteins and five percent of all prokaryotic proteins have been predicted to bear coiled-coil motifs (Liu and Rost 2001).

The most obvious advantage of coiled coils for their function as transcription factors appears to reside in their dynamic association–dissociation equilibrium (cf. Sect. 1.1). Another asset of coiled coils is their mechanical strength, and it can therefore be anticipated that they play an eminent role in many *scaffolding proteins*, most of which need to confer both stability and adaptability to supramolecular complexes, organelles, cells, and tissues. In fact, the first amino acid sequence of a coiled-coil motif was published in 1972 for tropomyosin, an actin-binding muscle contraction regulator protein (Hodges et al. 1972; Sodek et al. 1972). Other examples of scaffolding proteins containing coiled coils include cytoskeletal proteins, such as intermediate filaments (Steinert 1993), kinesin and other motor proteins, as well as occludin, an integral membrane protein implicated

in the organization of epithelial tight junctions. In this section, we will focus on two members of the vast and diverse class of coiled-coil-based scaffolding proteins, namely, protein complexes involved in mitosis and presynaptic vesicle docking and fusion.

2.1 The Ndc80 Complex

An example of a coiled coil that is structurally more sophisticated than and functionally drastically different from dimeric leucine zippers is provided by the heterotetrameric Ndc80 complex, an essential component of eukaryotic *kinetochores*. Each kinetochore consists of several dozen different proteins and is located on the centromere of a chromosome, where it dynamically connects the latter to the plus ends of microtubules from the mitotic spindle, thus mediating chromosome alignment and sister-chromatid segregation during mitosis and meiosis (Cleveland et al. 2003). Just like many other kinetochore proteins, the Ndc80 complex is highly conserved among eukaryotic organisms, even though kinetochores from different species vary largely with respect to their organizational complexity (Maiato et al. 2004).

The four proteins constituting the Ndc80 complex are named HEC1 (human homologue, highly expressed in cancer; yeast homologue known as Ndc80), Nuf2, Spc24, and Spc25 and comprise about 670, 460, 200, and 220 amino acid residues, respectively (Wigge and Kilmartin 2001). The heterotetrameric Ndc80 complex has a rod-like architecture in which a long coiled-coil shaft separates two relatively small globular heads, one formed by HEC1 and Nuf2 and the other one by Spc24 and Spc25 (Ciferri et al. 2005; Wei et al. 2005). All four protein subunits contain long stretches of leucine-zipper heptad repeats. HEC1 and Nuf2 form a stable heterodimeric subcomplex with an N-terminal globular domain and a C-terminal coiled-coil domain with a predicted length of about 35 nm. Of note, neither HEC1 nor Nuf2 is stable on its own, and the N-terminal globular parts of the two proteins do not associate in the absence of the coiled-coil regions. Likewise, Spc24 and Spc25 also heterodimerize to give rise to a second subcomplex with a smaller C-terminal globular head and an N-terminal coiled coil that possesses about half the number of residues and, consequently, is about half as long as that of the HEC1/Nuf2 subcomplex. The crystal structures of the two globular domains of the yeast complex have been solved (Wei et al. 2006, 2007), providing strong evidence that HEC1/Nuf2 and Spc24/Spc25 interact directly with microtubules and kinetochore proteins, respectively.

Such detailed structural insight is currently not available for the elongated coiled-coil domain of the Ndc80 complex, but several complementary approaches have shed considerable light on the molecular organization of the coiled-coil assembly. Electron micrographs readily visualize the yeast Ndc80 complex, whose total length has been estimated to amount to about 57 nm (Wei et al. 2005). Without the two globular domains, the coiled-coil shaft is about 51 nm long, which is in

excellent agreement with the summed lengths predicted for the coiled-coil domains of the HEC1/Nuf2 and Spc24/Spc25 subcomplexes (see above). This implies that the two subcomplexes assemble into the tetrameric Ndc80 complex by aligning in a linear tail-to-tail arrangement. Antibody labeling experiments using the yeast complex (Wei et al. 2005) and size-exclusion chromatography on its human homologue (Ciferri et al. 2005) have indeed demonstrated that the double-stranded coiled-coil domains of HEC1/Nuf2 on one hand and Spc24/Spc25 on the other hand avidly interact with each other in such a way. How exactly this tail-to-tail contact is established remains elusive, but it is intriguing that the polypeptide chain of HEC1 C-terminally extends for about 80 residues beyond that of Nuf2 and, consequently, beyond the C-terminus of the double-stranded HEC1/Nuf2 coiled coil. A similar overhang is observed for the N-terminus of the second subcomplex, where Spc24 extends for about 20 residues beyond Spc25 (Wei et al. 2005). Hence, the overhanging, single-stranded chains of both subcomplexes could act as "sticky ends" that intertwine with each other so that the shaft of the Ndc80 complex consists of a continuous double-stranded coiled coil. Alternatively, it is conceivable that a three-stranded *tetramerization domain* is formed in which either overhanging polypeptide chain is incorporated into the coiled-coil domain of the opposing subcomplex. The latter scenario is compatible with the agreement of measured and predicted coiled-coil lengths (see above), but a final answer will have to await further structural elucidation.

The overall structure of the Ndc80 complex reflects its function as a scaffolding protein in the kinetochore: While the two terminal globular domains provide attachment sites for microtubules and proteins located in the interior of the kinetochore, the central shaft bridges the outer plate of the kinetochore and thus transmits forces exerted between the mitotic or meiotic spindle apparatus and the chromosomal centromere. Whether, in addition to its static scaffolding function, the dynamics of association and dissociation of the Ndc80 complex plays a role in the regulation of the spindle apparatus has yet to be determined.

2.2 SNARE Proteins

Proteins, lipids, and other biomolecules are transferred among different cellular compartments by *transport vesicles*, which carry hydrophilic cargoes in the aqueous lumen or hydrophobic ones in the lipid membrane. Crucially, each vesicle must pinch off from the membrane of its parent compartment and recognize and fuse with that of its target compartment in a highly specific and tightly regulated way, such as to release its contents at the right place at the right time (Bennett 1995). In addition, very high activation energy barriers must be overcome during fusion of the vesicle membrane with the target membrane (Chernomordik and Kozlov 2005). In all eukaryotic cells, these tasks are accomplished by a family of proteins called soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (Söllner et al. 1993).

Although SNAREs differ substantially from one another with respect to size, structure, and cellular localization, they all have in common a conserved cytosolic coiled-coil domain of 60–70 residues known as the SNARE motif and a membrane-anchoring domain, such as a transmembrane sequence or cysteine-linked palmitoyl chains. SNARE proteins may be classified according to two different criteria: Traditionally, *vesicle* SNAREs (v-SNAREs) are distinguished from *target-membrane* SNAREs (t-SNAREs) because they are initially attached to different membranes. A more recent classification based on structural features (Fasshauer et al. 1998) distinguishes among four conserved subfamilies called R-, Qa-, Qb-, and Qc-SNARES, depending on whether a polypeptide chain contributes an arginine or a glutamine residue to a central region of the coiled coil termed the *zero ionic layer* (Sutton et al. 1998). By virtue of their SNARE motifs, and upon stimulation by a specific trigger, cognate v- and t-SNARE proteins avidly but reversibly assemble into a four-stranded coiled coil known as the SNAREpin, in which each of the above subfamilies is represented by one helix. Since the constituent protein subunits remain anchored to the membranes of different compartments or organelles, this assembly is said to be a *trans*-SNARE complex. Importantly, coiled-coil interactions first occur at the membrane-distal N-terminal end of the SNARE motif and then propagate towards the C-terminal membrane-binding domains, thus bringing the two membranes in closer contact. This zippering of the coiled-coil domain is energetically favorable and is thought to contribute substantially to surmounting the high activation energy barrier associated with membrane fusion (Weber et al. 1998). After fusion, all polypeptide chains reside in the same membrane, and the assembly is henceforth referred to as a *cis*-SNARE complex.

A very important and outstandingly well-studied SNARE protein complex is that mediating the Ca^{2+}-triggered fusion of acetylcholine-transporting neuronal vesicles with the presynaptic plasma membrane, thereby releasing the neurotransmitter into the synaptic cleft (Hanson et al. 1997). Here, synaptobrevin (also known as vesicle-associated membrane protein, VAMP) acts as v-SNARE and R-SNARE, residing in the membrane of presynaptic vesicles and contributing an arginine residue to the zero ionic layer. By contrast, syntaxin 1 and SNAP 25 (soluble *N*-ethylmaleimide-sensitive factor attachment protein of 25 kDa) assume the roles of t-SNAREs and Q-SNAREs, both being attached to the presynaptic plasma membrane and carrying glutamine residues in the center of their coiled-coil regions. Synaptobrevin and syntaxin 1 each contribute one helix to the coiled coil of the SNARE complex and are anchored to their respective membranes through their C-terminal transmembrane segments, whereas SNAP 25 participates with two helices in the complex and is bound to the presynaptic plasma membrane through palmitoyl chains linked to cysteine residues in between the two coiled-coil segments.

The structure of the trimeric core SNARE complex has been solved by X-ray crystallography (Sutton et al. 1998). The hydrophobic interface of the four-stranded coiled-coil region is commonly grouped into layers, each of which consists of four residues, one from each constituent helix. In the center of this elongated assembly, the zero ionic layer, containing one arginine (R56 from synaptobrevin) and three

glutamine residues (Q226 from syntaxin 1, Q53 and Q174 from SNAP 25), is flanked by two extended leucine-zipper regions whose layers are denoted +1, +2, etc. and −1, −2, etc., respectively. These coiled coils shield the zero ionic layer from water and thus enhance electrostatic attraction between the guanidinium group of the arginine and the carbonyl groups of the three glutamines. Without this stabilizing effect, the SNARE complex readily dissociates, which has been suggested to allow for recycling of SNARE proteins once membrane fusion is completed (Sutton et al. 1998).

Complex assembly upon neuronal stimulation by Ca^{2+} and disassembly upon acetylcholine release are controlled by an elaborate, but as yet poorly defined, network of *SNARE master proteins*. For instance, the vesicle-associated protein synaptotagmin acts as Ca^{2+} sensor and binds to certain membrane lipids as well as SNARE proteins, thereby modulating their oligomerization (Stein et al. 2007). Another regulatory mechanism is exerted by the N-terminal three-helix domain of syntaxin 1 itself, which can fold back onto the SNARE motif and thus prevent it from participating in coiled-coil assembly. Furthermore, this SNARE motif confers not only interaction with cognate SNARE proteins, but also binding to voltage-gated Ca^{2+} and K^+ channels in the presynaptic plasma membrane (Bezprozvanny et al. 1995) and self-association of syntaxin 1 into homomeric protein clusters comprising about 75 monomers (Sieber et al. 2006, 2007). Clustering is believed to be essential for providing the high syntaxin 1 densities required for vesicle docking and fusion, but a detailed understanding of this phenomenon and the mode of action of SNARE masters is just beginning to emerge. Nevertheless, neuronal v- and t-SNAREs can be reconstituted separately into artificial lipid bilayers and spontaneously trigger vesicle fusion (Schuette et al. 2004). Very recently, the energetics and dynamics of SNAREpin formation have been assessed by measuring the forces exerted between v- and t-SNAREs embedded in two opposing, solid-supported lipid bilayers (Li et al. 2007), suggesting that assembly of a single SNARE coiled coil can release sufficient energy to fuel hemifusion, but not complete fusion, of the membranes.

In summary, coiled-coil-dependent SNARE complexes fulfill multiple key functions in vesicular trafficking, accounting for target-membrane recognition, vesicle tethering, and energy supply. Moreover, SNAREs are likely to serve as scaffolds for the recruitment and assembly of other proteins involved in docking and fusion and could thus be crucial for integrating these two processes (Gillingham and Munro 2003).

3 Role of Coiled Coils in Viral Infection

Coiled-coil interactions are essential not only for vesicular trafficking during intracellular protein and lipid transport and intercellular communication using neurotransmitters (see Sect. 2.2), but they also constitute an indispensable part of the *fusion machinery* of enveloped viruses. Viruses have to overcome the membrane of

the host cell to deliver their genetic material into the cytosol and nucleus so that a new infectious cycle can be established. Viral entry into host cells proceeds via two functionally distinct steps, namely, recognition of the target cell and fusion of the viral membrane with the cell membrane of the host (for a recent review, see Weissenhorn et al. 2007). While recognition of target cells is appropriately specific and falls beyond the scope of this work, the basic steps leading to membrane fusion are believed to be common to all enveloped viruses, though differences exist as to the mode of activation, the structural motifs used, and the initial state of oligomerization of the viral fusion protein. For the purpose of this review, we restrict our considerations to class I fusion proteins, which are of extraordinary importance from a pharmacological viewpoint. We will first describe the generic functions of these fusion proteins during viral entry into the host cell and then turn our attention to two specific examples, influenza hemagglutinin and HIV gp41.

3.1 Class I Fusion Proteins

The first step in a fusion process triggered by a class I fusion protein is an activation event that releases a viral *fusion peptide*, which interacts with the target membrane. In the second step, a conformational change of the entire *fusion protein* brings the viral and target membranes into close proximity. Functional separation of these two steps ensures that the fusion mechanism is activated only when the target membrane is within reach. As implied in a very similar way for SNARE-mediated membrane fusion (see Sect. 2.2), the free energy difference between the pre- and post-fusion states of the fusion protein is the driving force for membrane deformation and ultimately fusion (Weissenhorn et al. 2007).

Recognition of target structures and membrane fusion are mediated by viral glycoproteins exposed at the surface of enveloped viruses. These glycoproteins contain a receptor-binding region and a part responsible for membrane fusion. Both are synthesized as a single polypeptide chain that assembles into a homotrimer before being proteolytically cleaved into two subunits, a receptor-binding protein and a fusion protein. The subunits remain closely associated and represent the metastable, fusion-competent state. The receptor-binding protein is entirely external to the viral membrane and contains specificity determinants for target-membrane receptor recognition. By contrast, the fusion protein is an integral membrane protein made up of a C-terminal endodomain, a transmembrane region, and an N-terminal ectodomain. The ectodomain contains two heptad repeat regions (HR1 and HR2), which are separated by a hinge domain, and an N-terminal hydrophobic sequence known as the fusion peptide, which makes contact with the target membrane. HR1 is present as a central trimeric coiled-coil stalk already in the pre-fusion complex. Activation of the fusion complex upon receptor binding leads to a number of sequential conformational changes (Fig. 3). First, the fusion protein is released from its complex with the receptor-binding protein, thereby exposing and extending the fusion peptide to establish interaction with the target membrane. Then, HR2 folds back onto the hydrophobic outer groove of the central trimeric coiled-coil stalk of HR1, thus forming a stable six-helix bundle. This refolding brings the viral

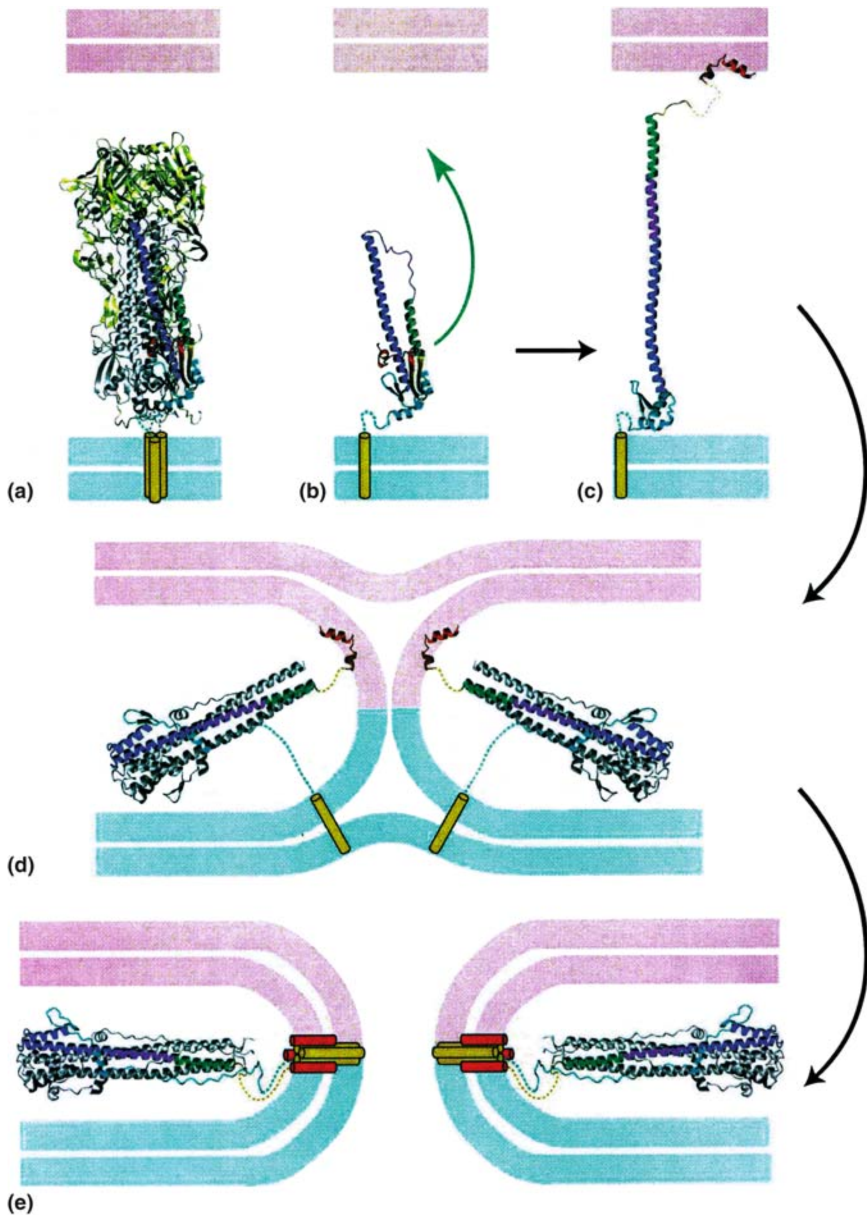

Fig. 3 Schematic of the different steps in viral fusion. **(a)** Trimeric HA in the pre-fusion state (Wilson et al. 1981; PDB accession code 1HGG). HA_1 is shown in *yellow*, HA_2 in *gray* (two monomers) and in *three different colors* (third monomer). The transmembrane anchor is schematically shown as *yellow ribbons*, the cellular membrane in *pink*. The fusion peptide (*red*) is sequestered in the complex. **(b)** Only one monomer of HA_2 is shown for clarity. The *arrow* indicates movement of the fusion peptide towards the cellular membrane after activation. **(c)** Hypothetical structure showing the HA_2-monomer with the fusion peptide inserted into the cellular membrane just before refolding of HR2 (*green*) onto HR1 (*blue*). **(d)** Refolding of HR2 and HR1 into the six-helix bundle brings the N-terminal fusion peptide and the C-terminal transmembrane anchor in close proximity, initiating hemi-fusion of the membranes. **(e)** Complete fusion of the cellular with the viral membrane allows transfer of viral genetic material through the pore. The interaction of the fusion peptide with the transmembrane region of HA_2 is hypothetical only (copyright Taylor & Francis Ltd, reproduced with permission from Schibli and Weissenhorn 2004)

and target membranes into sufficiently close apposition to initiate fusion according to the stalk hypothesis (Chernomordik and Kozlov 2005). The post-fusion state of the viral fusion protein is extremely stable, with unfolding temperatures >90°C (Baker et al. 1999). Hence, both the stability of coiled coils and their specificity are exploited in virus-induced membrane fusion.

3.2 *Influenza Hemagglutinin*

The best-characterized example of a viral fusion protein is influenza hemagglutinin (HA). In fact, the generic events described above derive almost entirely from studies on influenza HA, and it is still the only class I fusion protein for which structures of all relevant stages are available, that is, the unprocessed precursor HA_0 as well as the fusion protein itself in the pre- and post-fusion states (Chen et al. 1998; Wilson et al. 1981; Carr and Kim 1993; Bullough et al. 1994). HA_0 is cleaved into HA_1, the receptor-binding protein, and HA_2, the fusion protein, which together form the fusion-competent HA protein. In spite of this proteolytic step, HA_1 and HA_2 remain covalently connected by a disulfide bridge. HA_1 recognizes specific sialic acid residues of glycoproteins and glycolipids in the target host membrane. Organ specificity of viral infection is achieved through differential affinities to the major types of sialic acid–galactose linkages and their differential organ distribution (Ito et al. 1997). Binding of HA_1 to as yet unidentified target receptors leads to endocytosis of the HA_1/HA_2 complex by the host cell. The low pH of the late endosome triggers the release of the fusion protein HA_2 from the metastable HA_1/HA_2 complex to initiate membrane fusion (Skehel and Wiley 2000). In vitro, membrane fusion can also be initiated by elevated temperature (Wharton et al. 1986). Recombinantly expressed HA_2 lacking the fusion peptide and the C-terminal transmembrane region spontaneously folds into the stable post-fusion conformation, structurally and biochemically indistinguishable from that of full-length HA_2 in the post-fusion state (Chen et al. 1995). This indicates that this conformation is the lowest-free-energy state of the molecule, implying that the receptor-binding protein functions as a kinetic trap for the fusion protein, which is released upon lowering pH or raising temperature.

3.3 *Gp41 of HIV-1*

Human immunodeficiency virus 1 (HIV-1) is the causative pathogen of acquired immunodeficiency syndrome (AIDS). The precursor protein gp160 is cleaved into gp120, the receptor-binding protein, and gp41, the fusion protein. In contrast to HA, the association of the gp120/gp41 complex is noncovalent. The resulting protein complex, also referred to as Env protein, is composed of three gp120 and three gp41 subunits and is exposed at the surface of the virus. Recognition of CD4

receptors present on T-cells, macrophages, and monocytes by gp120 induces a first conformational change and enables binding of co-receptors, particularly chemokine receptors CCR5 and CXCR4. A second conformational change then leads to the release of gp41, which is sufficient for catalyzing membrane fusion. On the contrary to HA, no change in pH or temperature is required for activation of the fusion protein. Though no structure of the pre-fusion state of gp41 is available to date, the structural similarity of the recombinantly expressed ectodomain of gp41 with the post-fusion state of HA_2 suggests that the sequence of events leading to membrane fusion is similar for the two proteins (Chan et al. 1997; Tan et al. 1997; Weissenhorn et al. 1997). As detailed above (see Sect. 3.1), the structure of the fusion protein reveals a six-helix bundle containing in its center a trimeric coiled-coil stalk of HR1 immediately C-terminal to the fusion peptide. Three HR2 helices pack into the hydrophobic grooves formed by the HR1 trimer, which accommodate three hydrophobic amino acid residues of HR2, that is, W628, W631, and I635 (numbers refer to the precursor gp160).

4 Therapeutic Interference with Coiled-Coil Interactions

Among the structurally and functionally diverse coiled-coil proteins discussed so far, SNARE proteins (Sect. 2.2) and viral fusion proteins (Sect. 3) are of particular therapeutic relevance because they both represent targets of approved and widely employed drugs, botulinum toxins and fusion inhibitors. However, the specificity and reversibility of their association and the crucial roles they play in many central cellular processes suggest that many other coiled coils might also be considered promising targets for pharmacological interference.

4.1 Botulinum Toxins

Botulinum toxins from *Clostridium botulinum* are the most toxic proteins known to date but have, nevertheless, found widespread pharmaceutical and cosmetic use. Botulinum toxin type A (BTX-A) was approved in 1989 for the treatment of strabismus and in 2002 as a cosmetic against glabellar lines. Meanwhile, the use of BTX-A has been extended to the treatment of numerous other disorders resulting from hyperactive skeletal or smooth muscles, hypersecretory and painful disorders, and serotype B (BTX-B) was approved for treatment of cervical dystonia in 2000. Subcutaneous and intramuscular injections of BTX-A figure among the most popular cosmetic treatments worldwide (Lim and Seet 2007).

BTX-A consists of two polypeptide chains that are connected by a disulfide bridge. The 100-kDa heavy chain confers binding to proteins located in the presynaptic membrane of neuromuscular junctions and subsequent neuronal internalization

by endocytosis. Upon reduction of the disulfide bond, the 50-kDa light chain escapes from endocytotic vesicles and proteolytically degrades SNAP 25. Other botulinum toxins, known as serotypes B–G, share the same two-chain structure and a similar route of cellular uptake but proteolyze different components of the SNARE complex: type E also attacks SNAP 25, type C targets both SNAP 25 and synaptobrevin, whereas types B, D, F, and G degrade synaptobrevin (Jahn and Niemann 1994). Whichever SNARE protein is the target of the proteolytic attack, botulinum toxins consistently prevent presynaptic vesicles from docking to and fusing with the presynaptic plasma membrane to release acetylcholine neurotransmitter into the neuromuscular junction, which causes flaccid muscular paralysis that can persist for up to several months.

4.2 Viral Fusion Inhibitors

Until recently, only two proteins essential for the viral life cycle were targets of therapeutic intervention by three mechanistic classes of antiretroviral drugs. Reverse transcriptase is the target of nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs), while HIV protease is targeted by protease inhibitors (PIs). In March 2003, however, Enfuvirtide was approved as the first HIV fusion inhibitor. Enfuvirtide is a peptide consisting of residues 643–678 of the HIV-1 gp160 protein. These residues are part of the HR2 segment of gp41 that folds back onto the hydrophobic groove formed by the HR1 stalk. The mechanism of action is thought to involve competitive binding of Enfuvirtide to HR1 while gp41 is still in the pre-fusion state, thus inhibiting membrane fusion and viral entry. Interestingly, the first peptides inhibiting viral fusion were designed in the absence of high-resolution structural data on the fusion protein, and structural implications of the fusion process were merely based on secondary-structure prediction from the primary sequence of gp160 (Wild et al. 1992). Though Enfuvirtide lacks the three hydrophobic residues of HR2 that are in contact with HR1 (see Sect. 3.3), it has reasonably high in vitro antiviral activity with an IC_{50} value for HIV-1 entry of 50–60 nM (Wild et al. 1994). Peptides derived from the HR1 region also inhibit viral entry, indicating that the believed mechanism of action is essentially correct (Wild et al. 1992; Jiang et al. 1994).

