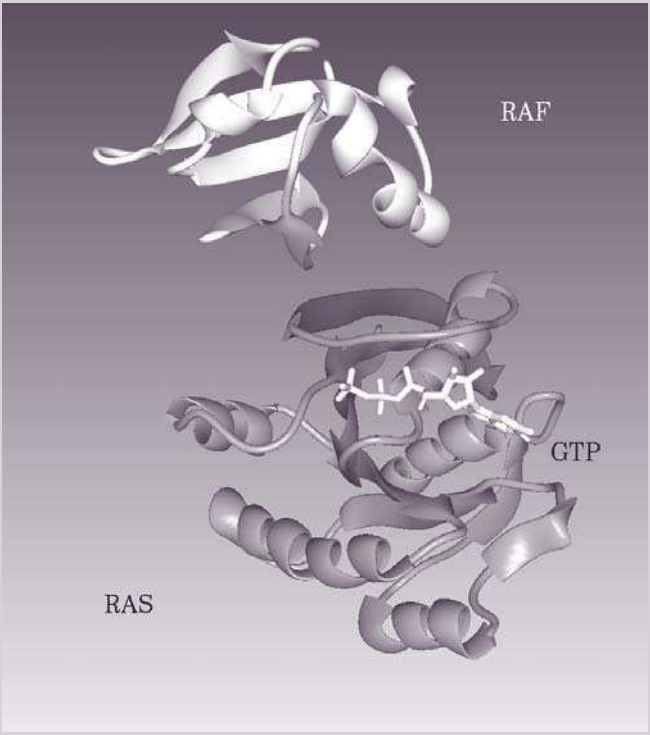
MOLECULAR BIOLOGY INTELLIGENCE UNIT 3

Hiroshi Maruta • Kazuhiro Kohama

G Proteins, Cytoskeleton and Cancer



R.G. LANDES
COMPANY

MOLECULAR BIOLOGY
INTELLIGENCE
UNIT 3

G Proteins, Cytoskeleton and Cancer

Hiroshi Maruta
Ludwig Institute for Cancer Research
Melbourne, Victoria, Australia

Kazuhiro Kohama
Department of Pharmacology
Gunma University School of Medicine
Maebashi, Gunma, Japan

R.G. LANDES
COMPANY
AUSTIN, TEXAS
U.S.A.

MOLECULAR BIOLOGY INTELLIGENCE UNIT 3

G Proteins, Cytoskeleton and Cancer

R.G. LANDES COMPANY
Austin, Texas, U.S.A.

Please address all inquiries to the Publishers:
R.G. Landes Company, 810 South Church Street, Georgetown, Texas, U.S.A. 78626
Phone: 512/ 863 7762; FAX: 512/ 863 0081

ISBN: 1-57059-551-8

Library of Congress Cataloging-in-Publication Data

G proteins, cytoskeleton, and cancer / [edited by] Hiroshi Maruta, Kazuhiro Kohama.
p. cm. -- (Molecular biology intelligence unit)
ISBN 1-57059-551-8 (alk. paper)
1. Carcinogenesis. 2. G proteins. 3. Cytoskeletal proteins.
I. Maruta, Hiroshi. II. Kohama, Kazuhiro. III. Series
[DNLM: 1. Neoplasms—etiology. 2. G-Proteins—metabolism.
3. G-Proteins—drug effects. 4. Cytoskeleton—metabolism.
5. Cytoskeleton—drug effects. QZ 202 G105 1998]
RC268.5.G2 1998
616.99'4071—dc21
DNLM/DLC
for Library of Congress 98-20518
CIP

Publisher's Note

Landes Bioscience produces books in six Intelligence Unit series: *Medical, Molecular Biology, Neuroscience, Tissue Engineering, Biotechnology* and *Environmental.* The authors of our books are acknowledged leaders in their fields. Topics are unique; almost without exception, no similar books exist on these topics.

Our goal is to publish books in important and rapidly changing areas of bioscience for sophisticated researchers and clinicians. We would like to thank our readers for their continuing interest and welcome any comments or suggestions they may have for future books.