The wealth of structural details and structure–activity relationships available for coiled-coil interactions make Enfuvirtide and other peptides obvious candidates for rational drug development. Indeed, several peptide sequences modified accordingly have been shown to be more active in inhibiting viral entry, with IC_{50} values in the upper femtomolar to lower nanomolar range (Otaka et al. 2002; Eron et al. 2004; Dwyer et al. 2007). A peptide termed T-1249 appeared to be the most promising candidate for development of second-generation fusion inhibitors because it possesses greater in vitro potency than Enfuvirtide and is active on most Enfuvirtide-resistant strains, too (Eron et al. 2004). However, further development was put on hold because its poor solubility and stability more than outweigh its

favorable safety, efficacy, and tolerability properties determined in phase I trials (Martínez-Carbonero 2004).

In an effort to design more potent inhibitors of viral fusion, Dwyer et al. (2007) applied modifications to an HR2 peptide known to increase helical content and oligomer stability of coiled-coil peptides. Having identified functionally critical residues by alanine scanning, they introduced glutamic acid and arginine residues at the core-flanking positions **e** and **g** such as to promote interhelical salt bridges stabilizing the coiled-coil assembly (see Sect. 1.2). Additionally, eight non-critical residues were replaced by alanine, which has one of the highest propensities to induce α-helix formation. In a last step, two alanine residues were included at **a** and **d** positions to increase the hydrophobic contact area of the core. The resulting peptide, referred to as T-2635, assembles into a homotrimeric coiled-coil structure at low micromolar concentrations and forms an extremely stable six-helix bundle with HR1, having a melting temperature of 86°C in 8 M urea. In vitro, T-2635 reveals greatly enhanced activity in comparison with Enfuvirtide against several, though not all, tested HIV-1 strains. Moreover, viral resistance evolves considerably slower for a close analogue of T-2635 as compared with Enfuvirtide (Dwyer et al. 2007).

Several small molecules inhibiting HIV-1 fusion with IC_{50} in the lower micromolar range have been discovered by high-throughput screening of molecular libraries. These compounds are believed to inhibit viral entry by interacting with the hydrophobic cavity of gp41-HR1, thereby destabilizing the central three-helix stalk and inhibiting formation of the post-fusion six-helix bundle (Jiang et al. 2004; Jin et al. 2005). Developing such small-molecule lead scaffolds into more potent HIV-1 fusion inhibitors is attracting enormous attention from both academic and industrial researchers as this could mark a major breakthrough in antiretroviral therapy.

5 Other Therapeutic Applications of Coiled Coils

Owing to their structural versatility, stability, and specificity, leucine zippers and other coiled coils have gained widespread attention as potential tools and building blocks for a number of model applications. Contegno et al. (2002) proposed a generalized method to inactivate self-associating molecular targets by creating a chimera of a coiled-coil domain and the selected target, creating interaction sites at both ends of the molecule. Because of the bifunctionality of the chimera, this leads to the formation of large-molecular-weight complexes and consequent inactivation of the target molecule. However, it is not clear from this work whether the chimera is stable in solution by itself or its application has to rely exclusively on genetic methods.

Coiled-coil systems have been considered as *drug-delivery vehicles* for the controlled release of drugs. An interesting approach to the reversible formation of a hydrogel has been described. An artificial recombinant polypeptide consisting of coiled-coil oligomerizing sequences and an unstructured part of hydrophilic amino

acids with large solvent retention functionality was constructed. Oligomerization via the coiled-coil region leads to formation of a hydrogel. Changes in pH or temperature lead to dissociation of the coiled-coil oligomers and dissolution of the hydrogel (Petka et al. 1998).

The specificity of coiled-coil oligomerization has also been used in *drug targeting*. In an approach known as pretargeting, a targeting compound, e.g., an antibody, is fused to a heterodimerizing coiled-coil sequence. The therapeutic agent, e.g., a radionuclide–chelate complex, is equipped with the complementary coiled-coil sequence. Administration of the targeting compound specifically labels the therapeutic target. Subsequent administration of the therapeutic agent and formation of the heterocomplex lead to targeted delivery of the drug to the site of interest. This is expected to increase therapeutic efficiency and reduce unwanted side reactions (Goodwin and Meares 2001; Goldenberg 2003). An advantage of using coiled-coil sequences for this approach is their small size with no concomitant loss of specificity.

Acknowledgements We thank Dr. Rudolf Volkmer, Dr. Michael Portwich, and Carsten Mahrenholz for stimulating discussions and Carolyn Vargas and Anja Sieber for helpful comments on the manuscript.

References

Alber T (1992) Structure of the leucine zipper. Curr Opin Genet Dev 2:205–210

Baker KA, Dutch RE, Lamb RA, Jardetzky TS (1999) Structural basis for paramyxovirus-mediated membrane fusion. Mol Cell 3:309–319

Bennett MK (1995) SNAREs and the specificity of transport vesicle targeting. Curr Opin Cell Biol 7:581–586

Bezprozvanny I, Scheller RH, Tsien RW (1995) Functional impact of syntaxin on gating of N-type and Q-type calcium channels. Nature 378:623–626

Biou V, Yaremchuk A, Tukalo M, Cusack S (1994) The 2.9 Å crystal structure of *T. thermophilus* seryl-tRNA synthetase complexed with tRNA(Ser). Science 263:1404–1410

Bullough PA, Hughson FM, Skehel JJ, Wiley DC (1994) Structure of influenza haemagglutinin at the pH of membrane fusion. Nature 371:37–43

Carr CM, Kim PS (1993) A spring-loaded mechanism for the conformational change of influenza hemagglutinin. Cell 73:823–832

Chan DC, Fass D, Berger JM, Kim PS (1997) Core structure of gp41 from the HIV envelope glycoprotein. Cell 89:263–273

Chen J, Wharton SA, Weissenhorn W, Calder LJ, Hughson FM, Skehel JJ, Wiley DC (1995) A soluble domain of the membrane-anchoring chain of influenza virus hemagglutinin (HA_2) folds in *Escherichia coli* into the low-pH-induced confirmation. Proc Natl Acad Sci USA 92:12205–12209

Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wiley DC (1998) Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell 95:409–417

Chernomordik LV, Kozlov MM (2005) Membrane hemifusion: crossing a chasm in two leaps. Cell 123:375–382

Ciferri C, De Luca J, Monzani S, Ferrari KJ, Ristic D, Wyman C, Stark H, Kilmartin J, Salmon ED, Musacchio A (2005) Architecture of the human ndc80-hec1 complex, a critical constituent of the outer kinetochore. J Biol Chem 280:29088–29095

Cleveland DW, Mao Y, Sullivan KF (2003) Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. Cell 112:407–421

Contegno F, Cioce M, Pelicci PG, Minucci S (2002) Targeting protein inactivation through an oligomerization chain reaction. Proc Natl Acad Sci USA 99:1865–1869

Conway JF, Parry DA (1990) Structural features in the heptad substructure and longer range repeats of two-stranded α-fibrous proteins. Int J Biol Macromol 12:328–334

Conway JF, Parry DA (1991) Three-stranded α-fibrous proteins: the heptad repeat and its implications for structure. Int J Biol Macromol 13:14–16

Crick FHC (1952) Is α-keratin a coiled coil? Nature 170:882–883

Crick FH (1953) The packing of α-helices: simple coiled coils. Acta Crystallogr 6:689–698

Cusack S, Berthet-Colominas C, Hartlein M, Nassar N, Leberman R (1990) A second class of synthetase structure revealed by X-ray analysis of *Escherichia coli* seryl-tRNA synthetase at 2.5 Å. Nature 347:249–255

Dwyer JJ, Wilson KL, Davison DK, Freel SA, Seedorff JE, Wring SA, Tvermoes NA, Matthews TJ, Greenberg ML, Delmedico MK (2007) Design of helical, oligomeric HIV-1 fusion inhibitor peptides with potent activity against enfuvirtide-resistant virus. Proc Natl Acad Sci USA 104:12772–12777

Ellenberger TE, Brandl CJ, Struhl K, Harrison, SC (1992) The GCN4 basic region leucine zipper binds DNA as a dimer of uninterrupted α helices: crystal structure of the protein–DNA complex. Cell 71:1223–1237

Eron JJ, Gulick RM, Bartlett JA, Merigan T, Arduino R, Kilby JM, Yangco B, Diers A, Drobnes C, DeMasi R, Greenberg M, Melby T, Raskino C, Rusnak P, Zhang Y, Spence R Miralles GD (2004) Short-term safety and antiretroviral activity of T-1249, a second-generation fusion inhibitor of HIV. J Infect Dis 189:1075–1083

Fasshauer D, Sutton RB, Brünger AT, Jahn R (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc Natl Acad Sci USA 95:15781–15786

Gillingham AK, Munro S (2003) Long coiled-coil proteins and membrane traffic. Biochim Biophys Acta 1641:71–85

Glover JN, Harrison SC (1995) Crystal structure of the heterodimeric bZIP transcription factor c-Fos–c-Jun bound to DNA. Nature 373:257–261

Goldenberg DM (2003) Advancing role of radiolabeled antibodies in the therapy of cancer. Cancer Immunol Immunother 52:281–296

Gonzalez L, Jr, Woolfson, DN, Alber T (1996) Buried polar residues and structural specificity in the GCN4 leucine zipper. Nat Struct Biol 3:1011–1018

Goodwin DA, Meares CF (2001) Advances in pretargeting biotechnology. Biotechnol Adv 19:435–450

Hanson PI, Heuser JE, Jahn R (1997) Neurotransmitter release–four years of SNARE complexes. Curr Opin Neurobiol 7:310–315

Harbury PB, Zhang T, Kim PS, Alber T (1993) A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants. Science 262:1401–1407

Harbury PB, Kim PS, Alber T (1994) Crystal structure of an isoleucine-zipper trimer. Nature 371:80–83

Hinnebusch AG (1992) General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in *Saccharomyces cerevisae*. In: Broach JR, Jones EW, Pringle JR (eds) The molecular and cellular biology of the yeast *Saccharomyces*: gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 319–414

Hinnebusch AG, Natarajan K (2002) Gcn4p, a master regulator of gene expression, is controlled at multiple levels by diverse signals of starvation and stress. Eukaryot Cell 1:22–32

Hodges RS, Sodek J, Smillie LB, Jurasek L (1972) Tropomyosin: amino acid sequence and coiled coil structure. Cold Spring Harbor Symp Quant Biol 37:299–310

Hodges RS, Zhu BY, Zhou NE, Mant CT (1994) Reversed-phase liquid chromatography as a useful probe of hydrophobic interactions involved in protein folding and protein stability. J Chromatogr A 676: 3–15

Hurst HC (1994) Transcription factors 1: bZIP proteins. Protein Profile 1(2):123–168

Hurst HC (1995) Transcription factors 1: bZIP proteins. Protein Profile 2(2):101–168

Ito T, Suzuki Y, Takada A, Kawamoto A, Otsuki K, Masuda H, Yamada M, Suzuki T, Kida H, Kawaoka Y (1997) Differences in sialic acid–galactose linkages in the chicken egg amnion and allantois influence human influenza virus receptor specificity and variant selection. J Virol 71:3357–3362

Jahn R, Niemann H (1994) Molecular mechanisms of clostridial neurotoxins. Ann NY Acad Sci 733:245–255

Jiang S, Lin K, Strick N, Neurath, AR (1994) HIV-1 inhibition by a peptide. Nature 365:113

Jiang S, Lu H, Liu S, Zhao Q, He Y, Debnath AK (2004) N-substituted pyrrole derivatives as novel human immunodeficiency virus type 1 entry inhibitors that interfere with the gp41 six-helix bundle formation and block virus fusion. Antimicrob Agents Chemother 48:4349–4359

Jin BS, Lee WK, Ahn K, Lee MK, Yu YG (2005) High-throughput screening method of inhibitors that block the interaction between two helical regions of HIV-1 gp41. Biomol Screen 10:13–19

Keller W, König P, Richmond TJ (1995) Crystal structure of a bZIP/DNA complex at 2.2 Å: determinants of DNA specific recognition. J Mol Biol 254:657–667

Knappenberger JA, Smith JE, Thorpe SH, Zitzewitz JA, Matthews CR (2002) A buried polar residue in the hydrophobic interface of the coiled coil peptide, GCN4-p1, plays a thermodynamic, not a kinetic role in folding. J Mol Biol 321:1–6

König P, Richmond TJ (1993) The X-ray structure of the GCN4-bZIP bound to ATF/CREB site DNA shows the complex depends on DNA flexibility. J Mol Biol 233:139–154

Krylov D, Mikhailenko I, Vinson C (1994) A thermodynamic scale for leucine zipper stability and dimerization specificity: e and g interhelical interactions. EMBO J 13:2849–2861

Langosch D, Heringa J (1998) Interaction of transmembrane helices by a knobs-into-holes packing characteristic of soluble coiled coils. Proteins 31:150–159

Lavigne P, Sonnichsen FD, Kay CM, Hodges RS, Lumb KJ, Kim PS (1996) Interhelical salt bridges, coiled coil stability, and specificity of dimerization. Science 271:1136–1138

Li F, Pincet F, Perez E, Eng WS, Melia TJ, Rothman JE, Tareste D (2007) Energetics and dynamics of SNAREpin folding across lipid bilayers. Nat Struct Mol Biol 14:890–896

Lim EC, Seet RC (2007) Botulinum toxin, Quo Vadis? Med Hypotheses 69:718–723

Liu J, Rost B (2001) Comparing function and structure between entire proteomes. Protein Sci 10:1970–1979

Lumb KJ, Kim PS (1995) Measurement of interhelical electrostatic interactions in the GCN4 leucine zipper. Science 268:436–439

Maiato H, DeLuca J, Salmon ED, Earnshaw WC (2004) The dynamic kinetochore–microtubule interface. J Cell Sci 117:5461–5477

Martínez-Carbonero L (2004) Discontinuation of the clinical development of fusion inhibitor T1249. AIDS Rev 6:61

McLachlan AD, Stewart M (1975) Tropomyosin coiled coil interactions: evidence for an unstaggered structure. J Mol Biol 98:293–304

Newman JR, Keating AE (2003) Comprehensive identification of human bZIP interactions with coiled coil arrays. Science 300:2097–2101

O'Shea EK, Rutkowski R, Kim PS (1989) Preferential heterodimer formation by isolated leucine zippers from fos and jun. Science 245:646–648

O'Shea EK, Klemm JD, Kim PS, Alber T (1991) X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. Science 254:539–544

O'Shea EK, Rutkowski R, Kim PS (1992) Mechanism of specificity in the Fos-Jun oncoprotein heterodimer. Cell 68:699–708
Otaka A, Nakamura M, Nameki D, Kodama E, Uchiyama S, Nakamura S, Nakano H, Tamamura H, Kobayashi Y, Matsuoka M, Fujii N (2002) Remodeling of gp41-C34 peptide leads to highly effective inhibitors of the fusion of HIV-1 with target cells. Angew Chem Int Ed 41:2937–2940
Petka WA, Harden J L, McGrath KP, Wirtz D, Tirrell DA (1998) Reversible hydrogels from self-assembling artificial proteins. Science 281:389–392
Portwich M, Keller S, Strauss HM, Mahrenholz CC, Kramer A, Kretzschmar I, Volkmer R (2007). A network of coiled-coil associations derived from synthetic GCN4 leucine-zipper arrays. Angew Chem Int Ed 46:1654–1657
Potekhin SA, Medvedkin VN, Kashparov IA, Venyaminov SY (1994) Synthesis and properties of the peptide corresponding to the mutant form of the leucine zipper of the transcriptional activator GCN4 from yeast. Protein Eng 7:1097–1101
Schibli DJ, Weissenhorn W (2004) class I and class II viral fusion protein structures reveal similar principles in membrane fusion. Mol Membr Biol 21:361–371
Schuette CG, Hatsuzawa K, Margittai M, Stein A, Riedel D, Küster P, König M, Seidel C, Jahn R (2004) Determinants of liposome fusion mediated by synaptic SNARE proteins. Proc Natl Acad Sci USA 101:2858–2863
Sieber JJ, Willig KI, Heintzmann R, Hell SW, Lang T (2006) The SNARE motif is essential for the formation of syntaxin clusters in the plasma membrane. Biophys J 90:2843–2851
Sieber JJ, Willig KI, Kutzner C, Gerding-Reimers C, Harke B, Donnert G, Rammner B, Eggeling C, Hell SW, Grubmüller H, Lang T (2007) Anatomy and dynamics of a supramolecular membrane protein cluster. Science 317:1072–1076
Skehel JJ, Wiley DJ (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 69:531–569
Sodek J, Hodges RS, Smillie LB, Jurasek L (1972) Amino-acid sequence of rabbit skeletal tropomyosin and its coiled-coil structure. Proc Natl Acad Sci USA 69:3800–3804
Söllner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature 362:318–324
Stein A, Radhakrishnan A, Riedel D, Fasshauer D, Jahn R (2007) Synaptotagmin activates membrane fusion through a Ca^{2+}-dependent *trans* interaction with phospholipids. Nat Struct Mol Biol 14:904–911
Steinert PM (1993) Structure, function, and dynamics of keratin intermediate filaments. J Invest Dermatol 100:729–734
Sutton RB, Fasshauer D, Jahn R, Brünger AT (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. Nature 395:347–353
Tan K, Liu, J, Wang J-H, Shen S, Lu M (1997) Atomic structure of a thermostable subdomain of HIV-1 gp41. Proc Natl Acad Sci USA 94:12303–12308
Vinson CR, Sigler PB, McKnight SL (1989) Scissors-grip model for DNA recognition by a family of leucine zipper proteins. Science 246:911–916
Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Söllner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. Cell 92:759–772
Wei RR, Sorger PK, Harrison SC (2005) Molecular organization of the Ndc80 complex, an essential kinetochore component. Proc Natl Acad Sci USA 102:5363–5367
Wei RR, Schnell JR, Larsen NA, Sorger PK, Chou JJ, Harrison SC (2006) Structure of a central component of the yeast kinetochore: the Spc24p/Spc25p globular domain. Structure 14:1003–1009
Wei RR, Al-Bassam J, Harrison SC (2007) The Ndc80/HEC1 complex is a contact point for kinetochore–microtubule attachment. Nat Struct Mol Biol 14:54–59
Weissenhorn W, Dessen A, Harrison SC, Skehel JJ, Wiley DC (1997) Atomic structure of the ectodomain from HIV-1 gp41. Nature 387:426–430
Weissenhorn W, Hinz A, Gaudin Y (2007) Virus membrane fusion. FEBS Lett 581:2150–2155

Wharton SA, Skehel JJ, Wiley DC (1986) Studies of influenza haemagglutinin-mediated membrane fusion. Virology 149:27–35

Wigge PA, Kilmartin JV (2001) The Ndc80p complex from *Saccharomyces cerevisiae* contains conserved centromere components and has a function in chromosome segregation. J Cell Biol 152:349–360

Wild C, Oas T, McDanal CB, Bolognesi D, Matthews T (1992) A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. Proc Natl Acad Sci USA 89:10537–10541

Wild C, Shugars DC, Greenwell TK, McDanal CB, Matthews TJ (1994) Peptides corresponding to a predictive α-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. Proc Natl Acad Sci USA 91:9770–9774

Wilson IA, Skehel JJ, Wiley DC (1981) Structure of the haemeagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. Nature 289:366–373

Direct AKAP-Mediated Protein–Protein Interactions as Potential Drug Targets

C. Hundsrucker and E. Klussmann(✉)

Contents

1 A-Kinase-Anchoring Proteins Are Platforms for Cellular Signal Integration 484
2 Elucidation of AKAP Functions 485
2.1 Disruption of AKAP Function by Gene Targeting. 486
2.2 Knockdown of AKAP Expression Using RNA_i Approaches. 487
2.3 Peptides as Disruptors of Protein–Protein Interactions 489
2.4 Peptides Interfering with Direct AKAP-Mediated Protein–Protein Interactions. . 489
2.5 Disruption of AKAP-PKA Interactions by Genetically Encoded Peptides 493
3 Direct AKAP-Mediated Protein–Protein Interactions in Compartmentalized cAMP Signalling as Potential Drug Targets 495
4 The Potential of Small Molecules for Pharmacological Interference with Direct AKAP-Mediated Protein–Protein Interaction 497
5 Summary and Conclusion 498
References 499

Abstract A-kinase-anchoring proteins (AKAPs) are a diverse family of about 50 scaffolding proteins. They are defined by the presence of a structurally conserved protein kinase A (PKA)-binding domain. AKAPs tether PKA and other signalling proteins such as further protein kinases, protein phosphatases and phosphodiesterases by direct protein–protein interactions to cellular compartments. Thus, AKAPs form the basis of signalling modules that integrate cellular signalling processes and limit these to defined sites. Disruption of AKAP functions by gene targeting, knockdown approaches and, in particular, pharmacological disruption of defined AKAP-dependent protein–protein interactions has revealed key roles of AKAPs in numerous processes, including the regulation of cardiac myocyte contractility and vasopressin-mediated water reabsorption in the kidney. Dysregulation

E. Klussmann
Leibniz-Institut für Molekulare Pharmakologie, Robert-Rössle-Str. 10, 13125 Berlin, Germany
Institut für Pharmakologie, Charité–Universitätsmedizin, Freie Universität Berlin, Berlin, Germany
klussmann@fmp-berlin.de

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.* Handbook of Experimental Pharmacology 186,
© Springer-Verlag Berlin Heidelberg 2008

of such processes causes diseases, including cardiovascular and renal disorders. In this review, we discuss AKAP functions elucidated by gene targeting and knock-down approaches, but mainly focus on studies utilizing peptides for disruption of direct AKAP-mediated protein–protein interactions. The latter studies point to direct AKAP-mediated protein–protein interactions as targets for novel drugs.

Abbreviations AKAP: A-kinase-anchoring protein; AVP: arginine-vasopressin; GEF: guanine nucleotide exchange factor; PKA: protein kinase A; SR: sarcoplasmic reticulum; PDE: phosphodiesterase; cAMP: cyclic adenosine monophosphate; PLN: phospholamban; RyR2: ryanodine receptors type 2; PP2B: protein phosphatase 2B/ calcineurin

1 A-Kinase-Anchoring Proteins Are Platforms for Cellular Signal Integration

The compartmentalization of cyclic adenosine monophosphate (cAMP)-dependent signalling is a prerequisite for numerous cellular processes, including cardiac myocyte contraction and exocytic processes, such as arginine-vasopressin (AVP)-mediated water reabsorption in renal collecting duct principal cells (Beavo and Brunton 2002; Houslay et al. 2005; Beene and Scott 2007; Szaszák et al. 2008).

Key players in compartmentalised cAMP signalling are A-kinase-anchoring proteins (AKAPs; see also Dodge-Kafka et al. this volume). They comprise a family of about 50 scaffolding proteins, which form the basis of cAMP-dependent signalling modules at defined cellular sites (Tasken and Aandahl 2004; Wong and Scott, 2004). AKAPs tether the main effector of cAMP, cAMP-dependent protein kinase (protein kinase A, PKA), to cellular compartments, and thereby limit its access to a subset of its substrates. Several AKAPs directly interact with PKA substrates. For example, AKAP18α, also termed AKAP15 or AKAP7α, directly interacts with L-type Ca^{2+} channels *via* a C-terminal leucine-zipper motif in cardiac myocytes and thereby facilitates β-adrenoceptor-dependent PKA phosphorylation of this channel. The phosphorylation increases channel open probability, enhances Ca^{2+} entry into the cytosol and thereby contributes to the β-adrenoceptor-mediated increase in cardiac myocyte contractility (Hulme et al. 2003).

The tethering of PKA through AKAPs by itself is not sufficient to compartmentalize and control a cAMP-dependent pathway since cAMP readily diffuses throughout the cell. Therefore, discrete cAMP signalling compartments are only conceivable if this diffusion is limited. Phosphodiesterases (PDE) establish gradients of cAMP by local hydrolysis of the second messenger and thereby terminate PKA activity locally (Lynch et al. 2006; Conti and Beavo 2007). Several AKAPs interact with PDEs and thus play a role at this level of control. For instance, AKAP450, mAKAP and AKAP18δ directly interact with PDE4D (Dodge et al. 2001; Tasken et al. 2001; Stefan et al. 2007). The AKAP18δ-PDE4D interaction, for example, appears to be

involved in the control of AVP-mediated water reabsorption in renal collecting duct principal cells (Stefan et al. 2007).

AKAPs also bind protein phosphatases dephosphorylating PKA substrates and thereby terminate PKA signalling. Phosphatase 2A (PP2A) is, for instance, bound by mAKAP and dephosphorylates Ca^{2+}-activated Ca^{2+} release channels (ryanodine receptors type 2, RyR_2) in cardiac myocytes. Dephosphorylation decreases channel open probability and contributes to lowering cytosolic Ca^{2+}, which in turn decreases cardiac myocyte contractility (Pare et al. 2005).

In addition to PKA, PDEs and protein phosphatases involved in cAMP signalling, AKAPs bind further signalling proteins, including other kinases, such as protein kinases C (PKC) and D (PKD), and other protein phosphatases, such as calcium/calmodulin-dependent phosphatase (calcineurin, protein phosphatase 2B, PP2B) (Carnegie et al. 2004; Oliveria et al. 2007). The activation of some of these signalling proteins depends on second messengers other than cAMP, e.g., Ca^{2+} activating calcineurin and PKC. Some AKAPs possess own catalytic activity, such as Rab32, which functions as an AKAP and possesses GTPase activity (Alto et al. 2002). A prime example of an AKAP integrating cellular signalling is AKAP-Lbc (Diviani et al. 2001). AKAP-Lbc catalyses the exchange of GDP for GTP on the small GTPase Rho through its Rho guanine nucleotide exchange factor (GEF) activity. The active, GTP-bound Rho induces the formation of F-actin-containing stress fibres. An increase of the cAMP level in response to direct activation of adenylyl cyclase with forskolin mediates activation of AKAP-Lbc-bound PKA. PKA in turn phosphorylates AKAP-Lbc. Subsequently, a protein of the 14-3-3 family binds to the phosphorylated site and inhibits the RhoGEF activity (Diviani et al. 2004). Conversely, lysophatidic acid, an agonist stimulating receptors coupled to the G protein G_{12}, increases the RhoGEF activity of AKAP-Lbc (Diviani et al. 2001). In addition, α_1-adrenoceptor-mediated activation of G_{12} with phenylephrine increases the RhoGEF activity of AKAP-Lbc in cardiac myocytes by elevating its expression. This causes cardiac myocyte hypertrophy (Appert-Collin et al. 2007).

2 Elucidation of AKAP Functions

Physiological functions of AKAPs have been elucidated by gene targeting in mice and knockdown experiments with RNA_i in various cultured cell types. Moreover, the disruption of direct AKAP-mediated protein–protein interactions with peptides in *in vitro* and cellular assays and animal models has revealed the involvement of AKAPs in a variety of cellular processes. In this chapter we will mainly focus on studies utilising peptides for disruption of direct AKAP-mediated protein–protein interactions. These studies point to AKAP-mediated protein–protein interactions as targets for novel drugs. Initially, we briefly discuss AKAP functions elucidated by gene targeting and gene silencing.

2.1 *Disruption of AKAP Function by Gene Targeting*

Although knockout and RNA_i strategies have revealed crucial functions of AKAPs in elementary processes whose dysregulation causes disease, it is not clear to which degree the loss of the AKAP function of a protein, i.e. its ability to interact with R subunits of PKA, contributes to the phenotypes because gene knockout deletes all functions of a protein.

Knockout of AKAP149 (also termed AKAP1) decreases fertility in female null mutant mice, but not in heterozygotes (Newhall et al. 2006). Oocytes from null mutant females are either degenerated or arrested in the germinal vesicle state. RIIα does not associate with mitochondria as in wild-type oocytes, suggesting that AKAP149-dependent localization of PKA at mitochondria is essential to drive oocyte maturation.

AKAP4, also termed fibrous sheath component 1, is sperm-specifically expressed and the major fibrous sheath protein of the principal piece of the sperm flagellum. AKAP4 knockout mice are infertile due to inhibition of sperm motility. Immotility is associated with the disruption of the fibrous sheath in spermatozoa and the redistribution of RIIα subunits of PKA from the particulate to the soluble fraction (Huang et al. 2005).

Disruption of D-AKAP2 increases the cardiac response to cholinergic stimuli. The mutant mice suffer from cardiac arrhythmias, and approximately 50% of homozygous and 25% of heterozygous mice die in the first year of life, pointing to a key role of D-AKAP2 in the control of heart rhythm (Tingley et al. 2007).

AKAP150 directly binds L-type Ca^{2+} channels in neurons (Hall et al. 2007; Oliveria et al. 2007). AKAP150 facilitates PKA phosphorylation of serine 1928 of the α_1 subunit of the channel upon β-adrenergic stimulation. This is abolished in the knockout mice, but does not cause detectable phenotypic changes of the animal. Consistently, knockin mice with a substitution of serine 1928 for alanine fail to upregulate channel activity in response to adrenergic stimuli (Gao et al. 1997).

WAVE-1, a member of the Wiskott-Aldrich syndrome protein (WASP) family, binds PKA and thus functions as an AKAP. Knockout of WAVE-1 reduces size of the mice and causes behavioural abnormalities, including deficits in sensorimotor function and cognition (Soderling et al. 2003). Approximately one third of the homozygous null progeny die within 24–48 h of birth. WAVE-1 knockout mice are characterised by a phenotype similar to the one observed in patients with a mental disorder, 3p syndrome. Analysis of WAVE-1 knockin mice lacking the interaction domain of WAVE-1 for mental disorder-associated GAP protein (MEGAP/WRP) display a similar phenotype as the knockout animals and the patients suffering from 3p syndrome. In the knockin mice the WAVE-1-dependent interaction of WRP with the small GTPase Rac1 does not occur, and thus WRP does not inactivate Rac1 (Soderling et al. 2002, 2007). The loss of the AKAP function in the knockout model may at least partially contribute to the phenotypic differences between the knockout and knockin models.

Microtubule-associated protein (MAP) 2 is a neuron-specific AKAP. Knockout mice lack gross abnormalities, which is most likely due to the compensation of the

loss by MAP1B (Teng et al. 2001). However, several subtle changes in hippocampal neurons are detectable. They include reduction in microtubule density in dendrites and of dendritic length, reduced amounts of catalytic, RIIα and RIIβ subunits of PKA in dendrites and reduced CREB phosphorylation in response to forskolin (Harada et al. 2002). A disease with a similar phenotype has not been identified.

Ezrin belongs to the ezrin-radixin-moesin (ERM) protein family. The ERM proteins connect the cytoskeleton with plasma membrane proteins (Bretscher et al. 2002). ERM proteins function as AKAPs binding RI and RII subunits of PKA with low affinity (e.g. K_D for binding of ezrin to RII ~30 μM) compared with other AKAPs (e.g. K_D for binding of RIIβ to AKAP18δ =20 nM) (Dransfield et al. 1997; Henn et al. 2004). Ezrin knockout causes growth retardation and high mortality. Only ~7% of the animals survive to adulthood. The cause of this early death is unclear. Postnatal day-1 mice show, for example, substantial retardation in the development of photoreceptors, reductions in the apical microvilli of retinal pigment epithelium (RPE) and of Müller cells, and reductions of basal infoldings in RPE cells (Bonilha et al. 2006). In addition, surviving mice suffer severe achlorhydria, caused by defects in the formation of canalicular apical membranes in gastric parietal cells (Tamura et al. 2005).

The protein encoded by myeloid translocation gene 8, MTG8, is an AKAP (Fukuyama et al. 2001b). The reciprocal translocation, t(8;21)(q22;q22), occurs almost always in leukaemic cells of patients with acute myeloid leukaemia (AML). The translocation disrupts the AML1 gene on chromosome 21 and the ETO (MTG8) gene on chromosome 8 (Miyoshi et al. 1991; Erickson et al. 1992; Nisson et al. 1992; Fukuyama et al. 2001a). MTG8 knockin mice heterozygous for an allele mimicking the human t(8;21) translocation die in mid-gestation from haemorrhage in the central nervous system and exhibit a severe block in foetal liver haematopoiesis (Yergeau et al. 1997).

Taken together, additional genetically modified animal models, where the PKA-binding domain is deleted in selected AKAPs, are required to understand the role of AKAP-PKA interactions in cellular function. Deletion of other interaction domains within AKAPs will elucidate the function of further direct AKAP-mediated protein–protein interactions.

2.2 Knockdown of AKAP Expression Using RNA$_i$ Approaches

AKAP350 targets PKA and a variety of interacting proteins to the Golgi apparatus in epithelial cells. Larocca et al. utilized RNA$_i$ directed against AKAP350, which reduced the expression level to 20% in HeLa cells and disperses the Golgi apparatus, indicating that the AKAP is important for the maintenance of the Golgi.

The human AKAP79 and its rat ortholog AKAP150 bind PKA, PKC and calcineurin. Knockdown of AKAP79 in HEK293-B2 cells, stably expressing the β_2-adrenoceptor, revealed that AKAP79-bound PKA phosphorylates the receptor and thereby induces switching of the receptor G protein coupling from G_s to G_i (Lynch et al. 2005).

The use of RNA_i also revealed that AKAP79/AKAP150 coordinate the regulation of AMPA receptors and M-type K^+ channels (Hoshi et al. 2005; Hoshi and Scott 2006). Endogenous AKAP79 in human-derived cells and AKAP150 in rat-derived cells were knocked down. Their expression was reconstituted with an ortholog version lacking a particular binding domain. For example, the lack of AKAP79 was rescued by an AKAP150 version lacking the PKA-binding domain. This work showed that in hippocampal neurons, AKAP150 positions PKA and calcineurin to modulate AMPA channels and maintains PKC inactive. In superior ganglial neurons, AKAP150 facilitates PKC phophorylation of M channels, while keeping PKA and calcineurin inactive. The difference in targeting is due to the interaction of AKAP150 with the scaffolding protein SAP97, which occurs in hippocampal neurons, but not in superior ganglial neurons.

The expression and thus RhoGEF activity of AKAP-Lbc and the expression of mAKAP are upregulated in cardiac myocyte hypertrophy. The AKAP-Lbc RhoGEF activity upregulation is due to constitutive α_1-adrenoceptor signalling (see above). Knockdown of either AKAP-Lbc or mAKAP prevents the hypertrophy (Pare et al. 2005; Appert-Collin et al. 2007).

AKAP18δ directly interacts with phospholamban (PLN) in cardiac myocytes. Knockdown of AKAP18δ decreases the velocity of Ca^{2+} reuptake from the cytosol into the sarcoplasmic reticulum (SR). Therefore, AKAP18δ participates in controlling the relaxation of the heart muscle (Lygren et al. 2007).

Willoughby et al. (2006) compared cAMP dynamics in microdomains beneath the plasma membrane with global cAMP dynamics in HEK293 cells. RNA_i directed against human gravin (also termed AKAP12 or AKAP250) reduces gravin expression by 80% and limits hydrolysis of subplasmalemmal cAMP, as gravin no longer tethers PDE4D to the plasma membrane. Rescue of gravin expression with the mouse ortholog Src-suppressed C kinase substrate (SSeCKS) of gravin reverses the effects of gravin knockdown (Willoughby et al. 2006).

Combined knockdown of MAP2 or tau in MAP1B-deficient mouse hippocampal neurons with antisense oligonucleotides arrests the cells in early stages of neuronal polarity development (compare MAP2 knockout mice, see Sect. 2.1; Gonzalez-Billault et al. 2002).

AKAP97 (radial spoke protein 3, RSP3) is a flagellar protein of *Trypanosoma brucei*. Its knockdown leads to immotility due to defective flagellar beat, loss of radial spokes and defects in cytokinesis (Ralston et al. 2006). In nasopharyngeal carcinoma 5–8 F cells, RNA_i directed against ezrin reduces the invasiveness of the cells (Peng et al. 2007).

Similar to the knockout approaches described above (Sect. 2.1), knockdown strategies have highlighted crucial roles of AKAPs in a variety of cellular functions that cause disease if they are dysregulated. However, like the knockout strategies, knockdown approaches do not define the roles of AKAP-PKA or other direct AKAP-mediated protein–protein interactions in a cellular process. In order to define the function of particular protein interactions, agents such as peptides or small molecules for their disruption are needed.

2.3 Peptides as Disruptors of Protein–Protein Interactions

Peptides mimicking binding domains disrupt protein–protein interactions with high selectivity by competitively binding to one of the interacting partners. Such peptides have been widely utilized to gain insight into the function of protein–protein interactions (Shin et al. 2005).

The generation of disruptor peptides requires mapping of the interacting domains. Recent studies took advantage of a combination of peptide spot technology (Kramer and Schneider-Mergener 1998; Frank 2002) and overlay assays with recombinant proteins (Kofler et al. 2005; Hundsrucker et al. 2006; Stefan et al. 2007; Sachs et al. 2007; Baillie et al. 2007). Here, overlapping peptides of up to 27 amino acids in length covering the entire sequence of a protein are spot-synthesized on cellulose membranes and overlaid with a potential interacting partner. This, together with analysis of 3D protein structures, reveals continuous and discontinuous interaction sites (Bolger et al. 2006). The influence of single amino acid contributions to the interaction is revealed by amino acid substitution arrays in which every amino acid is replaced with every amino acid found in native proteins (an example is shown in Fig. 1). This approach allows for optimisation of peptides, i.e., an increase in affinity and selectivity (Burns-Hamuro et al. 2003; Gold et al. 2006). Interactions detected by this technique need to be confirmed by other approaches, e.g., immunoprecipitation studies with truncated versions of the proteins.

Optimised peptides to be used for disruption of protein–protein interactions can be modified for visualisation, immobilisation or cell permeation by coupling to fluorescent dyes, affinity tags, cell penetrating tags or sequences (e.g., stearate, poly-arginine, penetratin, MAP- or Tat-peptide), respectively, (Vives, 2005; Zorko and Langel 2005; Lygren et al. 2007; Smith et al. 2007).

2.4 Peptides Interfering with Direct AKAP-Mediated Protein–Protein Interactions

Disruption of AKAP-PKA interactions with PKA-anchoring disruptor peptides has provided insight into the function of this interaction. Inactive PKA holoenzyme is a heterotetramer consisting of a homodimer of regulatory RI (RIα or RIβ) or RII (RIIα or RIIβ) subunits and two catalytic (Cα, Cβ or Cγ) subunits, each bound to one of the regulatory subunits (Kim et al. 2007; Wu et al. 2007). Depending on the presence of RI or RII subunits, PKA is designated type I or type II. AKAPs bind dimers of regulatory subunits, preferentially RII subunits, although some AKAPs also bind RI subunits. The PKA-binding domains of AKAPs (also termed RII-binding domains) are structurally conserved. They form amphipathic α-helices, which dock into a hydrophobic pocket formed by the R subunits dimers, the dimerisation and docking (DD) domain. The identity of amino acid sequences of RII-binding domains is <30%. The amphipathic helix

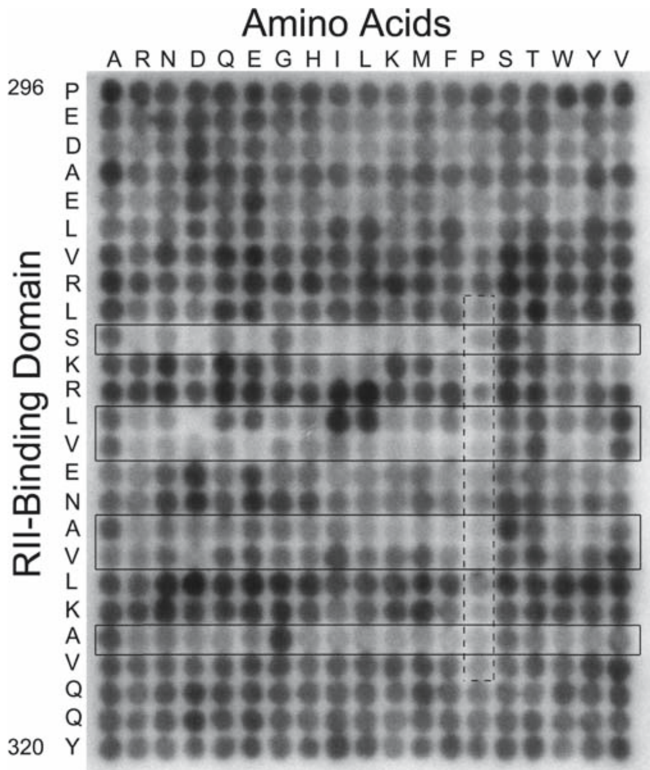

Fig. 1 Peptide substitution array of the RII-binding domain of AKAP18δ. Shown is a peptide array of spot-synthesized 25-amino-acid-long peptides, comprising the RII-binding domain of AKAP18δ (positions 296–320; vertical) in which every amino acid is replaced by the amino acids indicated at the top of the membrane. Peptides were spot-synthesised on cellulose membranes and probed for binding to ^{32}P-labelled RII subunits of PKA (RII-overlay assay). Binding of RII-subunits was detected by autoradiography. Amino acids indicated by *solid boxes* are conserved in RII-binding domains. Introduction of proline into the core RII-binding domain leads to disruption of the α-helical structure of the domain, which reduces or abolishes RII-binding (highlighted by the *dotted box*). Amino acids are indicated by *single letter code*

structure of RII-binding domains was initially predicted from a fragment of AKAP-Lbc, Ht31 (Carr et al. 1991) and confirmed by NMR studies for a peptide (Ht31) derived from the RII-binding domain of AKAP-Lbc (Table 1) and for a peptide derived from the RII-binding domain of AKAP79 (Newlon et al. 1999). Recent X-ray crystallography studies confirmed the amphipathic helix structures for $AKAP_{IS}$ (see below) and a peptide derived from the RII-binding domain of D-AKAP1 (Gold et al. 2006; Kinderman et al. 2006).

The dissociation constants for the interactions of the peptide Ht31 with RIIα and RI subunits are $K_D = 1.3 \pm 0.06$ μM and 2.2 ± 0.03 nM, respectively (Table 1) (Carr et al. 1992; Alto et al. 2003). Consistently, *in vitro* and cellular assays revealed that the peptide Ht31 functions as an effective disruptor of both AKAP-RI and AKAP-RII

Table 1 Peptides disrupting AKAP-dependent protein–protein interactions

Peptide	Sequence	K_d (nM, mean ± SEM)		Properties
		RI	RII	
Ht31	DLIEEAASRIVDAVIE-QVKAAGAY	1,300 ± 6.0	4.0 ± 1.2[a] 2.2 ± 0.03[b]	Global disruptor
Ht31-P	DLIEEAASRPVDAVPE-QVKAAGAY	n.b.	n.b.	Neg. control
AKB (dual)	VQGNTDEAQEELAWKIAKMIVSDVMQQ	48 ± 4	2.2 ± 0.2	Global disruptor[c]
AKB (RII)	VQGNTDEAQEELLWKIAKMIVSDVMQQ	2,493 ± 409	2.7 ± 0.1	
				RII-preferring[c]
AKB (RI)	FEELAWKIAKMIWSDVFQQ	5.2 ± 0.5	456 ± 33	RI-preferring[c]
AKB (null)	VQGNTDEAQEELAWKIEKMIWSDVMQQ	998 ± 66	>10,000	Neg. control[c]
$AKAP_{IS}$	QIEYLAKQIVDNAIQQA	0.23 ± 0.05	0.45 ± 0.07	Global disruptor[b]
AKAP18δ-wt-pep.	PEDAELVRLSKRLVENAVLKAVQQY	n.d.	0.4 ± 0.3	Global disruptor[d]
AKAP18δ-L314E	PEDAELVRLSKRLVENAVEKAVQQY	n.d.	0.7 ± 0.5	Global disruptor[d]
AKAP18δ-PP	PEDAELVRLSKRLPENAPLKAVQQY	n.d.	n.b.	Neg. control[d]
RIAD	LEQYANQLADQIIKEATE	1.0 ± 0.2	1,760 ± 290	RI-preferring[e]
Arg9-11-PLN	RRRRRRRRRRRASTIEMPQQ	n.a.	n.a.	AKAP18δ-PLN disruptor[f]
AKAP15-LZ	ENAVLKAVQQYLEETQN	n.a.	n.a.	AKAP18α-L type Ca^{2+} channel disruptor[g]

AKAP-derived PKA-anchoring disruptor peptides binding both regulatory RI and RII subunits of PKA globally inhibit PKA anchoring to cellular compartments. As indicated, a few peptides preferentially uncouple PKA RI subunits from AKAPs since their binding affinities for RI subunits are higher than for RII subunits. Examples of peptides disrupting other AKAP-dependent protein–protein interactions are also listed. In addition, several proline (P)-containing inactive control peptides are shown (e.g. Ht31-P for experiments with Ht31). K_D, equilibrium dissociation constants; n.b., no binding; n.d., not detected; n.a., not available

[a] Carr et al. (1991)
[b] Alto et al. (2003)
[c] Burns-Hamuro et al. (2003)
[d] Hundsrucker et al. (2006)
[e] Carlson et al. (2006)
[f] Lygren et al. (2007)
[g] Hulme et al. (2002)

interactions. Alto et al. (2003) utilized peptides derived from RII-binding domains of AKAP-KL, AKAP79, mAKAP, AKAP18α and AKAP-Lbc for amino acid substitution analysis at all positions within the peptides and a bioinformatics approach to develop a high-affinity peptide termed AKAP in silico ($AKAP_{IS}$). The dissociation constants for the binding of $AKAP_{IS}$ to regulatory RI and RII subunits of PKA are $K_D = 0.23 \pm 0.05$ nM and 0.45 ± 0.07 nM, respectively (Table 1). Consistent with this, $AKAP_{IS}$ disrupts AKAP-RI and AKAP-RII interactions. In HEK293 cells overexpressing glutamate GluR1 receptors, $AKAP_{IS}$ displaces PKA type II from the receptors and thereby evokes a rapid reduction of glutamate GluR1 receptor currents. Among the AKAPs binding RII subunits with high affinity is AKAP18δ (RIIα: K_D = 31 nM; RIIβ: K_D = 20 nM) (Henn et al. 2004). Truncated versions of AKAP18δ bind RII subunits with even higher affinity than the full-length protein (e.g. K_D of amino acids 124–353 for binding RIIβ = 4 nM; Henn et al. 2004). This observation led to the development of 25-amino-acid-long PKA-anchoring disruptor peptides derived from the RII-binding domain of AKAP18δ. The wild-type peptide AKAP18δ-wt binds RIIα subunits ($K_D = 0.4 \pm 0.3$ nM) with higher affinity than peptides derived from the RII-binding domains of other AKAPs and with a similar high affinity as $AKAP_{IS}$ (Table 1). Peptide substitution arrays and Biacore measurements revealed that several AKAP18δ-derived peptides bind RII subunits with a similar high affinity (Fig. 1). One of these is the peptide AKAP18δ-L314E, characterised by greater water solubility than AKAP18δ-wt. Therefore, it is widely applicable for the study of compartmentalized PKA signalling. The ability of AKAP18δ-derived peptides to displace RI subunits from AKAPs has not been investigated.

The dual specificity AKAPs D-AKAP1 and D-AKAP2 bind both RI and RII subunits of PKA. This observation initiated the development of peptides selectively disrupting AKAP-RI and AKAP-RII interactions. On the basis of the PKA-anchoring domain of D-AKAP2, Burns-Hamuro et al. (2003) generated the peptide AKB-RI (A-kinase-binding-RI. It binds RIα subunits with approximately 90-fold higher affinity than RIIα subunits ($K_D = 5.2 \pm 0.5$ nM versus 456 ± 33 nM) and thus preferentially disrupts AKAP–RI interactions. In addition, the peptide AKB-RII with opposite binding characteristics was developed (Burns-Hamuro et al. 2003). It binds RIα subunits with almost 1,000-fold lower affinity than RIIα subunits ($K_D = 2.4 \pm 0.4$ μM versus 2.7 ± 0.1 nM). Carlson et al. (2006) developed the peptide RIAD (RI-Anchoring Disruptor). It was optimised for RI binding based on the PKA-anchoring domains of the dual-specificity AKAPs D-AKAP1, AKAP149, AKAP82 and ezrin, which possess higher binding affinity for RIα than for RIIα subunits. The dissociation constants for the binding of RIAD to RIα and RIIα subunits of PKA are $K_D = 1.0 \pm 0.1$ nM and 1.76 ± 0.3 μM, respectively (Table 1). Compared with other peptides, RIAD is, therefore, the peptide with both the highest affinity and specificity for RIα subunits. Consequences of disruption of AKAP-RI interactions with RIAD in cultured T-cells are reduced phosphorylation and therefore inactivation of C-terminal Src kinase (Csk) by PKA type I. This in turn leads to a reduced phosphorylation of the lymphocyte-specific protein-thyosine kinase (Lck), which causes up-regulation of T cell receptor signalling. RIAD also reduced progesterone production in adrenocortical cells, which thus depends on the interaction of RI with AKAPs (Carlson et al. 2006).

Several PKA-anchoring disruptor peptides have been rendered cell-permeable (e.g. coupling Ht31 or AKAP18δ-derived peptides with stearate) and utilised to study functions of AKAP-PKA interactions in cultured cells (Table 2). Moita et al. (2002) carried out experiments with stearate-coupled Ht31 peptide in brains of rats. They infused the peptide into the lateral amygdala in order to examine the role of PKA anchoring in auditory fear memory. Behavioural tests revealed that anchoring of PKA is necessary for the consolidation, but not for the acquisition of conditioned fear.

A few peptides have been used to displace interaction partners other than PKA from AKAPs. For example, disruption of the interaction of AKAP18δ with PLN employing a peptide derived from the PLN interaction site for AKAP18δ reduces the velocity of Ca^{2+} reuptake into the SR of cardiac myocytes (Lygren et al. 2007). The effect of the peptides resembles the effect of RNA_i directed against AKAP18δ (see above). A peptide derived from the leucine zipper motif of AKAP18α that interferes with the interaction of AKAP18α with L-type Ca^{2+} channels prevents β-adrenoceptor-mediated increases of Ca^{2+} entry into cardiac myocytes (Hulme et al. 2003).

The properties of the peptides described here are summarized in Table 1. Functions of direct AKAP-mediated protein–protein interactions identified by the use of PKA-anchoring disruptor peptides and peptides disrupting interactions of AKAPs with other partners are listed in Table 2.