Judith Kemper
Production Manager
R.G. Landes Company

CONTENTS

Section I: Actin-Cytoskeleton

EDITORS

Hiroshi Maruta
Ludwig Institute for Cancer Research
Melbourne, Victoria, Australia
Chapters 10, 11

Kazuhiro Kohama
Department of Pharmacology
Gunma University School of Medicine
Maebashi, Gunma, Japan
Chapter 4

CONTRIBUTORS

Sohail Ahmed
Department of Neurochemistry
Institute of Neurology
London, England *and*
Glaxo-IMCB Group
Institute of Molecular and Cell Biology
National University of Singapore
Singapore
Chapter 16

Klaus Aktories
Institut für Pharmakologie
und Toxikologie
Universität Freiburg
Freiburg, Germany
Chapter 23

Avri Ben-Ze'ev
Department of Molecular Cell Biology
The Weizmann Institute of Science
Rehovot, Israel
Chapter 7

André Bernards
Massachusetts General Hospital
Cancer Center
and Harvard Medical School
Charlestown, Massachusetts, U.S.A.
Chapter 18

Hugh D. Campbell
Centre for Molecular Structure
and Function
Research School of Biological Sciences
The Australian National University
Canberra, Australia
Chapter 17

Marie F. Carlier
Laboratoire d'Enzymologie et
Biochimie Structurales, C.N.R.S.
Gif Sur Yvette, France
Chapter 1

Graham P. Côté
Department of Biochemistry
Queen's University
Kingston, Ontario, Canada
Chapter 8

Julian Downward
Imperial Cancer Research Fund
London, England, UK
Chapter 12

Hisakazu Fujita
Division of Gene Regulation
Cancer Institute
Hokkaido University School of Medicine
Sapporo, Japan
Chapter 9

Kiyoko Fukami
Department of Biochemistry
The Institute of Medical Science
The University of Tokyo
Tokyo, Japan
Chapter 8

John A. Hammer, III
Laboratory of Cell Biology
National Heart, Lung and Blood Institute
National Institutes of Health
Bethesda, Marylland, U.S.A.
Chapter 3

Sueharu Horinouchi
Department of Biotechnology
Graduate School of Agriculture and Life Sciences
The University of Tokyo
Tokyo, Japan
Chapter 22

Ryoki Ishikawa
Department of Pharmacology
Gunma University School of Medicine
Maebashi, Gunma, Japan
Chapter 4

Toshimasa Ishizaki
Department of Pharmacology
Kyoto University Faculty of Medicine
Yoshida, Kyoto, Japan
Chapter 15

Kenneth S. Koblan
Department of Cancer Research
Merck Research Laboratories
West Point, Pennsylvania, U.S.A.
Chapter 19

Nancy E. Kohl
Department of Cancer Research
Merck Research Laboratories
West Point, Pennsylvania, U.S.A.
Chapter 19

Robert Kozma
Department of Neurochemistry
Institute of Neurology
London, England *and*
Glaxo-IMCB Group
Institute of Molecular and Cell Biology
National University of Singapore
Singapore
Chapter 16

C. Chandra Kumar
Department of Tumor Biology
Schering-Plough Research Institute
Kenilworth, New Jersey, U.S.A.
Chapter 20

Noboru Kuzumaki, M.D.
Division of Gene Regulation
Cancer Institute
Hokkaido University School of Medicine
Sapporo, Japan
Chapter 9

Louis Lim
Department of Neurochemistry
Institute of Neurology
London, England *and*
Glaxo-IMCB Group
Institute of Molecular and Cell Biology
National University of Singapore
Singapore
Chapter 16

Yu-Tsueng Liu
University of Texas Southwestern Medical Center at Dallas
Dallas, Texas
Chapter 2

Yoshiaki Monden
Banyu Tsukuba Research Institute in collaboration with Merck Research Laboratories
Tsukuba, Ibaraki, Japan
Chapter 21

Brett P. Monia
Department of Molecular Pharmacology
Isis Pharmaceutcials
Carlsbad, California, U.S.A.
Chapter 24

Shuh Narumiya
Department of Pharmacology
Kyoto University Faculty of Medicine
Yoshida, Kyoto, Japan
Chapter 15

Susumu Nishimura
Banyu Tsukuba Research Institute in collaboration with Merck Research Laboratories
Tsukuba, Ibaraki, Japan
Chapter 21

Makoto Ohtsu, M.D.
Division of Gene Regulation
Cancer Institute
Hokkaido University School of Medicine
Sapporo, Japan
Chapter 9

D. Pantaloni
Laboratoire d'Enzymologie et Biochimie Structurales, C.N.R.S.
Gif Sur Yvette, France
Chapter 1

Anne J. Ridley
Ludwig Institute for Cancer Research
and Department of Biochemistry and Molecular Biology
University College of London
Gower Street
London, U.K.
Chapter 14

José Luis Rodríguez-Fernández
Cancer Research Unit
Clínica Universitaria
Facultad de Medicina
Universidad de Navarra
Pamplona, Spain
Chapter 6

Andrew L. Rozelle
University of Texas Southwestern Medical Center at Dallas
Dallas, Texas
Chapter 2

Enrique Rozengurt
Department of Medicine
UCLA School of Medicine
Los Angeles, California, U.S.A.
Chapter 6