2.5 *Disruption of AKAP–PKA Interactions by Genetically Encoded Peptides*

In order to study the function of PKA anchoring in cell and animal models, disruptor peptides have been genetically encoded for expression. Generally, this approach overcomes limitations of peptides, such as their short half-life if applied to animals parenterally. In addition, this technique allows for introduction of peptides into cells and possibly animals that are not or are only limitedly accessible to chemical synthesis. Furthermore, encoding peptides genetically permits labelling with chromophores, such as green fluorescent protein (GFP), permitting tracking of the peptides in living cells. For example, a GFP-$AKAP_{IS}$ fusion protein transiently expressed in HEK293 cells displaces PKA from the Golgi and was utilized to study co-localisation with PKA RII subunits (Alto et al. 2003). Similarly, the peptide RIAD was fused to GFP. Analysis of HEK293 cells expressing the fusion protein revealed binding of RIAD to PKA regulatory RI subunits and confirmed the involvement of PKA type I in T cell receptor signalling and progesterone production (see above).

Bond and co-workers introduced Ht31 peptides by adenoviral transfer into primary neonatal cardiac myocytes and observed an increase in contractility (see Sect. 3; Fink et al. 2001).

In order to investigate the influence of AKAP-PKA interactions on hippocampal synaptic plasticity and spatial memory, transgenic mice conditionally expressing the peptide Ht31 in forebrain neurons were generated (Nie et al. 2007). Analysis of the transgenes shows that at hippocampal Schaffer collateral CA3–CA1 synapses,

Table 2 Examples of functional consequences of disruption of AKAP-dependent protein–protein interactions with disruptor peptides (see Table 1)

Peptide disruptor	Cell type	Protein complex comprising	Functional consequence
Ht31	Mouse oocytes	PKA, AKAP?, maturation promoting factor	Stimulation of oocyte maturation[a]
S-Ht31	Renal inner medullary collecting duct (IMCD) cells	AKAP18δ, AKAP?, PKA	Inhibition of aquaporin-2 redistribution[b]
AKAP18δ-wt	Rat neonatal cardiac myocytes	PKA, AKAP18α, L-type Ca^{2+} channel	Reduction of L-type Ca^{2+} channel currents[c]
$AKAP_{IS}$	HEK293 cells	PKA, AKAP79, AMPA channel (GluR1 receptor subunits)	Reduction of ectopically expressed GluR1 receptor currents[d]
RIAD-Arg_{11}	T cells	PKA Type I, Ezrin	Reduction of Lck phosphorylation (activation of cAMP inhibited T cell signalling)[e]
RIAD-Arg_{11}	Mouse Y1 adrenocortical cells	PKA Type I, PBR	Reduction of progesterone production[e]
superAKAP-IS	Hippocampal neurons	PKA, AKAP150, GluR1 receptor subunits of AMPA channel, PP2B	Reduction of AMPA-receptor currents[f]
AKAP15-LZ	Mouse skeletal muscle cells (MM14, DZ1A)	AKAP18α, PKA, L-type Ca^{2+} channel (Ca_V1.1)	Reduction of voltage-dependent potentiation of Ca^{2+} channels[g]
Arg9-11-PLN	Rat neonatal and adult cardiac myocytes	PKA, AKAP18δ, PLN, SERCA2	Reduction of Ca^{2+} re-uptake into the SR[h]

Listed are the peptides utilized (peptide disruptors), components comprising the macromolecular complexes targeted by the peptides and the cell types in which the peptides elicited the indicated functional consequences. The peptides Ht31, S-Ht31, AKAP18δ-wt, AKAP18δ-L304T, AKAP18δ-L314E, RIAD-Arg_{11} and SuperAKAP-IS disrupt AKAP-PKA interactions. The peptides AKAP15-LZ and Arg9-11-PLN disrupt AKAP18α-L type Ca^{2+} channel and AKAP18δ-PLN interactions, respectively. *?*, unidentified AKAP; *AMPA*, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; *PAP7*, PBR-associated protein; *PBR*, peripheral-type benzodiazepine receptor; *PLN*, phospholamban; *PP2B*, protein phosphatase 2B/calcineurin A1; *SERCA2*, sarcoplasmic reticulum Ca^{2+}-ATPase 2; *SR*, sarcoplasmic reticulum

[a] Newhall et al. (2006)

[b] Klussmann et al. (1999), Henn et al. (2004)

[c] Hundsrucker et al. (2006)

[d] Alto et al. (2003)

[e] Carlson et al. (2006)

[f] Gold et al. (2006)

[g] Hulme et al. (2002)

[h] Lygren et al. (2007)

long-term potentiation (LTP) requires presynaptically anchored PKA if the neurons are stimulated by a theta-burst and postsynaptically anchored PKA if multiple high-frequency trains are used for stimulation. In addition, the data show that a pool of anchored PKA in CA3 neurons is required for spatial memory storage.

A role of compartmentalized cAMP signalling in memory formation of Drosophila was revealed by utilizing a peptide (eCOPR2) mimicking the AKAP-binding domain in the N terminus of Drosophila RII subunits (Schwaerzel et al. 2007). The peptide blocks interactions of RII subunits with AKAPs. It was expressed ubiquitously throughout Drosophila or in defined parts of the Drosophila nervous system. With this system, AKAP-bound pools of PKA were detected in distinct regions of the nervous system, and AKAP-dependent functions during aversive memory formation were elucidated.

3 Direct AKAP-Mediated Protein–Protein Interactions in Compartmentalized cAMP Signalling as Potential Drug Targets

The various approaches to elucidate AKAP functions described above have revealed a variety of cellular processes involving compartmentalised signal processing. A prime example showing the dependence of a biological process on AKAP-controlled compartmentalized cAMP signalling and the potential of direct AKAP-mediated protein–protein interactions as drug targets is the β-adrenoceptor-mediated increase in cardiac myocyte contractility. Activation of β-adrenoceptors causes a raise of cAMP and activation of PKA in microdomains at T-tubules, regions where the plasma membrane and the membrane of the sacroplasmatic reticulum (SR) are in close proximity (Zaccolo and Pozzan 2002). PDE4D isoforms, located at T-tubules, modulate the amplitude and duration of the cAMP response (Mongillo et al. 2004). At the T-tubules, PKA phosphorylates L-type Ca^{2+} channels and RyR_2, leading to an increased Ca^{2+} concentration in the cytosol, and thereby to an increase in contractility. PKA also phosphorylates PLN, a small protein regulating sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), which pumps Ca^{2+} from the cytosol into the SR. PKA phosphorylation of PLN induces its dissociation from SERCA and thereby SERCA activation. Thus through PKA phosphorylation of PLN, the velocity of Ca^{2+} reuptake into the SR increases. A prerequisite for the phosphorylations is the tethering of PKA to the substrates by AKAPs. AKAP18α anchors PKA to L-type Ca^{2+} channels (Hulme et al. 2003), mAKAP tethers it to RyR_2 (Kapiloff et al. 1999) and AKAP18δ to PLN (Lygren et al. 2007). Moreover, AKAP79 is involved in β-adrenoceptor switching from the G protein G_s to G_i, and AKAP250 plays a role in receptor desensitization (Malbon et al. 2004a, b; Lynch et al. 2005). The AKAP yotiao tethers PKA to K^+ channels (KCNQ1). β-adrenoceptor activation induces phosphorylation of the channel by yotiao-bound PKA, which contributes to repolarisation of the cardiac myocyte plasmalemma and thus relaxation (Marx et al. 2002).

When cardiac myocytes are to be protected from stimulation by adrenergic agonists, such as for the treatment of hypertension or coronary heart disease, β-adrenoceptor antagonists (β-blockers) are administered for preventing increases of contractility (Bristow 1993; Ungerer et al. 1994; Towbin and Bowles 2002); β-blockers prevent β-adrenoceptor-mediated increases of L-type Ca^{2+} channel currents in cardiac myocytes. This effect is mimicked by non-selective disruption of AKAP-PKA interactions with Ht31 and AKAP18δ-derived PKA-anchoring disruptor peptides (Hulme et al. 2003; Hundsrucker et al. 2006) and selective disruption of the interaction of AKAP18α and L-type Ca^{2+} channels with AKAP18α-derived peptides (peptide AKAP15-LZ; Table 1). Selective disruption of the interaction of AKAP18δ and PLN with PLN-derived peptides (Arg9-11-PLN) slows Ca^{2+} reuptake into the SR (see above; Lygren et al. 2007). The data suggest that, similar to β-blockers, all of the disruptor peptides reduce cardiac myocyte contractility. Unexpectedly, however, Ht31 peptides increased contractility of cultured cardiac myocytes (Fink et al. 2001). This apparent discrepancy with the data discussed above may be explained by the different experimental readouts. While L-type Ca^{2+} channel current measurements reproduce local events at the plasma membrane, determination of contractility reproduces measurements of whole cell responses and shows the final outcome of a manipulation such as global uncoupling of PKA from AKAPs. Experiments on isolated hearts and eventually in animals need to validate cardiac AKAPs as drug targets.

Another process depending on compartmentalization of cAMP signalling is the AVP-induced water reabsorption in renal collecting duct principal cells. On the molecular level, AVP activates vasopressin V_2 receptors and, *via* elevation of cAMP, PKA. PKA phosphorylates aquaporin-2 (AQP2). In resting cells, AQP2 resides on intracellular vesicles distributed throughout the cytoplasm. The phosphorylation triggers the redistribution of AQP2 to the plasma membrane, increasing the osmotic water permeability of the cells. Defects in the AVP-induced AQP2 redistribution cause nephrogenic diabetes insipidus (NDI), a disease characterized by a chronic loss of water (Klussmann et al. 2000; King et al. 2004; Valenti et al. 2005). Paradoxically, the patients are treated with thiazide diuretics, inhibiting Na^+ reabsorption in the distal tubule of the nephron. Drugs acting specifically on the affected principal cells, which for example would promote the AQP2 redistribution to the plasma membrane and thereby enhance water reabsorption, are not available. However, recent work points to novel strategies for targeting the AQP2 redistribution. In primary cultured rat inner medullary collecting duct (IMCD) cells, a model system for AVP-mediated water reabsorption, a decrease of local cAMP hydrolysis at AQP2-bearing vesicles through inhibition of vesicular PDE4 by rolipram and concomitant elevation of cAMP through direct activation of adenylyl cyclase with forskolin increases AQP2 in the plasma membrane and enhances water reabsorption (Stefan et al. 2007). PDE4D is tethered to AQP2-bearing vesicles by direct interaction with AKAP18δ (Stefan et al. 2007). Displacement of PDE4D from AQP2-bearing vesicles by disruption of the PDE4D interaction with AKAP18δ and concomitant elevation of the cAMP level may have a similar effect. Such approaches may lead to a novel strategy for the treatment of patients suffering from X-linked NDI, where the V2 receptor is defective, but the AQP2 redistribution is functional.

Chronic heart failure is associated with elevated AVP levels and consequent upregulation of AQP2 protein expression and water retention. Thus the first line of therapeutics includes diuretics (Schrier and Cadnapaphornchai 2003; Chen and Schrier, 2006). Clinical trials with vaptans, vasopressin V_2 receptor blockers, indicate that blocking the AVP-induced water reabsorption effectively improves the quality of life of the patients. Long-term studies with these new drugs will have to confirm that they act with fewer side effects than diuretics. Uncoupling of PKA from AKAPs with the PKA-anchoring disruptor peptide Ht31 or AKAP18δ-derived peptides (or inhibition of PKA) prevents the AVP-induced AQP2 redistribution in IMCD cells (Klussmann et al. 1999; Szaszak et al. 2007). Thus, pharmacological interference with AKAP-PKA interactions preventing the AVP-induced redistribution of AQP2 appears to be another alternative to conventional treatment (Klussmann et al. 1999; Stefan et al. 2007). In particular, the interaction of AKAP18δ with PKA appears relevant as AKAP18δ is one of the AKAPs tethering PKA in close proximity to AQP2 on intracellular vesicles. It is most likely involved in facilitating PKA phosphorylation of AQP2 (Henn et al. 2004; Stefan et al. 2007).

4 The Potential of Small Molecules for Pharmacological Interference with Direct AKAP-Mediated Protein–Protein Interaction

Peptides need to be administered parenterally, posses a short half-life and may evoke immune responses. Thus their therapeutic applications are limited. Peptides nevertheless may serve as the starting point for the generation of peptoids and peptidomimetics that eventually may become effective agents overcoming the limits of peptides. Recently developed peptide-to-peptoid transformation procedures may pave the way to reach this goal (Toogood 2002; Yin and Hamilton 2005; Hoffmann et al. 2006). Here linear peptides are transformed into peptomers and finally into peptoid analogs. A substitution analysis in which each amino acid of the peptide is exchanged by a set of different peptoid building blocks results in a peptidomimetic array. This approach permits the transformation of peptide lead structures into non-peptidic compounds.

Small molecules can be orally applied and have longer half-lives than peptides. Several examples show that small molecule development for the disruption of protein–protein interactions is feasible (Arkin and Wells 2004). The small molecule wiskostatin, for example, targets an intramolecular interaction in a manner that stabilizes neural-Wiskott-Aldrich syndrome protein (N-WASP) in its native autoinhibited conformation and thereby prevents its activation by the small GTPase cdc42 (Deacon and Peterson, this volume) (Peterson et al. 2004). An example for a disease-relevant protein–protein interaction is the direct binding of p53 to human double minute 2 (HDM2). p53 is a transcription factor acting as a tumour suppressor. It is mutated or deleted in 50% of human cancers.

Its overexpression induces cell cycle arrest or apoptosis. In normal cells p53 expression is low because HDM2 (ubiquitin ligase) ubiquitinates it and thereby directs it to proteasomal degradation. Disruption of the p53-HDM2 interaction with a hexapeptide derived from the binding site for HDM2 in p53 or with several small molecules, such as nutilins (or inhibition of HDM2 activity), stabilizes p53. Nutilin-3 blocks anchorage-independent growth of mammary epithelial cells transformed by cyclin D1/cyclin-dependent kinase (CDK) overexpression (Yin and Hamilton 2005; Dudkina and Lindsley 2007; Kan et al. 2007). Thus, drugs developed on the basis of such disruptors potentially have anti-cancer activity. Another small molecule with potential anti-cancer activity is a molecule disrupting the interaction of the small GTPase Rac1 with the Rac1-specific GEFs Trio and Tiam1. In human prostate cancer PC-3 cells, the molecule inhibits the proliferation, anchorage-independent growth and invasion phenotypes that require endogenous Rac1 activity (Gao et al. 2004).

5 Summary and Conclusion

Knockout mouse models, knockdown approaches with RNA_i and peptide-based disruption of direct AKAP-mediated protein–protein interactions in both cell and animal models have revealed crucial roles of AKAPs in compartmentalized cellular signalling in a variety of disease-relevant processes. Presently, AKAPs are not targets for drugs. However, based on the data obtained with peptides disrupting direct AKAP-mediated protein–protein interactions, the development of agents targeting such interactions for therapeutic purposes appears feasible (e.g. cardiovascular and renal diseases). Candidate agents include improved, stabilized peptides, peptidomimetica and small molecules. Such agents, interfering with signal transduction processes in defined cellular compartments by modulating selectively direct AKAP-mediated protein–protein interactions, may have fewer side effects than conventional drugs. Displacing selected proteins from complexes assembled by scaffolding proteins is likely to cause subtle local changes in signal processing that do not affect whole cell functions. Such agents are also likely to act more specifically than RNA_i, which downregulates protein expression and thereby interferes with all interactions of a protein. The new approach may lead to alternative strategies for the treatment of diseases associated with altered cAMP signalling that are not addressed by conventional pharmacotherapy. For a rational search of agents specifically interfering with the function of a given AKAP by modulating its interactions, it is necessary to solve the 3D structures of all relevant AKAPs. On the basis of structural differences between interacting domains, selective AKAP modulators can be developed.

Acknowledgments This work was supported by grants from Deutsche Forschungsgemeinschaft (Kl 1415/2-2, 3-1 and 4-1), the European Union (project thera cAMP–proposal 037189) and the GoBio programme of the German Ministry of Education and Science (BMBF).

References

Alto NM, Soderling J, Scott JD (2002) Rab32 is an A-kinase anchoring protein and participates in mitochondrial dynamics. J Cell Biol 158:659–668

Alto NM, Soderling SH, Hoshi N, Langeberg LK, Fayos R, Jennings PA, Scott JD (2003) Bioinformatic design of A-kinase anchoring protein-in silico: a potent and selective peptide antagonist of type II protein kinase A anchoring. Proc Natl Acad Sci USA 100:4445–4450

Appert-Collin A, Cotecchia S, Nenniger-Tosato M, Pedrazzini T, Diviani D (2007) The A-kinase anchoring protein (AKAP)-Lbc-signaling complex mediates alpha1 adrenergic receptor-induced cardiomyocyte hypertrophy. Proc Natl Acad Sci USA 104:10140–10145

Arkin MR, Wells JA (2004) Small-molecule inhibitors of protein–protein interactions: progressing towards the dream. Nat Rev Drug Discov 3:301–317

Baillie GS, Adams DR, Bhari N, Houslay TM, Vadrevu S, Meng D, Li X, Dunlop A, Milligan G, Bolger GB, Klussmann E, Houslay MD (2007) Mapping binding sites for the PDE4D5 cAMP-specific phosphodiesterase to the N- and C-domains of beta-arrestin using spot-immobilized peptide arrays. Biochem J 404:71–80

Beavo JA, Brunton LL (2002) Cyclic nucleotide research – still expanding after half a century. Nat Rev Mol Cell Biol 3:710–718

Beene DL, Scott JD (2007) A-kinase anchoring proteins take shape. Curr Opin Cell Biol 19:192–198

Bolger GB, Baillie GS, Li X, Lynch MJ, Herzyk P, Mohamed A, Mitchell LH, McCahill A, Hundsrucker C, Klussmann E, Adams DR, Houslay MD (2006) Scanning peptide array analyses identify overlapping binding sites for the signalling scaffold proteins, beta-arrestin and RACK1, in cAMP-specific phosphodiesterase PDE4D5. Biochem J 398:23–36

Bonilha VL, Rayborn ME, Saotome I, McClatchey AI, Hollyfield JG (2006) Microvilli defects in retinas of ezrin knockout mice. Exp Eye Res 82:720–729

Bretscher A, Edwards K, Fehon RG (2002) ERM proteins and merlin: integrators at the cell cortex. Nat Rev Mol Cell Biol 3:586–599

Bristow MR (1993) Changes in myocardial and vascular receptors in heart failure. J Am Coll Cardiol 22:61A–71A

Burns-Hamuro LL, Ma Y, Kammerer S, Reineke U, Self C, Cook C, Olson GL, Cantor CR, Braun A, Taylor SS (2003) Designing isoform-specific peptide disruptors of protein kinase A localization. Proc Natl Acad Sci USA 100:4072–4077

Carlson CR, Lygren B, Berge T, Hoshi N, Wong W, Tasken K, Scott JD (2006) Delineation of type I protein kinase a selective signaling events using an ri anchoring disruptor (RIAD). J Biol Chem 281:21535–21545

Carnegie GK, Smith FD, McConnachie G, Langeberg LK, Scott JD (2004) AKAP-Lbc nucleates a protein kinase D activation scaffold. Mol Cell 15:889–899

Carr DW, Stofko-Hahn RE, Fraser ID, Bishop SM, Acott TS, Brennan RG, Scott JD (1991) Interaction of the regulatory subunit (RII) of cAMP-dependent protein kinase with RII-anchoring proteins occurs through an amphipathic helix binding motif. J Biol Chem 266:14188–14192

Carr DW, Hausken ZE, Fraser ID, Stofko-Hahn RE, Scott JD (1992) Association of the type II cAMP-dependent protein kinase with a human thyroid RII-anchoring protein. Cloning and characterization of the RII-binding domain. J Biol Chem 267:13376–13382

Chen HH, Schrier RW (2006) Pathophysiology of volume overload in acute heart failure syndromes. Am J Med 119:S11–S16

Conti M, Beavo J (2007) Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu Rev Biochem 76:481–511

Diviani D, Soderling J, Scott JD (2001) AKAP-Lbc anchors protein kinase A and nucleates Galpha 12-selective Rho-mediated stress fiber formation. J Biol Chem 276:44247–44257

Diviani D, Abuin L, Cotecchia S, Pansier L (2004) Anchoring of both PKA and 14-3-3 inhibits the Rho-GEF activity of the AKAP-Lbc signaling complex. EMBO J 23:2811–2820

Dodge KL, Khouangsathiene S, Kapiloff MS, Mouton R, Hill EV, Houslay MD, Langeberg LK, Scott JD (2001) mAKAP assembles a protein kinase A/PDE4 phosphodiesterase cAMP signaling module. EMBO J 20:1921–1930
Dransfield DT, Bradford AJ, Smith J, Martin M, Roy C, Mangeat PH, Goldenring JR (1997) Ezrin is a cyclic AMP-dependent protein kinase anchoring protein. EMBO J 16:35–43
Dudkina AS, Lindsley CW (2007) Small molecule protein–protein inhibitors for the p53-MDM2 interaction. Curr Top Med Chem 7:952–960
Erickson P, Gao J, Chang KS, Look T, Whisenant E, Raimondi S, Lasher R, Trujillo J, Rowley J, Drabkin H (1992) Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML1/ETO, with similarity to Drosophila segmentation gene, runt. Blood 80:1825–1831
Fink MA, Zakhary DR, Mackey JA, Desnoyer RW, Apperson-Hansen C, Damron DS, Bond M (2001) AKAP-mediated targeting of protein kinase a regulates contractility in cardiac myocytes. Circ Res 88:291–297
Frank R (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports – principles and applications. J Immunol Methods 267:13–26
Fukuyama T, Sueoka E, Sugio Y, Otsuka T, Niho Y, Akagi K, Kozu T (2001a) MTG8 proto-oncoprotein interacts with the regulatory subunit of type II cyclic AMP-dependent protein kinase in lymphocytes. Oncogene 20:6225–6232
Fukuyama T, Sueoka E, Sugio Y, Otsuka T, Niho Y, Akagi K, Kozu T (2001b) MTG8 proto-oncoprotein interacts with the regulatory subunit of type II cyclic AMP-dependent protein kinase in lymphocytes. Oncogene 20:6225–6232
Gao T, Yatani A, Dell'Acqua ML, Sako H, Green SA, Dascal N, Scott JD, Hosey MM (1997) cAMP-dependent regulation of cardiac L-type Ca2+ channels requires membrane targeting of PKA and phosphorylation of channel subunits. Neuron 19:185–196
Gao Y, Dickerson JB, Guo F, Zheng J, Zheng Y (2004) Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. Proc Natl Acad Sci USA 101:7618–7623
Gold MG, Lygren B, Dokurno P, Hoshi N, McConnachie G, Tasken K, Carlson CR, Scott JD, Barford D (2006) Molecular basis of AKAP specificity for PKA regulatory subunits. Mol Cell 24:383–395
Gonzalez-Billault C, Engelke M, Jimenez-Mateos EM, Wandosell F, Caceres A, Avila J (2002) Participation of structural microtubule-associated proteins (MAPs) in the development of neuronal polarity. J Neurosci Res 67:713–719
Hall DD, Davare MA, Shi M, Allen ML, Weisenhaus M, McKnight GS, Hell JW (2007) Critical role of cAMP-dependent protein kinase anchoring to the L-type calcium channel Ca(v)1.2 via A-kinase anchor protein 150 in neurons. Biochemistry 46:1635–1646
Harada A, Teng J, Takei Y, Oguchi K, Hirokawa N (2002) MAP2 is required for dendrite elongation, PKA anchoring in dendrites, and proper PKA signal transduction. J Cell Biol 158:541–549
Henn V, Edemir B, Stefan E, Wiesner B, Lorenz D, Theilig F, Schmitt R, Vossebein L, Tamma G, Beyermann M, Krause E, Herberg FW, Valenti G, Bachmann S, Rosenthal W, Klussmann E (2004) Identification of a novel A-kinase anchoring protein 18 isoform and evidence for its role in the vasopressin-induced aquaporin-2 shuttle in renal principal cells. J Biol Chem 279:26654–26665
Hoffmann B, Ast T, Polakowski T, Reineke U, Volkmer R (2006) Transformation of a biologically active peptide into peptoid analogs while retaining biological activity. Protein Pept Lett 13:829–833
Hoshi N, Scott JD (2006) Contextual utilization of enzymes in discrete AKAP79/150 signalling complexes. Eur J Cell Biol 85:621–622
Hoshi N, Langeberg LK, Scott JD (2005) Distinct enzyme combinations in AKAP signalling complexes permit functional diversity. Nat Cell Biol 7:1066–1073
Houslay MD, Schafer P, Zhang KY (2005) Keynote review: Phosphodiesterase-4 as a therapeutic target. Drug Discov Today 10:1503–1519
Huang Z, Somanath PR, Chakrabarti R, Eddy EM, Vijayaraghavan S (2005) Changes in intracellular distribution and activity of protein phosphatase PP1gamma2 and its regulating proteins in spermatozoa lacking AKAP4. Biol Reprod 72:384–392

Hulme JT, Ahn M, Hauschka SD, Scheuer T, Catterall WA (2002) A novel leucine zipper targets AKAP15 and cyclic AMP-dependent protein kinase to the C terminus of the skeletal muscle Ca2+ channel and modulates its function. J Biol Chem 277:4079–4087
Hulme JT, Lin TW, Westenbroek RE, Scheuer T, Catterall WA (2003) Beta-adrenergic regulation requires direct anchoring of PKA to cardiac CaV1.2 channels via a leucine zipper interaction with A kinase-anchoring protein 15. Proc Natl Acad Sci USA 100:13093–13098
Hundsrucker C, Krause G, Beyermann M, Prinz A, Zimmermann B, Diekmann O, Lorenz D, Stefan E, Nedvetsky P, Dathe M, Christian F, McSorley T, Krause E, McConnachie G, Herberg FW, Scott JD, Rosenthal W, Klussmann E (2006) High-affinity AKAP7delta-protein kinase A interaction yields novel protein kinase A-anchoring disruptor peptides. Biochem J 396:297–306
Kan CE, Patton JT, Stark GR, Jackson MW (2007) p53-mediated growth suppression in response to nutlin-3 in cyclin D1 transformed cells occurs independently of p21. Cancer Res 67:9862–9868
Kapiloff MS, Schillace RV, Westphal AM, Scott JD (1999) mAKAP: an A-kinase anchoring protein targeted to the nuclear membrane of differentiated myocytes. J Cell Sci 112(Pt 16): 2725–2736
Kim C, Cheng CY, Saldanha SA, Taylor SS (2007) PKA-I holoenzyme structure reveals a mechanism for cAMP-dependent activation. Cell 130:1032–1043
Kinderman FS, Kim C, von Daake S, Ma Y, Pham BQ, Spraggon G, Xuong NH, Jennings PA, Taylor SS (2006) A dynamic mechanism for AKAP binding to RII isoforms of cAMP-dependent protein kinase. Mol Cell 24:397–408
King LS, Kozono D, Agre P (2004) From structure to disease: the evolving tale of aquaporin biology. Nat Rev Mol Cell Biol 5:687–698
Klussmann E, Maric K, Wiesner B, Beyermann M, Rosenthal W (1999) Protein kinase A anchoring proteins are required for vasopressin-mediated translocation of aquaporin-2 into cell membranes of renal principal cells. J Biol Chem 274:4934–4938
Klussmann E, Maric K, Rosenthal W (2000) The mechanisms of aquaporin control in the renal collecting duct. Rev Physiol Biochem Pharmacol 141:33–95
Kofler M, Motzny K, Freund C (2005) GYF domain proteomics reveals interaction sites in known and novel target proteins. Mol Cell Proteomics 4:1797–1811
Kramer A, Schneider-Mergener J (1998) Synthesis and screening of peptide libraries on continuous cellulose membrane supports. Methods Mol Biol 87:25–39
Lygren B, Carlson CR, Santamaria K, Lissandron V, McSorley T, Litzenberg J, Lorenz D, Wiesner B, Rosenthal W, Zaccolo M, Tasken K, Klussmann E (2007) AKAP complex regulates Ca(2+) re-uptake into heart sarcoplasmic reticulum. EMBO Rep 8:1061–1067
Lynch MJ, Baillie GS, Mohamed A, Li X, Maisonneuve C, Klussmann E, van Heeke G, Houslay MD (2005) RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with beta arrestin to control the protein kinase A/AKAP79-mediated switching of the beta2-adrenergic receptor to activation of ERK in HEK293B2 cells. J Biol Chem 280:33178–33189
Lynch MJ, Hill EV, Houslay MD (2006) Intracellular targeting of phosphodiesterase-4 underpins compartmentalized cAMP signaling. Curr Top Dev Biol 75:225–259
Malbon CC, Tao J, Shumay E, Wang HY (2004a) AKAP (A-kinase anchoring protein) domains: beads of structure-function on the necklace of G-protein signalling. Biochem Soc Trans 32:861–864
Malbon CC, Tao J, Wang HY (2004b) AKAPs (A-kinase anchoring proteins) and molecules that compose their G-protein-coupled receptor signalling complexes. Biochem J 379:1–9
Marx SO, Kurokawa J, Reiken S, Motoike H, D'Armiento J, Marks AR, Kass RS (2002) Requirement of a macromolecular signaling complex for beta adrenergic receptor modulation of the KCNQ1-KCNE1 potassium channel. Science 295:496–499
Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y, Ohki M (1991) t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc Natl Acad Sci USA 88:10431–10434

Moita MA, Lamprecht R, Nader K, LeDoux JE (2002) A-kinase anchoring proteins in amygdala are involved in auditory fear memory. Nat Neurosci 5:837–838
Mongillo M, McSorley T, Evellin S, Sood A, Lissandron V, Terrin A, Huston E, Hannawacker A, Lohse MJ, Pozzan T, Houslay MD, Zaccolo M (2004) Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. Circ Res 95:67–75
Newhall KJ, Criniti AR, Cheah CS, Smith KC, Kafer KE, Burkart AD, McKnight GS (2006) Dynamic anchoring of PKA is essential during oocyte maturation. Curr Biol 16:321–327
Newlon MG, Roy M, Morikis D, Hausken ZE, Coghlan V, Scott JD, Jennings PA (1999) The molecular basis for protein kinase A anchoring revealed by solution NMR. Nat Struct Biol 6:222–227
Nie T, McDonough CB, Huang T, Nguyen PV, Abel T (2007) Genetic disruption of protein kinase A anchoring reveals a role for compartmentalized kinase signaling in theta-burst long-term potentiation and spatial memory. J Neurosci 27:10278–10288
Nisson PE, Watkins PC, Sacchi N (1992) Transcriptionally active chimeric gene derived from the fusion of the AML1 gene and a novel gene on chromosome 8 in t(8;21) leukemic cells. Cancer Genet Cytogenet 63:81–88
Oliveria SF, Dell'Acqua ML, Sather WA (2007) AKAP79/150 anchoring of calcineurin controls neuronal L-type Ca2+ channel activity and nuclear signaling. Neuron 55:261–275
Pare GC, Bauman AL, McHenry M, Michel JJ, Dodge-Kafka KL, Kapiloff MS (2005) The mAKAP complex participates in the induction of cardiac myocyte hypertrophy by adrenergic receptor signaling. J Cell Sci 118:5637–5646
Peng S, Fan S, Li X, Wang L, Liu H, Zhou M, Wang L, Shen S, Li G (2007) The expression of ezrin in NPC and its interaction with NGX6, a novel candidate suppressor. Cancer Sci 98:341–349
Peterson JR, Bickford LC, Morgan D, Kim AS, Ouerfelli O, Kirschner MW, Rosen MK (2004) Chemical inhibition of N-WASP by stabilization of a native autoinhibited conformation. Nat Struct Mol Biol 11:747–755
Ralston KS, Lerner AG, Diener DR, Hill KL (2006) Flagellar motility contributes to cytokinesis in Trypanosoma brucei and is modulated by an evolutionarily conserved dynein regulatory system. Eukaryot Cell 5:696–711
Sachs BD, Baillie GS, McCall JR, Passino MA, Schachtrup C, Wallace DA, Dunlop AJ, MacKenzie KF, Klussmann E, Lynch MJ, Sikorski SL, Nuriel T, Tsigelny I, Zhang J, Houslay MD, Chao MV, Akassoglou K (2007) p75 neurotrophin receptor regulates tissue fibrosis through inhibition of plasminogen activation via a PDE4/cAMP/PKA pathway. J Cell Biol 177:1119–1132
Schrier RW, Cadnapaphornchai MA (2003) Renal aquaporin water channels: from molecules to human disease. Prog Biophys Mol Biol 81:117–131
Schwaerzel M, Jaeckel A, Mueller U (2007) Signaling at A-kinase anchoring proteins organizes anesthesia-sensitive memory in Drosophila. J Neurosci 27:1229–1233
Shin DS, Kim DH, Chung WJ, Lee YS (2005) Combinatorial solid phase peptide synthesis and bioassays. J Biochem Mol Biol 38:517–525
Smith KJ, Baillie GS, Hyde EI, Li X, Houslay TM, McCahill A, Dunlop AJ, Bolger GB, Klussmann E, Adams DR, Houslay MD (2007) (1)H NMR structural and functional characterisation of a cAMP-specific phosphodiesterase-4D5 (PDE4D5) N-terminal region peptide that disrupts PDE4D5 interaction with the signalling scaffold proteins, betaarrestin and RACK1. Cell Signal 19:2612–2624
Soderling SH, Binns KL, Wayman GA, Davee SM, Ong SH, Pawson T, Scott JD (2002) The WRP component of the WAVE-1 complex attenuates Rac-mediated signalling. Nat Cell Biol 4:970–975
Soderling SH, Langeberg LK, Soderling JA, Davee SM, Simerly R, Raber J, Scott JD (2003) Loss of WAVE-1 causes sensorimotor retardation and reduced learning and memory in mice. Proc Natl Acad Sci USA 100:1723–1728
Soderling SH, Guire ES, Kaech S, White J, Zhang F, Schutz K, Langeberg LK, Banker G, Raber J, Scott JD (2007) A WAVE-1 and WRP signaling complex regulates spine density, synaptic plasticity, and memory. J Neurosci 27:355–365

Stefan E, Wiesner B, Baillie GS, Mollajew R, Henn V, Lorenz D, Furkert J, Santamaria K, Nedvetsky P, Hundsrucker C, Beyermann M, Krause E, Pohl P, Gall I, MacIntyre AN, Bachmann S, Houslay MD, Rosenthal W, Klussmann E (2007) Compartmentalization of cAMP-dependent signaling by phosphodiesterase-4D is involved in the regulation of vasopressin-mediated water reabsorption in renal principal cells. J Am Soc Nephrol 18:199–212

Szaszák M, Christian F, Rosenthal W, Klussmann E (2008) Compartmentalized cAMP signalling in regulated exocytic processes in non-neuronal cells. Cell Signal 20:590–601

Tamura A, Kikuchi S, Hata M, Katsuno T, Matsui T, Hayashi H, Suzuki Y, Noda T, Tsukita S, Tsukita S (2005) Achlorhydria by ezrin knockdown: defects in the formation/expansion of apical canaliculi in gastric parietal cells. J Cell Biol 169:21–28

Tasken K, Aandahl EM (2004) Localized effects of cAMP mediated by distinct routes of protein kinase A. Physiol Rev 84:137–167

Tasken KA, Collas P, Kemmner WA, Witczak O, Conti M, Tasken K (2001) Phosphodiesterase 4D and protein kinase a type II constitute a signaling unit in the centrosomal area. J Biol Chem 276:21999–22002

Teng J, Takei Y, Harada A, Nakata T, Chen J, Hirokawa N (2001) Synergistic effects of MAP2 and MAP1B knockout in neuronal migration, dendritic outgrowth, and microtubule organization. J Cell Biol 155:65–76

Tingley WG, Pawlikowska L, Zaroff JG, Kim T, Nguyen T, Young SG, Vranizan K, Kwok PY, Whooley MA, Conklin BR (2007) Gene-trapped mouse embryonic stem cell-derived cardiac myocytes and human genetics implicate AKAP10 in heart rhythm regulation. Proc Natl Acad Sci USA 104:8461–8466

Toogood PL (2002) Inhibition of protein–protein association by small molecules: approaches and progress. J Med Chem 45:1543–1558

Towbin JA, Bowles NE (2002) The failing heart. Nature 415:227–233

Ungerer M, Parruti G, Bohm M, Puzicha M, DeBlasi A, Erdmann E, Lohse MJ (1994) Expression of beta-arrestins and beta-adrenergic receptor kinases in the failing human heart. Circ Res 74:206–213

Valenti G, Procino G, Tamma G, Carmosino M, Svelto M (2005) Minireview: aquaporin 2 trafficking. Endocrinology 146:5063–5070

Vives E (2005) Present and future of cell-penetrating peptide mediated delivery systems: "Is the Trojan horse too wild to go only to Troy?" J Control Release 109:77–85

Willoughby D, Wong W, Schaack J, Scott JD, Cooper DM (2006) An anchored PKA and PDE4 complex regulates subplasmalemmal cAMP dynamics. EMBO J 25:2051–2061

Wong W, Scott JD (2004) AKAP signalling complexes: focal points in space and time. Nat Rev Mol Cell Biol 5:959–970

Wu J, Brown SH, von Daake S, Taylor SS (2007) PKA type IIalpha holoenzyme reveals a combinatorial strategy for isoform diversity. Science 318:274–279

Yergeau DA, Hetherington CJ, Wang Q, Zhang P, Sharpe AH, Binder M, Marin-Padilla M, Tenen DG, Speck NA, Zhang DE (1997) Embryonic lethality and impairment of haematopoiesis in mice heterozygous for an AML1-ETO fusion gene. Nat Genet 15:303–306

Yin H, Hamilton AD (2005) Strategies for targeting protein–protein interactions with synthetic agents. Angew Chem Int Ed Engl 44:4130–4163

Zaccolo M, Pozzan T (2002) Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. Science 295:1711–1715

Zorko M, Langel U (2005) Cell-penetrating peptides: mechanism and kinetics of cargo delivery. Adv Drug Deliv Rev 57:529–545

Domains Mediate Protein–Protein Interactions and Nucleate Protein Assemblies

S. Costa and G. Cesareni(✉)

Contents

1 Networks of Protein–Protein Interactions 384
2 Proteins Can Be Decomposed in Functional Modules 385
3 Domain Identification Methods 387
4 Domains Can Be Organized Hierarchically in Families 388
5 Domain Interactions Mediate the Formation of Macromolecular Complexes. 390
6 Interaction Modules and Their Importance in Signaling Pathways. 391
7 Methods to Detect the Specificity of Interaction Modules 395
8 Methods to Infer Domain–Domain Interactions 398
9 Domain Interactions as Potential Drug Targets 400
10 Conclusion 402
References 402

Abstract Cell physiology is governed by an intricate mesh of physical and functional links among proteins, nucleic acids and other metabolites. The recent information flood coming from large-scale genomic and proteomic approaches allows us to foresee the possibility of compiling an exhaustive list of the molecules present within a cell, enriched with quantitative information on concentration and cellular localization. Moreover, several high-throughput experimental and computational techniques have been devised to map all the protein interactions occurring in a living cell. So far, such maps have been drawn as graphs where nodes represent proteins and edges represent interactions. However, this representation does not take into account the intrinsically modular nature of proteins and thus fails in providing an effective description of the determinants of binding. Since proteins are composed of domains that often confer on proteins their binding capabilities, a more informative description of the interaction network would detail, for each pair of interacting proteins in the network, which domains mediate the binding. Understanding how protein domains combine to mediate protein interactions would allow one to add important features to the protein interaction network, making it possible to discriminate between simultaneously occurring and mutually exclusive

G. Cesareni
University of Rome Tor Vergata, Via della Ricerca Scientifica, Rome, Italy
cesareni@uniroma2.it

E. Klussmann, J. Scott (eds.) *Protein–Protein Interactions as New Drug Targets.*
Handbook of Experimental Pharmacology 186,

© Springer-Verlag Berlin Heidelberg 2008

interactions. This objective can be achieved by experimentally characterizing domain recognition specificity or by analyzing the frequency of co-occurring domains in proteins that do interact. Such approaches allow gaining insights on the topology of complexes with unknown three-dimensional structure, thus opening the prospect of adopting a more rational strategy in developing drugs designed to selectively target specific protein interactions.

1 Networks of Protein–Protein Interactions

The completion of the sequencing of the genome of numerous organisms has helped realizing that the observed increase in genome complexity is inadequate to satisfactorily explain the greater morphological, physiological and behavioral complexity of higher organisms. An increase in the intricacy of the network that controls the expression of gene products and their interactions has been invoked as a further level of complexity that might explain the differences observed at the organismal level.

Thus, compiling an exhaustive list of the genes and the proteins they encode is merely the first step towards a full understanding of cell functioning. In the great majority of biological processes, from signal transduction to immune response, from uptake and metabolism of nutrients to cell movement, from cytoskeleton organization to regulation of gene transcription and translation, proteins and protein complexes play a key role. In carrying out their functions, proteins form either stable or transient interactions, assembling macromolecular complexes that are often essential to fulfill their biological role. Mapping all the interactions among proteins in a cell would represent a fundamental starting point to explore the mechanisms regulating living systems behavior. The network comprising all possible physical protein–protein interactions naturally occurring within a cell is called an “interactome.” The interactome constitutes a scaffold to which other genomic scale data may be attached to further elucidate cell physiology. The interactome plays a major role in stimulating the growth of a new branch of biology, called systems biology. Systems biology aims at gaining insights into the dynamics governing the behavior of complex biological systems, viewed as the result of the coordinated action of many elementary components connected to each other, eventually building an abstract model of the system capable of predicting the system’s response to any variation of input: internal parameters and environmental conditions. The availability of a map of all physical connections among proteins in the cell is mandatory to develop useful, biologically relevant mathematical models of cellular processes. However, the information provided by standard experimental techniques for detecting protein–protein interactions may not be enough for the level of detail required by modeling. For instance, interaction data often contain no indication of the post-translational modifications (PTM) that protein partners may have undergone, and it is not clear whether such modifications favor or hinder the interaction. Moreover, quantitative information on

interaction affinity and kinetic parameters, crucial for describing the dynamic of assembly and disassembly processes, is only very rarely available. Finally, while the genome is essentially static and does not change during the life of an individual, the proteome and, consequently, the interactome are far more dynamic, varying their composition according to the cell cycle and environmental conditions. In other words, interaction detection techniques can only take a snapshot of the possibly occurring interactions in a particular moment of the life of a specific type of cell: by no means do they provide an exhaustive description of the temporal and spatial evolution of the interactome. Another severe limitation of current high-throughput approaches is that they are generally unsuited to provide information on the protein regions involved in binding. Elucidating the molecular details of how proteins interact would allow us to pursue ambitious goals, such as modeling competitive binding or designing in a rational way synthetic molecules targeted at inhibiting specific interactions.

Although atomic level details on protein interactions will only come from crystallographic studies of the 3D structures of multi-protein complexes (Aloy and Russell 2006), valuable insights into the mechanisms underlying the formation and functioning of protein networks can be achieved by considering the intrinsic modular nature of proteins. It is the domain composition of a protein that determines both the functions it can perform and its binding ability to form complexes with partner proteins. We will advocate in this review a paradigm shift from a protein-centric to a domain-centric view of protein interaction networks. This would represent a finer resolution description of the dynamic protein mesh in a cell, and it would favor a deeper understanding of the logic underneath the wiring of protein networks.

2 Proteins Can Be Decomposed in Functional Modules

Although protein interaction graphs represent proteins as nodes, mathematical abstractions devoid of any structural detail, viewing a protein as a monolithic unit designed to perform a single, specific biological function is a too simplistic description of reality. In fact, many proteins can be thought of as the assembly of smaller building blocks, termed "domains", and therefore show an intrinsic modular nature. Figure 1 shows an example of a protein composed of three clearly distinguishable domains. Although no universally agreed definition of domain exists, protein domains are usually identified and classified according to structural, functional and evolutionary criteria. A domain can thus be described as a compact, autonomously folding structural unit that is conserved across evolution and is capable of performing a function, independently of the context of the protein it belongs to. Proteins can be composed of one (single-domain proteins) or more (multi-domain proteins) domains. Genome-wide structural assignments of domains have shown that domain composition complexity increases in proteins of higher order organisms: about two thirds of prokaryotic proteins are multi-domain, compared to 80% of eukaryotic

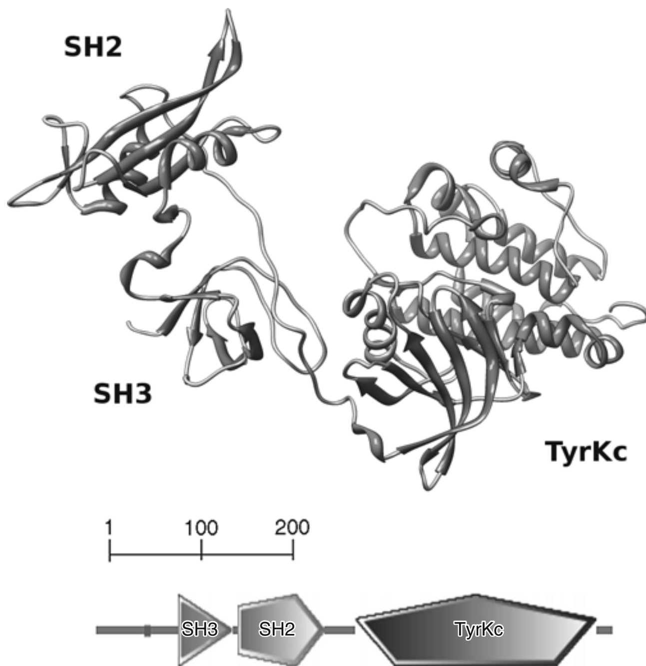

Fig. 1 Structure of the human Src kinase (UniProt AC: P12931). It is easy to notice that the protein is composed of three structurally distinct and loosely associated domains: SH3, SH2 and tyrosine kinase. In the lower part of the picture, a schematic representation of the protein highlighting its domain composition is depicted

proteins (Liu and Rost 2004). The variety we observe in the proteomes of living organisms was likely generated from a limited set of ancestral domains with different functions. Then, the evolution of protein repertoires may have been driven primarily by three forces: (1) duplication of gene sequences coding for one or more domains; (2) sequence differentiation by mutation and genetic drift, eventually resulting in the acquisition of new functions; (3) gene recombination, which promotes rearrangements in the sequential order of domains (Chothia et al. 2003, Bornberg-Bauer 2005). This interpretation leaves us with a question: how did novel polypeptide domains arise in the beginning? Recently, the observation of species-specific exons in the genomes of closely related species has suggested that the "exonization" of species-specific intron sequences may play an important role in the genesis of domains. Furthermore, insertions and deletions occurring in already existing protein-coding sequences or modifications to the length of sequence repeats may contribute to the process of acquiring new structural features of increased complexity in proteins, ultimately resulting in the creation of new polypeptide domains (Schmidt and Davies 2007).

3 Domain Identification Methods

A domain maintains the overall structural characteristics of the whole protein (compactness, hydrophobic core, autonomous folding) and, in most cases, performs its function even after being removed from the context of the protein. Decomposing proteins into simpler, modular building blocks helps realizing that proteins are the result of the combinatorial rearrangement of domains, and it shifts the focus of structural and functional analysis from proteins to domains. Therefore, the non-ambiguous identification of biologically relevant domains is a key issue for a proteomics strategy fully aware of the intrinsic modular nature of proteins.

When the three-dimensional structure of a protein is available, domains can be moderately easily spotted by eye. Manual inspection, however, is affected by two major drawbacks: (1) experts do not always fully agree on domain definitions, even after careful cross-inspection, and domain boundaries may remain concealed with the mist of subjectivity; (2) human processing of structures has become quickly unpractical, as the amount of data from crystallographic studies has been growing at a considerable pace for years. However, automatic methods produce uniform and reproducible definitions and can deal with the increasing volume of available data without much difficulty. Some objective criteria are required to be able to split proteins into substructures in an automated fashion. The most widely used criterion is compactness: from the definition of domains as small, compact structural units, it follows that residue-residue contacts within a domain should be more numerous than the contacts with the rest of the protein. Since compactness is not enough for reliable domain assignment, other criteria are evaluated, such as integrity of secondary structures, domain size and domain fragmentation.

As is common when we consider trusting computational and automatic approaches, doubts arise about the performance and accuracy of such methods. A thorough comparison of four domain assignment algorithms was performed by Veretnik et al. (2004) and Holland et al. (2006). The authors came to the conclusion that each method has its own strengths and weaknesses, and they partially complement each other. None of them is able to match the performance of expert-based methods, although good results can still be achieved in many cases, and significant improvements may come from a meta-method merging multiple methods, summing the benefits and hopefully limiting the defects of each method considered separately. Finally, the very concept of domain may require some degree of flexibility to best accommodate the needs of the context we are in: if we focus on evolution, what we will care about is the part of the domain that is conserved across different proteins and species; if we are more interested in structural features, we will define a domain simply as a compact folding subunit; from a functional point of view, we may regard as domain only the part of the protein involved in performing the function (e.g., for interaction domains, binding). Each definition is correct in its particular context, though they may not always provide coincident domain boundaries.

4 Domains Can Be Organized Hierarchically in Families

The domain space is not flat: rather, it can be organized in a hierarchical fashion by grouping together similar domains in the same family. Several classification strategies, relying on sequence, structure and functional evidence, have been proposed to accomplish this task. In general, structure-based approaches are more robust and comprehensive: due to the higher conservation of structure compared to sequence information, even distant relationships between domains can be detected. On the one hand, the application of such methods is limited to proteins whose three-dimensional structure has been solved or proteins whose spatial conformation can be related to some known structure through homology. On the other hand, thanks to the deluge of sequencing data, sequence-based approaches can easily operate on a proteome-wide scale, but hard-to-find relationships are sometimes missed and require additional information to be spotted.

Over the past 10–15 years, several databases have emerged with the purpose of storing classifications of domain families. Two of the most widely used ones are structural classification of proteins (SCOP) (Andreeva et al. 2004) and class architecture topology homology (CATH) (Pearl et al. 2003). The classification schemes of SCOP and CATH are both based on protein structures and aim at grouping together proteins sharing a common ancestor. The classification unit is the domain. Domains are defined with the aid of automatic tools, whose output is manually checked by visual inspection before being entered in the database. The SCOP classification is structured into four hierarchical levels:

– FAMILY: proteins with sequence identity greater than or equal to 30% and proteins with sequence identity lower than 30%, but whose function and structure similarity confirms a common evolutionary origin are clustered together in the same family.
– SUPERFAMILY: proteins with low sequence identity, but whose likely common evolutionary origin is suggested by structure and functional evidence belong to the same superfamily.
– FOLD: superfamilies and families are grouped together in a common fold if the major secondary structures present in their member proteins are arranged in the same order and form identical topological connections.
– CLASS: different folds have been grouped into five classes according to the type of secondary structure composition: all alpha (alpha helices only), all beta (beta sheets only), alpha and beta (alpha helices and beta strands are highly interspersed), alpha plus beta (alpha helices and beta strands are mostly segregated) and multi-domain (proteins with domains of a different fold).

The CATH classification is similar to the SCOP.