Norio Sakai, M.D.
Division of Gene Regulation
Cancer Institute
Hokkaido University School of Medicine
Sapporo, Japan
Chapter 9

Klaus Scheffzek
Max-Planck-Institut für Molekulare Physiologie
Dortmund, Germany
Chapter 13

Nobuko Shindo-Okada
Biology Division
National Cancer Center Research Institute
Tokyo, Japan
Chapter 21

Dennis Strand
Department of Developmental Genetics
Deutsches Krebsforschungszentrum
Heidelberg, Germany
Chapter 5

Tadaomi Takenawa
Department of Biochemistry
The Institute of Medical Science
The University of Tokyo
Tokyo, Japan
Chapter 8

Motoyoshi Tanaka, M.D.
Division of Gene Regulation
Cancer Institute
Hokkaido University School of Medicine
Sapporo, Japan
Chapter 9

Naoki Watanabe
Department of Pharmacology
Kyoto University Faculty of Medicine
Yoshida, Kyoto, Japan
Chapter 15

Alfred Wittinghofer
Max-Planck-Institut für Molekulare Physiologie
Dortmund, Germany
Chapter 13

Helen L. Yin, Ph.D.
University of Texas Southwestern Medical Center at Dallas
Dallas, Texas
Chapter 2

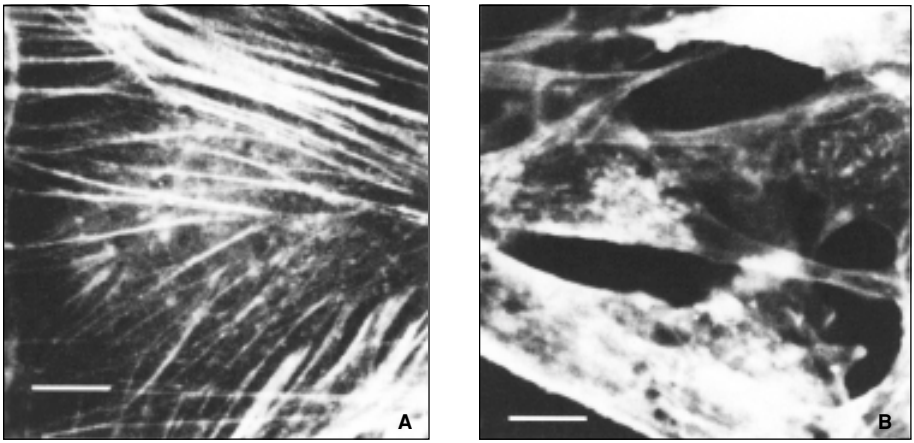
Minoru Yoshida
Department of Biotechnology
Graduate School of Agriculture and Life Sciences
The University of Tokyo
Tokyo, Japan
Chapter 22

PREFACE

The malignant transformation of normal epithelial, endothelial or fibroblastic cells by tumor virus infection or oncogenic (gain-of-function) mutations of cellular proto-oncogenes or loss-of-function mutations of tumor suppressor genes causes two characteristic changes in their growth behavior: (i) a loss of contact inhibition in a liquid culture, and (ii) anchorage-independent growth in a suspension culture such as soft agar. When normal fibroblasts such as NIH 3T3 cells reach a confluence in a liquid culture on a solid substratum, they stop growing and form a confluent monolayer. This phenomenon is called "contact inhibition of cell growth" (Abercrombie 1962). However, when cells are transformed by SV40 virus or oncogenes such as Ras, they are no longer contact-inhibited, and continue to grow and form foci by piling up on top of each other. In other words, transformed cells ignore the contact by other cells. Furthermore, unlike normal fibroblasts which require their adhesion to (or anchorage on) the solid substratum for their growth, and therefore cannot grow in soft agar, transformed cells no longer require their anchorage on the substratum, and therefore can grow in soft agar and form colonies. The molecular mechanism of the transformation-induced loss of both contact inhibition and anchorage-dependency still remains a big "unknown" to be solved by molecular oncologists during the coming new century.