Another database of protein domain families that has gained large consensus in the scientific community is Pfam (Protein FAMilies) (Sonnhammer et al. 1997). Unlike SCOP and CATH, the Pfam classification of domain families is based solely on sequence data and relies on the construction of accurate Hidden Markov Models

from multiple sequence alignments. First, a non-redundant set of proteins known to be representative of a certain family is collected from various data sources (Swissprot, Prosite, published alignments, etc.), and a seed alignment is built. The quality of the alignment is manually checked to verify that all important features (key residues, active sites, etc.) are present. Then, an HMM profile is built from the alignment and is compared with all sequences in sequence databases (e.g., Swissprot) to find all family members: by aligning all family members to the HMM, a full alignment is obtained. Finally, both seed and full alignments must pass strict quality controls before being annotated and stored in the database. Being focused primarily on sequence homology, Pfam's methodology operates at the family or superfamily level, where sequence similarity can still be successfully employed to detect clear evolutionary relationships among proteins. Recently (Finn et al. 2006), Pfam has introduced the concept of clans, with the purpose of grouping together families sharing a common evolutionary origin. Clans are mainly defined on the basis of structural similarity and profile–profile comparisons. Although a one-to-one correspondence cannot always be traced, there is substantial overlap between Pfam families and SCOP families, and also between Pfam clans and SCOP superfamilies (Elofsson and Sonnhammer 1999; Finn et al. 2006). Pfam clans have the advantage of comprising also families of unknown three-dimensional structure, while SCOP superfamilies are in some cases more comprehensive, due to the larger informative content of structure with respect to sequence data.

In 2000, some of the major groups working on characterizing protein families joined their efforts and skills, giving birth to the InterPro consortium (Apweiler et al. 2000), a documentation resource aiming at integrating the different and, to some extent, complementary methodologies employed by the founder groups to derive accurate and comprehensive protein signatures. Up to now (Mulder et al. 2007), ten databases have become members of the consortium: PROSITE (Hulo et al. 2006), PRINTS (Attwood 2002), ProDom (Servant et al. 2002), Pfam (Finn et al. 2006), SMART (Letunic et al. 2006), TIGRFAMs (Haft et al. 2003), PIRSF (Wu et al. 2004), SUPERFAMILY (Gough et al. 2001), Gene3D (Buchan et al. 2003) and PANTHER (Thomas et al. 2003). Furthermore, InterPro cross-references to SCOP, CATH and ModBase (Pieper et al. 2006). The integration of multiple data sources allows InterPro to reach extensive coverage of the proteins reported in UniProt (Wu et al. 2006): release 15.1 of InterPro covers 92.5% of the SwissProt part of UniProt. This figure decreases a little considering also TrEMBL records (76.9%). Table 1 reports some statistics about the number of different protein families stored in SCOP, CATH, Pfam and InterPro.

Uncovering sequence and structure similarities among protein domain families is no void, purely intellectual effort: structural genomics studies may consider giving greater priority to solving the structures of protein domains that belong to highly populated families and superfamilies. In fact, since putative structures can be assigned by homology mapping to members of the same family for which no structural information is available yet, solving a single structure in a family with hundreds of members may shed some light over the structural features of a high number of protein domains.

Table 1 The table shows the content of four of the major databases storing protein family definitions

	SCOP 1.71	CATH 3.1.0	Pfam 21.0	InterPro 15.1
Folds	971	1,084	–	–
Superfamilies	1,589	2,091	264 (clans)	–
Families	3,004	7,794	8,957	14,671

A "fold" groups proteins whose major secondary structures are arranged in the same order and form identical topological connections. A "superfamily" groups proteins with a clear common evolutionary origin. A "family" groups proteins sharing a good level of sequence identity. Care should be taken comparing SCOP and CATH superfamily and family statistics to those regarding Pfam and InterPro, because the classification schemes adopted by the databases are quite different. Pfam only recently introduced clans, which are somewhat similar to SCOP's superfamilies, and InterPro organizes its entries hierarchically in the superfamily/family/subfamily levels by defining parent/child relationships among them

5 Domain Interactions Mediate the Formation of Macromolecular Complexes

Interactions between domains can be divided in two main classes: intra-chain interactions, occurring only in multi-domain polypeptide chains, and inter-chain interactions, involving domains that reside in different polypeptide chains, as is the case for multi-subunit proteins or, more generically, for protein complexes. Since many protein–protein interactions are mediated by one or more inter-chain domain interactions, understanding how domain interfaces are formed and detailing their properties at the atomic level may shed some light on the inner workings of protein–protein interaction networks.

Inter-chain interactions show differences in their nature and, consequently, in their behavior: as we know, proteins spontaneously aggregate to form macromolecular complexes, either stably or transiently. Stable multi-protein complexes act as molecular machines that coordinate their action to carry out almost any biological process occurring in the cell. snRNP particles, responsible for directing splicing events in eukaryotes, the proteasome and nuclear pores, are good examples of molecular machines. Besides forming obligate complexes, the molecular components of which cannot exist separately (e.g., hemoglobin), proteins may associate to assemble transient complexes. Since the action of transient complexes is generally more confined in terms of space and time, the affinity of the bonds holding them together must be modulated so as to allow rapid assembly and disruption of the complex. Such capability to quickly re-model a protein complex is especially important in signaling pathways, where interaction events activate and direct the cell response to the possibly abrupt occurrence of external stimuli and swift changes in environmental conditions.

Permanent and non-obligate interfaces differ in size and polarity: permanent interfaces are usually large and hydrophobic, while non-obligate ones are smaller and more polar. Remarkably, intra-chain interfaces have physical and chemical properties that closely resemble those of inter-chain interfaces, usually intermediate

between permanent and non-obligate interfaces (Park et al. 2001): this suggests that interactions within and between monomers are governed by the same underlying principles and, theoretically, no substantial difference should exist between an intra-chain domain interaction and an interaction between a pair of identical domains residing in different polypeptide chains. The phenomenon of domain swapping (one of the two domains forming an intra-chain interface is substituted by an identical domain belonging to another polypeptide chain) (Bennett et al. 1995), which is known to occur in the formation of some oligomeric proteins, is an example of such interchangeability among identical domains, regardless of the polypeptide chain they belong to. In fact, for domain swapping to occur, within monomer and between monomer interfaces must present similar interaction sites and must share similar characteristics (Jones et al. 2000).

6 Interaction Modules and Their Importance in Signaling Pathways

As we have seen in the previous paragraph, proteins associate to form either stable or transient multi-protein complexes. The assembly of stable macromolecular complexes often requires high-affinity interactions, involving large portions of the protein surfaces in the formation of the contact area. Such high-energy bonds are not easily disrupted and are thus well suited to confer stability on molecular machines, such as the centrosome, the proteasome and others (Brooijmans et al. 2002). However, when flexibility and responsiveness are needed, e.g., in signaling pathways, such great stability would be a limitation. In fact, in highly dynamic contexts protein–protein interactions frequently involve a modular protein domain binding to a short amino acid sequence, termed a “motif” (Pawson et al. 2002).

A motif is a sequence of 3–12 amino acids that acts as a recognition element for a modular protein domain that binds to it. Since these motifs are thought to be unstructured in their unbound form, they are often referred to as “linear” motifs: nonetheless, they may acquire a well-defined three-dimensional structure upon binding (for example, the proline-rich motifs recognized by SH3 domains adopt a left-handed poly-proline II helix conformation). Short linear motifs often lie within disordered regions of the protein; for example, they may reside in exposed flexible loops.

Binding motifs are recognized by a set of conserved protein interaction modules playing a major role in many biological processes: as an example, SH3, SH2, WW, EVH1 and PDZ domains are involved in protein trafficking and degradation, cytoskeletal organization, cell-cycle progression, cell survival and regulation of gene expression; plus, they can mediate the assembly of multi-protein complexes (PDZ domain proteins often act as scaffolds). Graphical representations of these and other interaction modules are illustrated in Fig. 2, and their properties are summarized in Table 2.

Each family of interaction modules has a defined conserved structure forming one or more “recognition pockets” to which ligands bind. A small number of highly conserved amino acids have their side chains protruding outward in the recognition

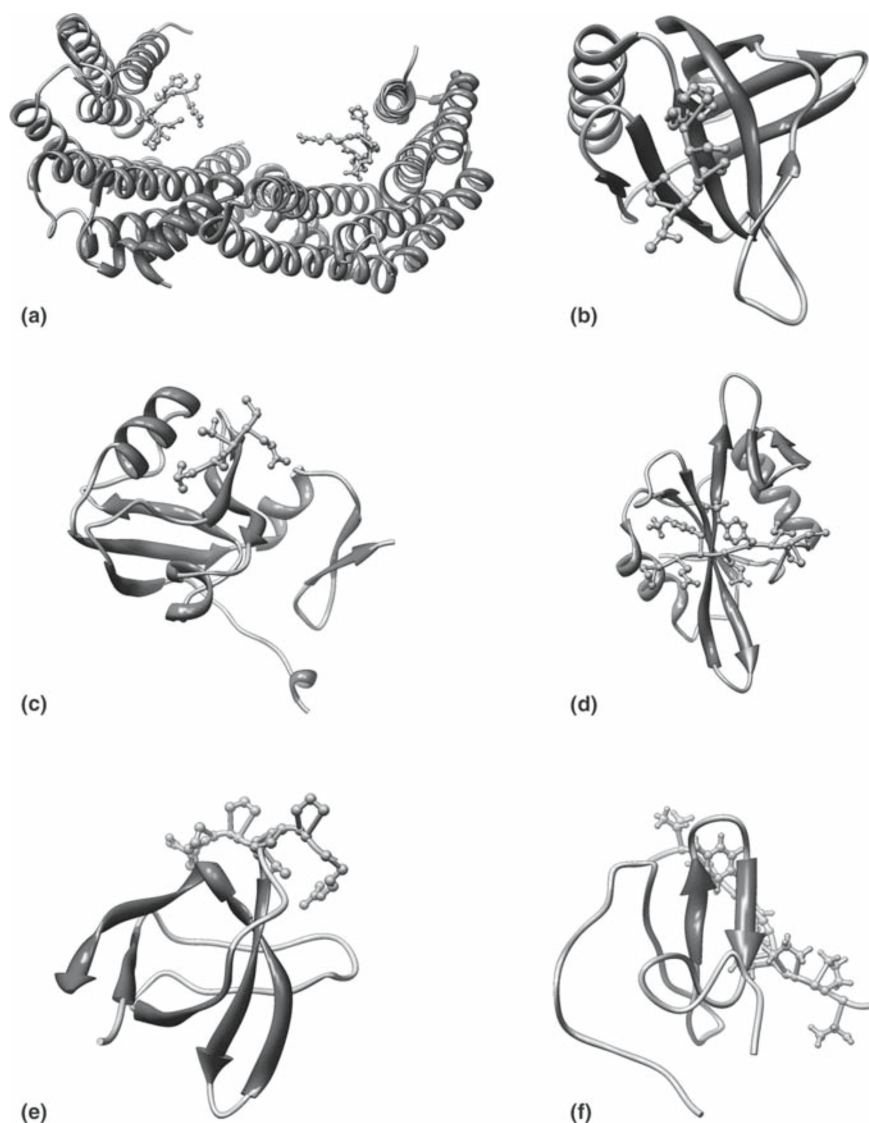

Fig. 2 Three-dimensional structures of six protein interaction modules. **(a)** A 14-3-3 Zeta/Delta dimer in complex with phospho-peptides (PDB code: 1QJB). **(b)** EVH1 domain of Homer protein homolog 1 in complex with peptide TPPSPF derived from metabotropic glutamate receptor 5 (PDB code: 1DDV). **(c)** The third PDZ domain from the synaptic protein Psd-95 in complex with a C-terminal peptide derived from CRIPT (PDB code: 1BE9). **(d)** Src SH2 domain bound to doubly phosphorylated peptide PQpYEpYIPI (PDB code: 1NZL). **(e)** C-terminal Sem-5 SH3 domain bound to a proline-rich peptide from mSos (Ac-PPPVPPRRR) (PDB code: 1SEM). **(f)** Yap65 (L30K mutant) WW domain in complex with GTPPPPYTVG peptide (PDB code: 1JMQ)

Table 2 The table summarizes the properties of six of the most relevant and well-studied interaction modules

Domain	Size	Structure	Target motifs	Binding pocket	Function
14-3-3	~30 kDa proteins	Nine anti-parallel helices forming an L-shaped structure	Phosphothreonine or phosphoserine motifs consensus: RSxpSxP	Four helices containing hydrophobic residues form a concave amphipatic groove	Regulation of many pathways (e.g., apoptosis, cell cycle)
EVH1	~110 amino acids	Compact parallel beta-sandwich, closed along one edge by a long alpha-helix	Proline-rich peptides consensus: E/DFPPPPXD/E	Highly conserved cluster of three surface-exposed aromatic side-chains	Scaffolding, signaling, nuclear transport and cytoskeletal organization
PDZ	80–90 amino acids	Six beta-strands and two alpha-helices compactly arranged in a globular structure	C-terminal motifs, several PDZ domains bind to phosphoinositide PIP2	Elongated surface groove forms as an antiparallel beta-strand, interacts with the betaB strand and the B helix	Scaffolding, localization of proteins to the plasma membrane, regulation of intracellular signaling
SH2	~100 amino acids	A central hydrophobic anti-parallel beta-sheet, flanked by two short alpha-helices forming a compact flattened hemisphere	Phosphotyrosine motifs consensus: p-YxxΨ	Ligand binds perpendicular to the beta-sheet and interacts with the loop between strands 2 and 3 and a hydrophobic binding pocket that interacts with a pY+3 side chain	Regulation of intracellular signaling cascades found in adaptor proteins and non-receptor tyrosine kinases
SH3	~50 amino acids	Five or six beta-strands arranged as two tightly packed anti-parallel beta sheets	Proline-rich motifs consensus: PxxP	Flat, hydrophobic pocket consisting of three shallow grooves defined by conservative aromatic residues, ligand adopts an extended left-handed helical arrangement	Signaling, cytoskeletal organization, assembly of macromolecular complexes found in adaptor proteins

(continued)

Table 2 (continued)

Domain	Size	Structure	Target motifs	Binding pocket	Function
WW	~40 amino acids	Stable, triple stranded beta-sheet	Phosphoserine-phosphothreonine motifs, proline-rich motifs, consensus: PPxY	WW or WWP name takes after the residues responsible for binding: two tryptophan residues that are spaced 20–23 amino acids apart and a conserved proline	Involved in a variety of signal transduction processes

The descriptions are mainly taken from InterPro version 15.1 (Mulder et al., 2007). *Ψ* indicates a hydrophobic amino acid, *x* indicates any amino acid, and *p-* indicates phosphorylation

pocket. These mediate recognition of a specific signature in the ligand molecule, a short "core" motif of a few amino acids whose side-chain chemical groups are complementary to those in the domain recognition pocket. Within a single domain family, the broad specificity encoded in the core motif is further refined by a few flanking residues. Thus, the residues surrounding the core motif in the target peptide contribute to defining which domains it will preferentially attach to and modulate the binding affinity. Domain-motif interactions involve a small portion of the proteins' surface area and are characterized by relatively low affinities, ranging from 0.1 to 100 μM. This characteristic confers on domain-motif interactions a dynamic nature and explains their ubiquitous presence in signaling pathways (Bhattacharyya et al. 2006), where protein complexes associate and disassociate in response to any arbitrarily varying stimulus. Some interaction modules bind their ligands dependently of some type of covalent modification, the most common being phosphorylation: for instance, SH2 domains bind with high affinity phosphotyrosine-containing peptides, while 14-3-3 proteins prefer phospho-serine-containing motifs.

The widespread distribution of proteins containing interaction modules and the specificity of the interaction mechanism, closely resembling a key-lock model, has led scientists to postulate the existence of a "protein recognition code" analogous to the genetic code (Sudol 1998). Such a code would be composed of a set of rules that may be encoded by relatively simple regular expressions, determining how protein interactions mediated by interaction modules can occur. However, caution should be taken in following this analogy too strictly to avoid overlooking some relevant differences between the two. Perhaps the most noteworthy features of the genetic code are its universality and its absoluteness: a three-letter codon encodes a specific amino acid always and anywhere (or at least with very few exceptions), independently of the organism and the cellular context. Even though protein interaction modules are present in a wide range of species, from unicellular to multi-cellular organisms, from plants to animals, their behavior is by no means universal or

context-independent. The in vivo binding of a potential ligand peptide is always conditioned to local concentrations, subcellular localizations and, in some cases, to the coordinated action of other interaction modules (e.g., adaptor proteins, Pawson 2007). Furthermore, domains belonging to the same family frequently share a considerable number of cognate ligands (Castagnoli et al. 2004), and a certain degree of overlap also exists between the target recognition rules of different families: this indicates that the protein recognition code presents a high level of degeneracy. This is known to be also an important feature of the genetic code (even if the level of redundancy in the genetic code is certainly lower) and may help confer robustness on the system by resisting the detrimental effect of mutations.

7 Methods to Detect the Specificity of Interaction Modules

In trying to unveil the rules governing the mechanisms of interaction between protein modules and peptides, one would like to explore the sequence space as exhaustively as possible, ideally testing the binding of all representatives of each domain family occurring in the proteome of a living cell to all possible amino acid sequences of reasonable length. However, this brute force strategy is not feasible due to the technical limitations of current techniques. Fortunately, the impossibility to reach perfect generality and completeness in searching potential ligands may be partially overcome by taking into account some a priori knowledge about the binding determinants for any given domain. Capitalizing on the characterization of a large number of target peptides and on the detailed structural information contained in high-resolution three-dimensional structures of interaction modules in complex with their ligand peptides, the sequence characteristics of binding peptides can be determined and confirmed by mutagenic analysis.

This information defines a biased sequence space where a few pre-determined positions contain specific amino acids, while the others are allowed to vary in a combinatorial fashion. Thus, the new search space is reduced in size and can be explored experimentally with currently available techniques. This approach may be concretized by constructing an "oriented peptide library," a biased collection of peptides of degenerate sequence, but having fixed amino acids in the "orienting" positions (e.g., for SH2 domains, the phospho-tyrosine residue would be the orienting amino acid, whereas for SH3 domains the two prolines in the PxxP motif) (Yaffe and Cantley 2000). The peptide mixture is then incubated with the domain of interest. Subsequent sequencing of the adsorbed peptides allows determining the positions showing enrichment for any particular amino acid. Although very powerful in theory, this approach has not been widely employed, mainly because of the technical expertise required to perform complicated peptide biochemistry (Santonico et al. 2005).

An alternative approach is based on phage display (Scott and Smith 1990): a library containing a large number (in the range of 10^9–10^{10}) of short (10–15 amino acids) random peptides is displayed on bacteriophage capsids and is panned against

a domain used as bait. The peptides having affinity with the domain can be purified, and their sequence can be easily obtained by sequencing the DNA of the capsid gene. It is then possible to derive a consensus sequence by aligning a reasonable number of interacting peptides. Although this technique has been successfully employed to profile several families of interaction modules (Sparks et al. 1994; Rickles et al. 1994; Vaccaro et al. 2001; Cestra et al. 1999; Paoluzi et al. 1998; Romano et al. 1999; Dente et al. 1997; Freund et al. 2003), there are some limitations to it: first, whereas it is fairly easy to identify which residues are highly conserved among ligands, it is often problematic to uncover statistical correlations in less conserved positions; second, some peptides are actually able to bind to the domain, even if they do not perfectly match the consensus. When the derived consensus is used to infer physiological partners in the proteome, these two problems inevitably give rise to the occurrence of false positives and false negatives.

A third approach, called SPOT synthesis (Frank 2002), successfully addresses the limitations of phage display. The technique is based on the chemical synthesis of a high number of peptides on a cellulose membrane or a glass slide in an array format: the binding of the domain to each spot is detected by a fluorescent probe, whose intensity is measured by a laser scanner. Since binding of each and every peptide in the collection is tested independently and is semi-quantitatively described by a figure correlating with the dissociation constant, we are able to tell readily which peptides bind and which do not, eliminating the inference step that represents the major drawback of the previously described techniques. However, the number of peptides that can be spotted together on a chip with current facilities is in the order of 10^4, limiting the applicability of this approach to a rather restricted search space.

Recently, the complementarity of the phage-display and the SPOT synthesis approaches has been exploited in the design of a two-step strategy, named WISE (Whole Interactome Scanning Experiment) (Landgraf et al. 2004), that aims at combining the strengths of the two methods into a single general purpose procedure (see Fig. 3). The first step involves the definition of a “strict” consensus sequence by panning a phage displayed peptide library against the selected domain. Then, a “relaxed” consensus is obtained with the aid of computational tools, and it is used to select a number of peptides between 5,000 and 10,000 from all the protein sequences in the proteome that match the consensus. Finally, the peptides are synthesized on a chip and are probed with the domain of interest. The selection step makes it possible to test all potential domain targets in a proteome.

Though the task of discovering new binding motifs cannot be satisfactorily addressed without the fundamental contribution of experimental approaches, it would be a mistake to underestimate the valuable support computational methods can provide. In fact, *in silico* predictions may be employed to drive wet lab experiments by formulating rationally founded initial guesses, thus speeding up research (Yaffe 2006). As an example of this, Neduva et al. (2005) developed a smart algorithm to detect linear motifs potentially mediating protein interactions. They start from the assumption that proteins sharing a common interaction partner must also share a feature mediating binding, either a domain or a linear motif: for this reason,

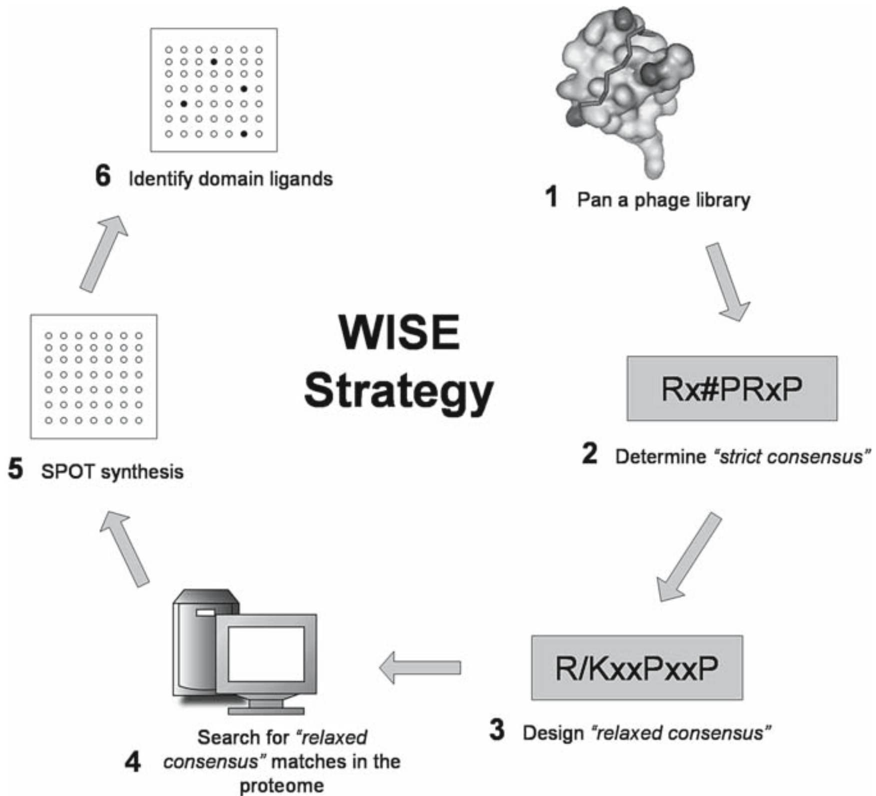

Fig. 3 Overview of the WISE strategy

the analysis focuses on groups of proteins sharing a common interaction partner. Since linear motifs often lie in low complexity regions of the protein, all globular domains, coiled coils, trans-membrane regions, collagen regions and signal peptides are removed from the protein sequences in the set. If homologous segments are detected comparing the sequences with each other, only one representative per region is left, to avoid any bias. Next, all three to eight residue motifs present in the remaining sequence space are found and are scored by their level of over-representation, measured as the binomial probability of observing the motif *N* times or more in a random set of similar sequences (where *N* is the number of times the motif occurs in the sequence set). Information from closely related species is also taken into account by looking at conservation of the motifs in orthologous proteins and combined into a final likelihood score.

The algorithm was applied to genome-scale protein interaction datasets and produced tens of candidate linear motifs in yeast, fly, nematode and human. Three of the novel predictions were also confirmed experimentally via fluorescence anisotropy (Neduva et al. 2005).

8 Methods to Infer Domain–Domain Interactions

Unfortunately, no well-established experimental method is available to detect domain-domain interactions on a large scale. In principle, one could use common interaction detection techniques, e.g., yeast two hybrid, on collections of engineered constructs expressing only a portion of the full-length protein: if a smaller construct maintains the same ability to interact as the full-length protein, one may safely conclude that the region mediating the interaction lies within the domain(s) present in the fragment protein. This approach has been successfully employed by two different groups to explore the protein interaction network of two micro-organisms, *P. falciparum* (Lacount et al. 2005) and *H. pylori* (Rain et al. 2001), by two hybrid screenings of a library of protein fragments. However, generally speaking, determining the domains mediating a protein interaction is a time-consuming task.

This prompted the development of several computational methods to identify pairs of putative interacting domains. Although many different algorithms have been devised for this purpose, so far most of them rely on the same basic assumption: if a pair of domains co-occurs in interacting protein pairs significantly more frequently than in non-interacting ones, they are likely to interact (see Fig. 4). Based on this hypothesis, statistical methods may be employed to search domain pairs recurring in interacting protein pairs. Sprinzak and Margalit (2001) scored putative interacting domain pairs by computing the log-odds of the two domains co-occurring in interacting pairs to the co-occurrence expected on a random base. Ng et al. (2003) developed a scoring system aimed at integrating the information from protein–protein interactions, multi-protein complexes and domain fusion events. The results of their predictions were stored in an online database called InterDom (http://interdom.lit.org.sg). Nye et al. (2005) adopted a rigorous statistical approach and applied a sophisticated simulation technique to assign to each pair of domain superfamilies occurring in a generic protein interaction dataset a *p*-value reflecting the likelihood that they are able to interact. Deng et al. (2002) developed a Maximum Likelihood Estimation (MLE) and an Expectation Maximization (EM) algorithm to infer probabilities of the domain interactions underlying a set of protein interactions; in a more recent paper (Lee et al. 2006), they extended their method by integrating interaction probabilities with information from protein fusions and Gene Ontology (Ashburner et al. 2000) functions through a Bayesian approach. Riley et al. (2005) modified the first version of the algorithm by Deng et al. (2002) and improved it by introducing the E-score, a measure reflecting the importance of a specific domain–domain interaction to explain a set of protein–protein interactions. Jothi et al. (2006) opted for a different approach, looking at the relative degree of co-evolution of domains in interacting protein pairs: they provided evidence that pairs of domains mediating the protein interaction are more likely to co-evolve with respect to non-interacting domain pairs.

Although some of the aforementioned methods show promising results, all of them are far from perfect. Since all methods invariantly require as input a protein interaction dataset, their performance is strictly dependent on the quality of the interaction data, which are often affected by high false-positive and false-negative

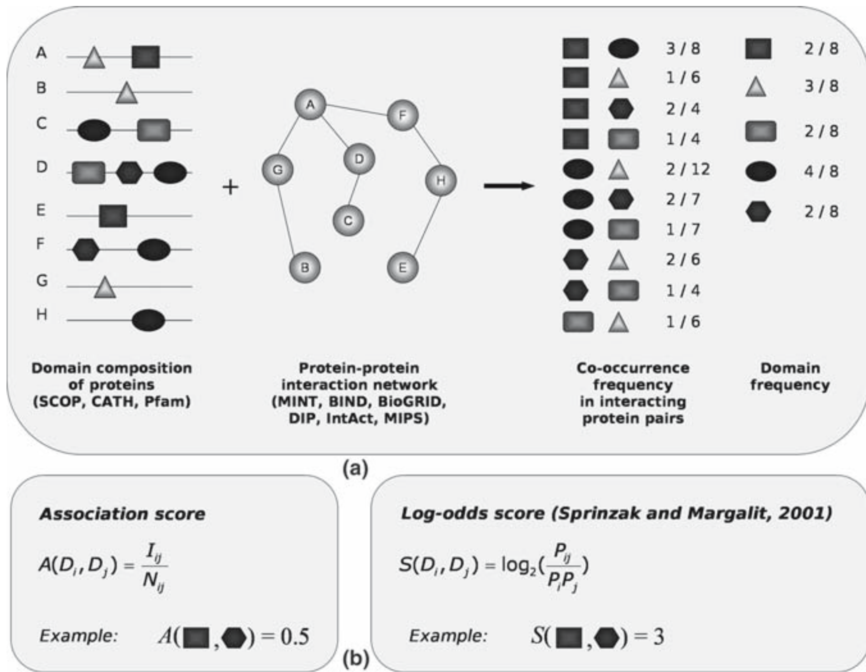

Fig. 4 The figure shows the basic idea underlying algorithms to infer domain–domain interactions. The algorithms need as input a protein interaction network along with information about the domain composition of the proteins present in the network. Given these data, it is possible to compute the co-occurrence frequency of domain pairs in interacting protein pairs as the ratio between the number of interacting protein pairs containing the domain pair of interest and the total number of protein pairs containing it. Also the domain frequency in the set of proteins appearing in the protein network can be calculated. Next, these frequencies can be combined to assign domain pairs an interaction probability. Panel **(b)** shows two different methods to rank domain pairs according to the likelihood that they interact: the fairly simple association score and the log-ratio score devised by Sprinzak and Margalit. The first method simply ranks domain pairs by their co-occurrence frequency in interacting protein pairs, whereas the second relates this value to the co-occurrence frequency that would be expected on a random base (P_i represents the frequency of domain i in the proteome). Several algorithms of various complexity levels have extended this basic procedure to improve the accuracy and reliability of the predicted domain–domain interactions

rates. Another major issue is validation: how can the reliability of predictions be assessed? Accurate estimation of the algorithm's performance would recommend the comparison of the output predictions with a reference set of trusted positives and negatives. The definition of such a reference set is greatly impaired by the scarcity of known interacting and non-interacting domain pairs. It is common practice to consider as true interacting domain pairs those that have been observed to interact at least in one solved three-dimensional structure and as true non-interacting pairs those that never come into contact in known structures. Given the small number of non-redundant three-dimensional structures contained in the PDB

(Protein Data Bank, Berman et al. 2000), many true interacting domain pairs may not be represented at all in the positive set, whereas the number of non-interacting pairs in the negative set is likely to be overestimated. Accuracy of the reference set should also be questioned: in many cases it is hard to tell whether two residues come close because a biologically meaningful interaction between the two has occurred or merely due to crystal packing. For this reason, Shoemaker et al. (2006) have recently developed a strategy, based on structural criteria, to discriminate biologically relevant protein domain interactions from artifactual ones.

9 Domain Interactions as Potential Drug Targets

Protein–protein interactions preside over the physiological and pathological processes in living organisms. Therefore, the ability to intervene in a controlled manner in the protein interaction network, altering its wiring by specifically favoring certain interactions and blocking others, is crucial for molding the cell response into a desired phenotype. Examples of the efficacy of such strategy is witnessed by the *modus operandi* of some known pathogens, such as the Epstein–Barr virus (EBV) (Longnecker and Miller 1996) and the enteropathogen *E. coli* (Gruenheid et al. 2001): specific viral and bacterial proteins possess recognition motifs that are able to recruit host proteins, thus reorganizing the wiring of the protein network to better fit the pathogen needs (Pawson et al. 2001). In these cases, the perturbation of the cellular interaction network leads to disease onset.

The outstanding importance of a tight regulation of protein interactions, governed by the underlying domain interactions, for proper cell functioning has led scientists to regard them as prominent targets for drug design. Research in this direction has been carried on the following two complementary approaches: (1) designing molecules to induce protein–protein interactions (dimerizers); (2) designing molecules to inhibit protein–protein interactions (inhibitors).

Since many signaling pathways are initiated by a dimerization event, pathway activation may be stimulated artificially by small molecules endowed with anchors having affinity for the protein partners that are to be brought into proximity (Archakov et al. 2003). The anchors are separated by a linker region of variable length. To avoid undesirable side effects and increase dimerizer specificity, a mutant domain may be inserted in the construct expressing the wild-type target proteins so as to properly tune their affinity to the anchor part of the interfering molecule. Over the last decade, several dimerizers have been proposed and tested in the laboratory (Austin et al. 1994; Clemons 1999; Belshaw et al. 1996; Kopytek et al. 2000). Theoretically, dimerizers could be used in cancer therapy to induce apoptosis in abnormally proliferating cells (Amara et al. 1997; MacCorkle et al. 1998).

When the challenge of inhibiting the formation of a protein complex is faced, different strategies must be adopted depending on the type of protein contact surface we are dealing with. As we have seen in paragraph 0, the domain–domain interfaces

of permanent complexes have different physical and chemical characteristics than those of temporary complexes. Namely, the shaping of contact surfaces in permanent oligomers involves the burial of large areas of the protein surface within the complex core, resulting in a tightly packed structure held together by strong non-covalent interactions. This process can be thought of as a continuation of protein folding (Archakov et al. 2003). The high-energy bonds involved in the interaction and the high level of complementarity between the domain surfaces make it difficult to design small molecules that would effectively block the formation of the macromolecular complex by competitive binding. However, some degree of success has been reached thanks to a mechanism called "interfacial inhibition," whose main idea is to trap macromolecules into dead-end complexes while they are undergoing conformational changes by letting artificial or natural compounds bind to "hot spots" that are present during folding transition states only. For instance, it has been shown that two natural inhibitors, brefeldin A (BFA) and camptothecin (CPT), have the power to freeze the targeted complexes (respectively, an Arf-GDP-Sec7 complex and a TOP1-DNA complex) in a permanently inactive conformation, preventing them from completing their biological function (Pommier and Cherfils 2005).

On the contrary, transient interactions nucleating the assembly of temporary protein complexes resemble receptor-ligand interactions. Many of these interactions are mediated by protein interaction modules binding to short peptides exposed on the surface of the target proteins. The contact area between the recognition pocket of the interaction module and the target peptide is usually small, allowing for competitive disruption of the complex. Using site-directed mutagenesis, it has been possible to determine the amino acid residues important for binding, and thus peptides resembling the dimer interface have been designed. The binding ability of the construct can be subsequently optimized by combinatorial chemistry techniques in order to individuate the amino acid composition that would confer to the synthetic construct a binding affinity that is possibly higher than that of the physiological partner. Peptidomimetics and small organic molecules have also been tested as inhibitors with good success rates (Zutshi et al. 1998; Jiang et al. 2006; Arkin and Wells 2004; Turkson et al. 2004).

Due to their pervasiveness and fundamental role in biological processes, protein interactions certainly hold enormous potential as drug targets. With respect to antibiotic design, targeting protein–protein interfaces rather than enzyme active sites has evident advantages: protein–protein interfaces have structural peculiarities that facilitate discrimination between the host proteins and the pathogen ones, whereas enzyme active sites show little difference. As a consequence, a potential drug directed against a pathogen enzyme active site may end up blocking the action of the cell's own enzymes (Singh et al. 2001). Furthermore, whereas pathogens can acquire antibiotic resistance by mutating a few residues in an enzyme active site, while maintaining the catalytic ones unaltered, in order to neutralize the action of a protein interaction inhibitor the pathogen would have to mutate simultaneously a few amino acid positions in both interacting surfaces to preclude drug binding and, at the same time, to preserve the ability of the partner proteins to interact. This is, of course, statistically more improbable.

10 Conclusion

Over the past decade, experimental and theoretical approaches have contributed to stressing the importance of interpreting protein interaction networks in the context of domain–domain and domain–peptide interaction preference. Unraveling the mechanisms by which modular domains mediate protein interactions will allow gaining a more insightful understanding of cell functioning in both physiological and pathological conditions. Only when this first step has been achieved will it be possible to progress towards ambitious goals, such as predicting the cell response to any given stimulus and attenuating undesirable phenotypes by selectively inducing or blocking protein interactions (Dueber et al. 2004).

References

Aloy P, Russell RB (2006) Structural systems biology: modelling protein interactions. Nat Rev Mol Cell Biol 7:188–197

Amara JF, Clackson T, Rivera VM, Guo T, Keenan T, Natesan S, Pollock R, Yang W, Courage NL, Holt DA, Gilman M (1997) A versatile synthetic dimerizer for the regulation of protein–protein interactions. Proc Natl Acad Sci USA 94:10618–10623

Andreeva A, Howorth D, Brenner SE, Hubbard TJP, Chothia C, Murzin AG (2004) SCOP database in 2004: refinements integrate structure and sequence family data. Nucleic Acids Res 32: D226–D229

Apweiler R, Attwood TK, Bairoch A, Bateman A, Birney E, Biswas M, Bucher P, Cerutti L, Corpet F, Croning MD, Durbin R, Falquet L, Fleischmann W, Gouzy J, Hermjakob H, Hulo N, Jonassen I, Kahn D, Kanapin A, Karavidopoulou Y, Lopez R, Marx B, Mulder NJ, Oinn TM, Pagni M, Servant F, Sigrist CJ, Zdobnov EM, Consortium I (2000) InterPro – an integrated documentation resource for protein families, domains and functional sites. Bioinformatics 16:1145–1150

Archakov AI, Govorun VM, Dubanov AV, Ivanov YD, Veselovsky AV, Lewi P, Janssen P (2003) Protein–protein interactions as a target for drugs in proteomics. Proteomics 3:380–391

Arkin MR, Wells JA (2004) Small-molecule inhibitors of protein–protein interactions: progressing towards the dream. Nat Rev Drug Discov 3:301–317

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. Gene Ontol Consort Nat Genet 25:25–29

Attwood TK (2002) The PRINTS database: a resource for identification of protein families. Brief Bioinform 3:252–263

Austin DJ, Crabtree GR, Schreiber SL (1994) Proximity versus allostery: the role of regulated protein dimerization in biology. Chem Biol 1:131–136

Belshaw PJ, Ho SN, Crabtree GR, Schreiber SL (1996) Controlling protein association and subcellular localization with a synthetic ligand that induces heterodimerization of proteins. Proc Natl Acad Sci USA 93:4604–4607

Bennett MJ, Schlunegger MP, Eisenberg D (1995) 3D domain swapping: a mechanism for oligomer assembly. Protein Sci 4:2455–2468

Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE (2000) The protein data bank. Nucleic Acids Res 28:235–242

Bhattacharyya RP, Reményi A, Yeh BJ, Lim WA (2006) Domains, motifs, and scaffolds: the role of modular interactions in the evolution and wiring of cell signaling circuits. Annu Rev Biochem 75:655–680

Bornberg-Bauer E, Beaussart F, Kummerfeld SK, Teichmann SA, Weiner J (2005) The evolution of domain arrangements in proteins and interaction networks. Cell Mol Life Sci 62:435–445
Brooijmans N, Sharp KA, Kuntz ID (2002) Stability of macromolecular complexes. Proteins 48:645–653
Buchan DWA, Rison SCG, Bray JE, Lee D, Pearl F, Thornton JM, Orengo CA (2003) Gene3D: structural assignments for the biologist and bioinformaticist alike. Nucleic Acids Res 31:469–473
Castagnoli L, Costantini A, Dall'Armi C, Gonfloni S, Montecchi-Palazzi L, Panni S, Paoluzi S, Santonico E, Cesareni G (2004) Selectivity and promiscuity in the interaction network mediated by protein recognition modules. FEBS Lett 567:74–79
Cestra G, Castagnoli L, Dente L, Minenkova O, Petrelli A, Migone N, Hoffmüller U, Schneider-Mergener J, Cesareni G (1999) The SH3 domains of endophilin and amphiphysin bind to the proline-rich region of synaptojanin 1 at distinct sites that display an unconventional binding specificity. J Biol Chem 274:32001–32007
Chothia C, Gough J, Vogel C, Teichmann SA (2003) Evolution of the protein repertoire. Science 300:1701–3
Clemons PA (1999) Design and discovery of protein dimerizers. Curr Opin Chem Biol 3:112–115
Deng M, Mehta S, Sun F, Chen T (2002) Inferring domain-domain interactions from protein–protein interactions. Genome Res 12:1540–8
Dente L, Vetriani C, Zucconi A, Pelicci G, Lanfrancone L, Pelicci PG, Cesareni G (1997) Modified phage peptide libraries as a tool to study specificity of phosphorylation and recognition of tyrosine containing peptides. J Mol Biol 269:694–703
Dueber JE, Yeh BJ, Bhattacharyya RP, Lim WA (2004) Rewiring cell signaling: the logic and plasticity of eukaryotic protein circuitry. Curr Opin Struct Biol 14:690–699
Elofsson A, Sonnhammer EL (1999) A comparison of sequence and structure protein domain families as a basis for structural genomics. Bioinformatics 15:480–500
Finn RD, Mistry J, Schuster-Böckler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R, Eddy SR, Sonnhammer ELL, Bateman A (2006) Pfam: clans, web tools and services. Nucleic Acids Res 34:D247–D251
Frank R (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports–principles and applications. J Immunol Methods 267:13–26
Freund C, Kühne R, Park S, Thiemke K, Reinherz EL, Wagner G (2003) Structural investigations of a GYF domain covalently linked to a proline-rich peptide. J Biomol NMR 27:143–149
Gough J, Karplus K, Hughey R, Chothia C (2001) Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. J Mol Biol 313:903–919
Gruenheid S, DeVinney R, Bladt F, Goosney D, Gelkop S, Gish GD, Pawson T, Finlay BB (2001) Enteropathogenic E. coli Tir binds Nck to initiate actin pedestal formation in host cells. Nat Cell Biol 3:856–859
Haft DH, Selengut JD, White O (2003) The TIGRFAMs database of protein families. Nucleic Acids Res 31:371–373
Holland TA, Veretnik S, Shindyalov IN, Bourne PE (2006) Partitioning protein structures into domains: why is it so difficult? J Mol Biol 361:562–590
Hulo N, Bairoch A, Bulliard V, Cerutti L, Castro ED, Langendijk-Genevaux PS, Pagni M, Sigrist CJA (2006) The PROSITE database. Nucleic Acids Res 34:D227–D230
Jiang S, Li P, Peach ML, Bindu L, Worthy KW, Fisher RJ, Burke TR, Nicklaus M, Roller PP (2006) Structure-based design of potent Grb2-SH2 domain antagonists not relying on phosphotyrosine mimics. Biochem Biophys Res Commun 349:497–503
Jones S, Marin A, Thornton JM (2000) Protein domain interfaces: characterization and comparison with oligomeric protein interfaces. Protein Eng 13:77–82
Jothi R, Cherukuri PF, Tasneem A, Przytycka TM (2006) Co-evolutionary analysis of domains in interacting proteins reveals insights into domain–domain interactions mediating protein-protein interactions. J Mol Biol 362:861–875

Kopytek SJ, Standaert RF, Dyer JC, Hu JC (2000) Chemically induced dimerization of dihydrofolate reductase by a homobifunctional dimer of methotrexate. Chem Biol 7:313–321
Lacount DJ, Vignali M, Chettier R, Phansalkar A, Bell R, Hesselberth JR, Schoenfeld LW, Ota I, Sahasrabudhe S, Kurschner C, Fields S, Hughes RE (2005) A protein interaction network of the malaria parasite Plasmodium falciparum. Nature 438:103–107
Landgraf C, Panni S, Montecchi-Palazzi L, Castagnoli L, Schneider-Mergener J, Volkmer-Engert R, Cesareni G (2004) Protein interaction networks by proteome peptide scanning. PLoS Biol 2:E14
Lee H, Deng M, Sun F, Chen T (2006) An integrated approach to the prediction of domain-domain interactions. BMC Bioinform 7:269
Letunic I, Copley RR, Pils B, Pinkert S, Schultz J, Bork P (2006) SMART 5: domains in the context of genomes and networks. Nucleic Acids Res 34:D257–D260
Liu J, Rost B (2004) CHOP proteins into structural domain-like fragments. Proteins 55:678–688
Longnecker R, Miller CL (1996) Regulation of Epstein-Barr virus latency by latent membrane protein 2. Trends Microbiol 4:38–42
MacCorkle RA, Freeman KW, Spencer DM (1998) Synthetic activation of caspases: artificial death switches. Proc Natl Acad Sci USA 95:3655–3660
Mulder NJ, Apweiler R, Attwood TK, Bairoch A, Bateman A, Binns D, Bork P, Buillard V, Cerutti L, Copley R, Courcelle E, Das U, Daugherty L, Dibley M, Finn R, Fleischmann W, Gough J, Haft D, Hulo N, Hunter S, Kahn D, Kanapin A, Kejariwal A, Labarga A, Langendijk-Genevaux PS, Lonsdale D, Lopez R, Letunic I, Madera M, Maslen J, McAnulla C, McDowall J, Mistry J, Mitchell A, Nikolskaya AN, Orchard S, Orengo C, Petryszak R, Selengut JD, Sigrist CJA, Thomas PD, Valentin F, Wilson D, Wu CH, Yeats C (2007) New developments in the InterPro database. Nucleic Acids Res 35:D224–D228
Neduva V, Linding R, Su-Angrand I, Stark A, de Masi F, Gibson TJ, Lewis J, Serrano L, Russell RB (2005) Systematic discovery of new recognition peptides mediating protein interaction networks. PLoS Biol 3:e405
Ng S-K, Zhang Z, Tan S-H (2003) Integrative approach for computationally inferring protein domain interactions. Bioinformatics 19:923–9
Nye TMW, Berzuini C, Gilks WR, Babu MM, Teichmann SA (2005) Statistical analysis of domains in interacting protein pairs. Bioinformatics 21:993–1001
Paoluzi S, Castagnoli L, Lauro I, Salcini AE, Coda L, Confalonieri SFaS, Pelicci PG, Fiore PPD, Cesareni G (1998) Recognition specificity of individual EH domains of mammals and yeast. EMBO J 17:6541–6550
Park J, Lappe M, Teichmann SA (2001) Mapping protein family interactions: intramolecular and intermolecular protein family interaction repertoires in the PDB and yeast. J Mol Biol 307:929–938
Pawson T (2007) Dynamic control of signaling by modular adaptor proteins. Curr Opin Cell Biol 19:112–116
Pawson T, Gish GD, Nash P (2001) SH2 domains, interaction modules and cellular wiring. Trends Cell Biol 11:504–511
Pawson T, Raina M, Nash P (2002) Interaction domains: from simple binding events to complex cellular behavior. FEBS Lett 513:2–10
Pearl FMG, Bennett CF, Bray JE, Harrison AP, Martin N, Shepherd A, Sillitoe I, Thornton J, Orengo CA (2003) The CATH database: an extended protein family resource for structural and functional genomics. Nucleic Acids Res 31:452–455
Pieper U, Eswar N, Davis FP, Braberg H, Madhusudhan MS, Rossi A, Marti-Renom M, Karchin R, Webb BM, Eramian D, Shen M-Y, Kelly L, Melo F, Sali A (2006) MODBASE: a database of annotated comparative protein structure models and associated resources. Nucleic Acids Res 34:D291–D295
Pommier Y, Cherfils J (2005) Interfacial inhibition of macromolecular interactions: nature's paradigm for drug discovery. Trends Pharmacol Sci 26:138–145
Rain JC, Selig L, Reuse HD, Battaglia V, Reverdy C, Simon S, Lenzen G, Petel F, Wojcik J, Schächter V, Chemama Y, Labigne A, Legrain P (2001) The protein-protein interaction map of Helicobacter pylori. Nature 409:211–215

Rickles RJ, Botfield MC, Weng Z, Taylor JA, Green OM, Brugge JS, Zoller MJ (1994) Identification of Src,Fyn,Lyn,PI3K and Abl SH3 domain ligands using phage display libraries. EMBO J 13:5598–5604

Riley R, Lee C, Sabatti C, Eisenberg D (2005) Inferring protein domain interactions from databases of interacting proteins. Genome Biol 6:R89

Romano AMMPR, Panni S, Mendoza M, Wong WT, Musacchio A, Cesareni G, Fiore PPD (1999) A novel peptide-SH3 interaction. EMBO J 18:5300–5309

Santonico E, Castagnoli L, Cesareni G (2005) Methods to reveal domain networks. Drug Discov Today 10:1111–1117

Schmidt EE, Davies CJ (2007) The origins of polypeptide domains. Bioessays 29:262–270

Scott JK, Smith GP (1990) Searching for peptide ligands with an epitope library. Science 249:386–390

Servant F, Bru C, Carrère S, Courcelle E, Gouzy J, Peyruc D, Kahn D (2002) ProDom: automated clustering of homologous domains. Brief Bioinform 3:246–251

Shoemaker BA, Panchenko AR, Bryant SH (2006) Finding biologically relevant protein domain interactions: conserved binding mode analysis. Protein Sci 15:352–361

Singh SK, Maithal K, Balaram H, Balaram P (2001) Synthetic peptides as inactivators of multimeric enzymes: inhibition of Plasmodium falciparum triosephosphate isomerase by interface peptides. FEBS Lett 501:19–23

Sonnhammer EL, Eddy SR, Durbin R (1997) Pfam: a comprehensive database of protein domain families based on seed alignments. Proteins 28:405–420

Sparks AB, Quilliam LA, Thorn JM, Der CJ, Kay BK (1994) Identification and characterization of Src SH3 ligands from phage-displayed random peptide libraries. J Biol Chem 269:23853–23856

Sprinzak E, Margalit H (2001) Correlated sequence-signatures as markers of protein–protein interaction. J Mol Biol 311:681–692

Sudol M (1998) From Src homology domains to other signaling modules: proposal of the ‘protein recognition code’. Oncogene 17:1469–1474

Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, Diemer K, Muruganujan A, Narechania A (2003) PANTHER: a library of protein families and subfamilies indexed by function. Genome Res 13:2129–2141

Turkson J, Kim JS, Zhang S, Yuan J, Huang M, Glenn M, Haura E, Sebti S, Hamilton AD, Jove R (2004) Novel peptidomimetic inhibitors of signal transducer and activator of transcription 3 dimerization and biological activity. Mol Cancer Ther 3:261–269

Vaccaro P, Brannetti B, Montecchi-Palazzi L, Philipp S, Citterich MH, Cesareni G, Dente L (2001) Distinct binding specificity of the multiple PDZ domains of INADL, a human protein with homology to INAD from Drosophila melanogaster. J Biol Chem 276:42122–42130

Veretnik S, Bourne PE, Alexandrov NN, Shindyalov IN (2004) Toward consistent assignment of structural domains in proteins. J Mol Biol 339:647–678

Wu CH, Apweiler R, Bairoch A, Natale DA, Barker WC, Boeckmann B, Ferro S, Gasteiger E, Huang H, Lopez R, Magrane M, Martin MJ, Mazumder R, O’Donovan C, Redaschi N, Suzek B (2006) The Universal Protein Resource (UniProt): an expanding universe of protein information. Nucleic Acids Res 34:D187–D191

Wu CH, Nikolskaya A, Huang H, Yeh L-SL, Natale DA, Vinayaka CR, Hu Z-Z, Mazumder R, Kumar S, Kourtesis P, Ledley RS, Suzek BE, Arminski L, Chen Y, Zhang J, Cardenas JL, Chung S, Castro-Alvear J, Dinkov G, Barker WC (2004) PIRSF: family classification system at the Protein Information Resource. Nucleic Acids Res 32:D112–D114

Yaffe MB (2006) “Bits”and pieces. Sci STKE 2006:pe28

Yaffe MB, Cantley LC (2000) Mapping specificity determinants for protein–protein association using protein fusions and random peptide libraries. Methods Enzymol 328:157–170

Zutshi R, Brickner M, Chmielewski J (1998) Inhibiting the assembly of protein–protein interfaces. Curr Opin Chem Biol 2:62–66

Index

A

Accumulation assays, 288–289
Acetylcholine, 470, 471, 476
AC isoforms, 175
Actin filament nucleators, 450
α-Actinin, 308, 311
Acute lymphoblastic leukemia (ALL), 267
Adenomatous polyposis coli (APC), 262–265, 268, 270, 271, 273, 274
Adenylyl cyclase (ACs), 168, 172, 175, 286, 288, 289, 307, 314
β-Adrenergic, 4, 5, 8–11
AKAP4, 486
AKAP18α, 484, 491–495
AKAP18δ, 484, 488, 492, 494–496
AKAP79, 487, 488, 492, 494, 495
AKAP82, 492
AKAP97, 488
AKAP149, 486, 492
AKAP150, 486–488, 494
AKAP250, 488, 495
AKAP350, 487
AKAP450, 484
$AKAP_{IS}$, 490–492
AKAP-KL, 492
AKAP-Lbc, 313, 317–319, 485, 488, 490, 492
A-Kinase anchoring proteins (AKAPs), 4–7, 10–12, 126, 146–148, 286, 287, 291, 292, 303–308, 310, 312–317, 319, 328, 341, 342, 352, 483–485
Allosteric inhibitors, 441, 443, 444, 446, 451
Alpha1-adrenergic receptors, 304, 306, 317
Alu repeat, 72
AMPA receptor, 370, 374
Angiogenesis, 53, 54
Ankyrin repeats, 367
Anti-capping, 43, 48, 50
AP-1, 328, 331, 339, 345, 350
AP-2, 106–118
Apoptosis, 89–92, 94, 138, 139
Arp2/3 complex, 436–439, 447, 450
Arrestin, 16–33
β-Arrestin, 136, 137, 141–145, 151, 152, 154, 304, 306–308, 344, 345
AT hook, 77, 92
Atherosclerosis, 54
Atherosclerotic lesions, 51
Autoinhibition, 432, 438, 440–443, 447–453
Autoinhibitory domain, 442, 449, 453

B

BAR/PDZ domains, 192
Base excision repair (BER), 88
Base-unpaired region (BUR), 70, 71, 74, 76–78, 86, 92–94
B cell receptor, 248
B cell regulator of IgH transcription (BRIGHT), 76, 77, 95
BCL10, 346
BCL-X1, 339
Beta-adrenergic receptors, 302–308, 312, 313, 315, 316
Botulinum toxins, 462, 475–476
BRCA1/2, 90–91
Breakpoint-cluster regions (BCR), 73
Breast cancer susceptibility protein C-terminal (BRCT), 85
BRIGHT. *See* B cell regulator of IgH transcription
Brugada syndrome, 312
BUR, 70, 74, 76, 77, 92–94

C
C1/C2 domains, 188
Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), 312
CAAX motif, 174
Calcineurin (PP2B), 307–309, 314
Calcium channel, 372
Calmodulin, 20, 22, 24, 27, 31, 309, 312, 314
Calsarsin, 308
cAMP. *See* 3′–5′-Cyclic adenosine monophosphate
Camptothecin, 253
Cancer, 49, 52–54, 174, 176–178
CapZ, 311
Cardiomyopathy, 49, 54–55
Cardiovascular diseases, 51
CARMA1, 340, 346–348
Caspase-3, 138, 139
Caspase-mediated apoptotic cleavage, 76
Caveolae, 168–179, 189, 212, 213, 303, 310
Caveolin, 169–178, 303, 310, 314
Caveolin scaffolding domain (CSD), 170–178
Caveolin signaling hypothesis, 171, 173
Caveolin signaling particle, 171
Caveosome, 171
CBM complex, 249
Cbp/PAG, 334–336, 342, 343
CD28, 339, 343–345
CDC37, 245
Cdc42, 373, 432–442, 445–447, 449
Cell adhesion, 262, 264, 273
Cell–cell adhesions, 44, 45
Cerebellum, 368
CFP, 290–293, 296
Chaperones, 245
Chemical genetics, 433–437
Chemical inhibitor, 432, 433, 443, 450, 453
Chibby (Cby), 265, 269
Cholesterol, 169, 175
Chromatin, 265–268
Chromatin domains, 71, 74, 77, 85, 86, 97
Chromosome territories, 68
Clathrin , 16, 19, 20, 22, 26, 27, 106–108, 111–118, 306, 308
Co-activator, 262–264, 266, 268, 269, 274, 275
Coiled-coil motif, 42
Coiled coils, 461–478
Colchicine, 175, 176
Collision coupling, 169
Colorectal (colon) cancer, 261, 268–274
Compartmentation, 168, 179
Complex multi-protein, 385, 390, 391, 398
Conformational changes, 20, 438, 439, 441, 442, 449, 452
β′-COP, 309, 311
Co-repressor, 263, 265, 269
Core-unpairing element (CUE), 93
Cortex, 366, 368
CREB-binding protein (CBP), 263, 265–267, 269, 273, 274
Csk, 328, 329, 334–336, 341–343
C-value, 69
3′–5′-Cyclic adenosine monophosphate (cAMP), 4–12, 286–296, 302–308, 313, 314, 316, 317, 328, 339–345, 353
Cyclic AMP signaling, 287–288
Cyclic nucleotide-gated (CNG) ion channels, 286, 287, 289, 292, 296
Cyclic nucleotide phosphodiesterases (PDEs), 175, 176
Cyclooxygenase-2 (COX-2), 272
Cypher, 308, 311
Cytochalasin D, 175
Cytoskeletal components, 176
Cytoskeleton, 78, 96

D
D-AKAP1, 490, 492
D-AKAP2, 486, 492
Database
  CATH, 388–390
  InterPro, 389, 390, 394
  PDB, 392, 399–400
  Pfam, 388–390
  Prosite 389
  SCOP, 388–390
  Swissprot, 389
  UniProt, 386, 389
Dendrite, 366, 369, 373–376
Desensitization, 16, 25, 26, 28, 31, 133, 141, 142
Diacylglycerol (DAG), 186–191, 194, 196
Diaphanous-related formins, 440, 441, 447–449, 451
Dilated cardiomyopathy, 193
DISC1, 136, 140–141, 151
Disruptor peptide, 489, 494
Dlgh1, 346, 348
DNA binding, 463
DNA damage
  ATM kinase, 253
  p53, 252

DNA methylation, 95, 96
DNAse I hypersensitive sites, 86
Domain
EVH1, 391–393
PDZ, 391–393
SH2, 386, 391–395
SH3, 386, 391–393, 395
WW, 391, 392, 394
Domain composition, 385–386, 399
Domain evolution, 386
Double-strand break (DSB) repair, 90
Drug delivery, 477
Drug design, 384, 400, 401, 476, 477
Drug targeting, 478

E
EBP50, 328, 343
EGF
EGFR, 206, 214, 215, 227
Embryonic development, 195
Emesis, 135, 150
Endocytosis, 106–108, 111, 113, 115–118
Endocytosis inhibitors, 117
Endoplasmic reticulum, 215, 216
Endosomes, 214, 215, 218, 219, 226, 227
eNOS, 172, 174–176
EPAC. *See* Exchange proteins activated by *c*AMP
Epac1, 316–318
Epac-based sensors, 293–295
Epacs - Exchange protein directly activated by cAMP, 286
Epigenetic code, 95
Epigenetics, 95
Epigenetic silencing, 271
Episome, 97
ERK, 130–133, 142–146, 148, 149, 151, 154, 155
ERK5, 7–9, 11
ERM proteins, 336, 338
Etoposide, 253
EVH1 domain, 40–41, 44, 55, 56
EVH2 domain, 40–43
Exchange proteins activated by cAMP, 126, 127, 146, 148–149, 302
Extracellular matrix, 96, 97
Extracellular signal-Regulated Kinase 5/big mitogen-activated protein kinase 1 (ERK5/BMK1), 316
Ezrin, 304–306, 328, 336, 342, 343, 352, 487, 492

F
F-actin, 372, 373, 375
Familial adenomatous polyposis (FAP), 270, 272
Family
domain, 388–391, 394, 395
protein, 388–390
Feedback loops, 131–133
Filopodia, 44, 48, 50, 51
FKBP12.6 (calstabin2), 314, 315
Flotillins/reggies, 170
Fluid mosaic model, 169
Fluorescence polarization, 452,
Fluorescence resonance energy transfer (FRET), 290–296, 447
Focal adhesions, 43, 178
Formin, 432, 440, 447, 450
FOXO3a, 340
FQF ERK specificity domain, 148, 149
Fusion peptide, 472–475
Fusion protein, 461, 472–476
Fyn, 328, 334–336, 342, 349, 350

G
GADS, 330–332, 337
GEF activity, 337, 338
Geldamycin, 245
Gene deserts, 69, 71
Gene therapy, 28, 29, 31
Genomic insulators, 68, 73
GFP, 290
GKAP, 366, 368–370, 372, 375, 376
GLUT4, 195
Glycogen synthase kinase 3 (GSK3), 263–265, 271, 272
Glycohydrolase (PARG), 83, 87
Glycosylphosphatidylinositol (GPI)-linked proteins, 171
Golgi, 215, 216, 218, 220, 226–228
GPCRs. *See* G-protein coupled receptors
GPCR desensitization, 173
G-protein-coupled receptor kinases (GRKs), 304–308
G-protein coupled receptors (GPCRs), 16–20, 24–28, 31, 168–173, 175–179
G-proteins, 168, 172, 175, 302–307, 309, 310, 343
Gravin, 488
Grb2, 330–334, 338
Groucho (Gro), 263, 265, 266
Guanine-nucleotide exchange proteins, 287, 292, 293, 295, 296

H
Haemostasis, 49, 51, 52
Haploinsufficiency, 368, 369
HARBS, 134
HDM2, 497, 498
Heart disease, 10, 12
Helical-wheel diagram, 464, 465
Helicase, 267
Hemagglutinin, 472, 474
Hepatocellular carcinoma, 271
Heptad repeat, 464, 468, 472
Heterogeneous nuclear ribonucleoproteins (hnRNPs), 69, 80–81
Heterotrimeric G-proteins, 168, 172, 174
High-mobility group (HMG) box, 265, 269
High-throughput assays, 446
Hippocampus, 366, 368
Histone acetyltransferase, 70, 77, 81, 266
Histone code, 95
Histone deacetylases (HDACs), 96, 265, 319
Histone-oligo(ADPribosyl)ation, 86
HMM profile, 389
hnRNP-U, 76, 77, 80
Homer, 366, 368, 370–372
Homology sequence, 388, 389
H-ras, 174
HSP90, 245–246
Ht31, 6, 7, 11, 12
Hydrophobic core, 462, 464–466
Hydroxyl radical, 92
Hypoxia-inducible transcription factor-1 (HIF-1), 89, 92

I
IκB, 237
IκB kinase (IKK), 345–347, 351
  activation, 241–243
  complex, 241
  structure, 241–243
ICD compartment, 68, 70
IKK&alphabdot;, 238, 241, 242
IKKβ, 238, 242
IKKγ/NEMO, 238, 242–245
IL-13, 349
IL-4, 349
Immunological synapse, 45–46, 332, 336–338, 348
Immunoreceptor tyrosine-based activation motifs (ITAMs), 327, 328
Inflammation, 84, 91, 92
Inhibitory complexes, 333
Integrin-dependent adhesion, 46
Integrin receptors, 336
Interaction
  domain mediated, 383, 390–391, 394, 396–398, 401, 402
  detection method, 385, 398
  domain-motif, 394, 402
  inhibition, 400
  protein-protein, 384–385, 390, 391, 394, 396–402
Interaction interface, 25
Interactome, 384, 385
Interchromatin domain compartment, 68, 70, 75
Intermediate filament network, 78
Intermediate filaments, 78, 96, 467
Internalization, 19, 25, 26, 31
IP3 receptor, 368, 371
IRSp53, 372, 373
Ischemic injury, 178
Itk, 337, 339

J
JNK2, 346, 348

K
KAP15/18, 11
KCNQ1, 313, 315
KIM docking domain, 148, 149
Kinase inhibitors, 434, 442, 443, 446, 453
Kinesin, 467
Kinetochore, 468, 469

L
Lamellipodia, 44
Lamina, 78, 79, 87
Lamin networks, 78–79
Laminopathies, 79–80
Lamins A-C, 78
LARBS, 134
LAT, 329–334, 336, 337, 339
Lck, 328, 329, 337, 342, 348
Leucine zipper motif, 313, 317, 462–468, 471, 477
Ligand recognition pocket, 391, 394, 401
LIM kinase, 444, 445
Lipid (membrane) rafts, 168–172
Lipid rafts, 169, 186, 189, 213, 214
Listeria monocytogenes, 42, 43, 45, 56
Long, interspersed nuclear elements (LINEs), 72
Long QT syndrome, 312, 315
L-type Ca^{2+} channel, 494–496
L-type voltage-dependent calcium channels (L-VDCC), 312

Lymphocyte antigen stimulation, 248
Lymphoproliferative, 332, 334, 350

M
mAKAP, 7–11, 136, 146, 149, 313–318, 484, 492, 495
MAP2, 486, 488
MAP kinase cascade, 92
MAP kinases (MAPKs), 17, 19, 20, 27, 328, 338, 348, 351
  ERK, 206–208, 210, 211, 213–221, 223, 224, 226
  JNK, 206, 207, 209, 216, 224, 227
  MEK, 206–211, 214, 216–221, 223, 228
  oncogenes, 220–223
  p38, 206, 207, 209, 223–225, 228
  Raf, 208, 210, 211, 213–219, 221–223, 228
  scaffolds, 206, 212, 216–218, 226–229
mDia1, 448
Meiosis, 462, 468
Melanoma, 271
Membrane-associated guanylate kinase (MAGUK), 346, 348
Membrane fusion, 468–472, 474–476
  fusion inhibitors, 475–477
Membrane/lipid, 168, 169
Membrane/lipid rafts, 168, 169
Membrane rafts, 169, 171, 172, 174–176, 178
Membrane tubulation, 192
Merlin, 445
Metastasis, 52, 53
Methyl-β-cyclodextrin (MβCD), 175
Microtubules, 17–24, 27, 29–31
Minicircles, 97
Mitosis, 462, 468
Module
  functional, 385–386
  interaction, 391–397, 401
Motif, 391, 393–397, 400
MTG8, 487
Muscle degeneration, 178
MyD88, 350, 351
Myomegalin, 136, 150

N
Na channel - SCN5A, 311–312, 315
Na^+-H^+ exchanger regulatory factor-1 (NHERF-1), 305
Na^+-H^+ exchanger regulatory factor-2 (NHERF-2), 305
Necrosis, 84, 91, 92
Nephritis, 49, 55–56.
Network interaction, 383–385, 390, 398–400, 402
Neurotransmitters, 461, 470, 471, 476
NFAT. *See* Nuclear factor of activated T cells
NF-κB, 89, 92, 328, 331, 338–340, 345–348, 350, 351
  family, 238
  pathway, 238–241, 252
NF-μNR, 95
NGF TrKA, 211, 213, 215
Nitric oxide, 92
NKT cells, 350
NMDA receptor, 370, 372, 374
Non-receptor tyrosine kinases, 178
Nonsteroidal anti-inflammatory drug (NSAID), 272, 275
NTAL, 329, 333, 334
Nuclear actin, 82
Nuclear factor of activated T cells (NFAT), 308, 309, 314, 328, 331, 339, 345, 346, 348
Nuclear hormone receptor, 81
Nuclear matrix, 68–70, 74–78, 80–83, 88, 89, 91, 95–97
Nuclear matrix-intermediate filament (NM-IF), 96
Nuclear matrix protein 1(NMP-1), 88
Nuclear matrix-targeting sequence (NMTS), 82, 83, 96
Nuclear mitotic apparatus protein (NuMA), 82–83
Nuclear myosin, 82
Nuclear scaffold, 68, 74, 79, 96
N-WASP, 432, 435–443, 449, 452, 497

O
Obscurin, 309
Occludin, 467–468
Origin-of-replication (ORI), 73

P
p21-activated kinases, 432, 440–447
p38 MAPK, 178
p42/44 ERK, 178
p42/44 MAP kinases, 178
p53, 76, 85, 88–90, 93, 497, 498
PAG, 328, 329, 333–336, 342, 343
Pak1, 432, 441–447, 449, 451, 453
Palmitoylation, 170, 173, 174, 333, 334
PARG, 83, 87

PARP
automodification, 85–87
inhibitors, 84, 89, 91
PARP-1, 69, 71, 76, 79–81, 83–89, 91–93
Pathogen-associated molecular patterns (PAMPS), 350, 351
PDE4, 334, 344, 345
and AKAPs, 126, 127, 136, 144–148, 151
and β-arrestin, 136, 137, 141–145, 151, 152, 154
and caspase-3, 138, 139
dimerisation, 131, 132, 140
and DISC1, 136, 140–141, 151
dominant negative, 128, 143, 144, 147, 151, 153–155
and EPAC, 126, 127, 146, 148–149
and ERK, 130–133, 142–146, 148, 149, 151, 154, 155
ERK phosphorylation, 132, 148
gene structure, 129–130, 141
isoform diversity, 128
lipid-protein interactions, 137
mRNA stability, 130, 133
and myomegalin, 136, 150
and phosphatidic acid, 131, 137
PKA phosphorylation, 131–133, 140, 142, 144–147, 152, 154
promoters, 130, 132, 133
protein-protein interactions, 125, 128, 132, 135–138, 140, 145, 151, 153, 155
and RACK1, 132, 134, 136, 137, 143–145, 151, 152
and SH3 domains, 132, 134, 137–139
siRNA, 145, 151
targeting, 125, 128, 133, 135–137, 143, 153
and Xap2/Ara9, 139
PDE4A1, 135–137
PDE4A1 N-terminal structure, 135–137
PDE4D, 495, 496
PDE4D3, 7–9
PDE4D3 and AKAPs, 146–148, 151
PDEs. *See* Phosphodiesterases
PDK-1, 340
PDZ domains, 303–306, 311, 336, 367, 369–373
Peptide AKAP18δ-L314E, 492
Peptide AKAP18δ-wt, 492
Peptide AKB-RI, 492
Peptide arrays, 143, 152
Peptide Ht31, 490–491, 493
Peptides, 352
Peptidomimetics, 352
Peptoids, 56
Peptomers, 56
Phagocytosis, 46
Phorbol esters 186,190, 196
Phosphatidic acid, 131, 137
Phosphatidylinositol 4,5-bisphosphate, 435
Phosphatidylinositol-3-kinase/protein kinase B (PI3K/PKB, Akt), 177
Phosphatidylinositols, 332, 339
Phosphodiesterases (PDEs), 286, 289, 292, 293, 295, 302–304, 306–308, 310, 314–318, 339, 341, 344
Phosphoinositide 3-kinases (PI3Ks), 339
Phosphoinositides, 188, 193
Phospholamban, 488, 494, 495
Phosphorylation, 16, 22–24, 30, 31, 44–52, 54, 394
Phosphorylation of caveolin, 178
Physical properties of membranes, 188, 189
PI3K, 339
PI3-kinase/Akt pathway, 92
PKA. *See* Protein kinase A
PKA-anchoring disruptor, 489, 492, 493
PKA-based sensors, 290–293
PKA type I, 341–343, 345
PKC. *See* Protein kinase C
PKCθ, 340, 346, 347
Plasma membrane, 167–170, 172, 173
Platelet aggregation, 48, 50, 51
PLCγ, 330, 331, 334
Poly(ADP-ribose) (PAR), 83–85, 89, 95
Poly(ADP-ribosyl)ation, 79, 80, 87
Postsynaptic density, 366–376
Postsynaptic density protein 95 (PSD-95), 305
PRM-binding domains (PRDs), 409–416, 419–422
Proline-rich domain, 40, 41
Proline-rich motifs (PRM), 409–422
ProSAP, 366, 367
Proteasomal degradation, 263, 264, 271
Protein fold, 388, 390
Protein
multi-domain, 385, 388, 390
single-domain, 385
Protein kinase A (PKA), 4–12, 46–52, 172, 173, 175, 176, 286–288, 290–292, 295, 302–308, 312–319
Protein kinase C (PKC), 172, 177, 248, 307, 309–311, 317–319
Protein kinase D (PKD), 317–319
Protein phosphatase 1 (PP1), 313–315
Protein-protein interactions (PPI), 25, 29, 408–410, 422
14-3-3 Proteins, 318
PSD-95, 366, 367, 369, 370, 372, 375

R
Rab32, 485
Rac, 373, 374, 432, 434, 441, 442, 445, 446, 449
Rac1, 496, 498
RACK1, 132, 134, 136, 137, 143–145, 151, 152
RAD51, 90
Rap1, 211
Rap1 (Rap2), 316, 317
Ras, 207, 208, 210–217, 220–223, 225, 226, 228
Real-time detection of cAMP, 288– 289, 292
Receptor internalization/endocytosis, 173
Receptors for activated C kinase (RACK), 307, 309–311
Receptor specificity, 28
Receptor tyrosine kinase (RTK), 170, 172, 177–178
Recombination hotspots, 73
Regulatory T cells (Treg), 332, 348
Retroelements, 72
Rho, 309, 317, 318, 373
Rho GTPases, 432–435, 440–442, 447–453
RIAD, 491–493
Ribosomes, 191
RIP1, 247, 249
RNA interference (RNAi), 269
RNA polymerase II (Pol ll), 267
RNA silencing, 128, 142, 154, 155
Rolipram, 129–134, 137, 138, 140, 141, 145, 154
Ruboxistaurin, 197
Ryanodine receptor (RyR2), 312–315, 485
RyR, 8–11

S
Salt bridges, 464, 465, 477
SAM domain, 367–370
SAP, 333, 349, 350
SAP97, 304, 305
SAPAP, 366, 368–370, 372, 375, 376
Scaffold attachment factor A (SAF-A), 77, 80–83
Scaffold-attachment factor A (SAF-A/ hnRNP-U), 76, 80
Scaffold/matrix attachment regions (S/MARs), 69–81, 83, 84, 86, 87, 90–97
Scaffolding, 432–434, 436, 438, 444, 452, 453
Scaffolds, 346, 352
Shank, 366–376
SH3, 41, 48
SH3 domain(s), 132, 134, 137–139, 332, 335, 336, 338, 348, 349, 373, 374
Short, interspersed nuclear elements (SINEs), 72
Signal transduction, 168, 169, 171–174, 176, 178, 179
Similarity
  sequence, 389
  structure, 388, 389
Signaling pathway, 390–395, 400
Single cAMP-binding domains, 295–296
SLAM, 333, 349, 350
SLAM-associated proteins or SAPs, 349
Slp-76, 331, 332, 336, 337, 339
Small-interfering RNA (siRNA), 275
S/MAR-binding potential, 90
SNARE proteins, 469–471, 475, 476
  SNARE masters, 471
Special AT-rich DNA binding protein 1 (SATB1), 93–94
Spindle apparatus, 468, 469
Spine, dendritic, 366, 368, 371–373, 376
Src, 176–178
Stem cell, 269, 270, 275
Stress fibres, 43
Stress-induced duplex destabilization (SIDD), 70, 71, 74, 76, 77, 86, 93
Subcellular fractionation, 168
Sumoylation, 94, 95
Superfamily protein, 388–390
Synapse, (not found in Chapter 19)
Systems biology, 186, 187, 197, 198, 384

T
TAK1, 347, 348, 351
TAPAS1 domain, 137
T-cell receptor (TCR), 248, 328–348, 352
Tensegrity, 96, 97
Tetramerization domain, 469
Thrombosis, 49–52
Tiam1, 498
TIRAP, 350, 351
TNFα, 240, 246–248
Toll/IL-1 receptor (TIR) domain, 350
Toll-like receptors (TLRs), 350, 351
TRAF2, 247, 249, 346, 347
TRAF6, 249, 346, 347, 351
TRAM, 350, 351
Transcription, 345
Transcription factors, 462, 463, 465, 467
  function, 463
  structure, 464–467
Transmembrane adaptor proteins (TRAPs), 329–330, 333, 334
Transport vesicles, 469–471
Trio, 498
Tropomyosin, 467

Tumor suppressor, 262, 268, 270
Tyrosine-phosphorylated, 48
Tyrosine phosphorylation, 190

U
Ubiquitin, 249, 263, 264
Ubiquitination, 246
  deubiquitinase (DUB), 251
  regulatory, 249–251
  RING E3 ligases, 250
Ubiquitin ligase, 16, 18, 20, 22, 30
Ubiquitinylation, 94
UCR1, 129, 130–132, 140, 146, 147
UCR2, 130–132, 138, 140, 141, 150
Unpairing elements (UEs), 70, 76, 77, 81, 86, 97

V
Vav, 329, 331, 332, 336–339
VEGF, 206, 213
Viruses, 471, 472, 474
  HIV, 472, 474–477
  influenza, 472, 474

W
WAVE-1, 486
WD40 domains, 191
Werner's syndrome, 79
Wilms' tumor, 269, 271
WISE, 396, 397
Wiskostatin, 435, 437–441, 452, 453
WW domain, 41, 48

X
Xap2/Ara9, 139
X-linked lymphoproliferative (XLP), 350

Y
YFP, 290–293, 296
Yin Yang 1 (YY1), 70, 85, 87–88, 93
Yotia, 495
Yotiao, 313, 315

Z
Zap-70, 328–331, 337, 346, 348
Z disc, 308–309, 311