However, an important key to our understanding of this transformation mechanism was discovered by Klaus Weber and his colleagues more than two decades ago (Weber et al 1974). They found that actin stress fiber or cable, a complex of actin filament (F-actin) and double-headed myosin (myosin II), rapidly disappears when 3T3 fibroblasts are transformed by SV40 virus. Later many other scientists confirmed that both the disruption of actin cables and subsequent induction of membrane ruffling commonly take place with the malignant transformation by many other oncogenes such as v-Ha-Ras and v-Src. This finding certainly sparked the mind of many enthusiastic young scientists including Helen Yin and myself and urged us to isolate and characterize a variety of actin-binding proteins (ABPs) including gelsolin, vinculin, alpha-actinin, tensin and single-headed myosins (myosin I). Interestingly, these distinct ABPs are localized with the focal adhesion plaques (FAPs), cellular tiptoes, where cells adhere to the solid substratum, and actin cables are originated. Furthermore, a similar set

Klaus Weber, ca. 1974



Actin stress fibers disappear upon malignant transformation. (A) normal fibroblasts and (B) transformed fibroblasts. Reprinted with permission from Weber K et al, Cold Spring Harbor Symp. Quant. Biology 1974; 39:367.

of ABPs is also localized at the cell-cell contact (CCC) sites, and the membrane ruffling region. These observations strongly suggest that these ABPs play a critical role in both the organization of actin-cytoskeleton and the growth control by the CCC sites and FAPs.

Finally, a few years ago, Avri Ben-Ze'ev and his colleagues for the first time demonstrated that over-expression of either vinculin or alpha-actinin suppresses SV40-induced malignant transformation of NIH 3T3 cells, and restores both their "contact inhibition" and "anchorage-dependency" of growth (Fernandez et al 1992; Glueck et al 1993). Independently Noboru Kuzumaki and his colleagues also showed that over-expression of a gelsolin mutant also reverses v-Ha-Ras-induced malignant transformation of the same fibroblasts (Muellauer et al 1993). These findings directly proved that these ABPs localized at FAPs and CCC sites are responsible for the anchorage-dependency and contact inhibition of cell growth, respectively. However, the detailed molecular mechanism underlying tumor suppression by these ABPs still remains to be further elucidated. One clue to our understanding of the molecular mechanism of Ras-induced changes in actin-cytoskeleton organization has emerged recently: Ras-transformation blocks the cross-linking of actin filaments by the SH3 protein EMS1/cortactin at least in part through a unique acidic phospholipid called PIP_2 (He 1997). Interestingly, over-expression of an EMS1-related PIP_2-binding protein called HS1 restores the ability of EMS1 to cross-link actin filaments, and reverses Ras-transformation. Most importantly, a mutant of cofilin that no longer binds F-actin but still binds PIP_2, is able to suppress Ras-induced malignancy (Maruta 1996), supporting a previous notion that PIP_2 is essential for both Ras-induced malignancy and changes in actin-cytoskeleton (Fukami et al 1988). So far all F-actin-binding proteins that suppress SV40/Ras-induced malignancy bind PIP_2, without any exception.

How does the monomeric G protein Ras induce the production of PIP_2? Julian Downward and his colleagues recently found that Ras activates PI-3 kinase that in turn activates another monomeric G protein Rac through a Rac GDS (Rodrigez-Viciana et al 1997). John Hartwig and his colleagues have found that Rac activates PI-4/PI-5 kinases which produce PIP_2, and PIP_2 in turn uncaps the plus-end of actin filament to induce a rapid actin polymerization by inactivating the plus-end capping proteins such as gelsolin, CapG and tensin (Hartwig et al 1995). Eventually Rac induces membrane ruffling (Ridley et al 1992), although the precise mechanism still remains to be determined.

Importantly, in addition to Rac (Qui et al 1995), at least two other members of Rho family G proteins (RhoB and CDC42) are also required for both Ras-induced malignancy and changes in actin cytoskeleton (Lebowitz et al 1995; Qui et al 1997). Unlike other Rho GTPases which are responsible for the formation of stress fibers (Ridley and Hall 1992), RhoB appears to be responsible for the disruption of actin stress fibers. Louis Lim and his colleagues found that CDC42 is involved in the formation of microspikes (Kozma et al 1996).

Furthermore, his group and other groups, in particular Shuh Narumiya's and Kozo Kaibuchi's, identified several distinct effectors of these Rho family GTPases such as the CDC42/Rac-activated kinase PAK and the Rho-activated kinase Rock. Rock induces both actin stress fiber formation and focal adhesions (Amano et al 1997), whereas PAK causes the loss of both stress fibers and focal adhesions (Manser et al 1997). In addition, using the bacterial exotoxin C3 that inactivates selectively Rho GTPases by ADP-ribosylation (Ohashi et al 1987; Aktories et al 1987), Yoshimi Takai's and Issei Mabuchi's groups found that Rho is required for the contractile ring formation during cytokinesis (Kishi et al 1993; Mabuchi et al 1993). Interestingly Ras, Rac and CDC42 are also required for the cytokinesis. Like stress fibers, the contractile ring is an actomyosin II-based complex. Recently a few distinct families of proteins called IQGAPs, Myr5 and Flightless I that bind both F-actin and Rho/Ras family GTPases (Campbell et al 1993; Reinhard et al 1995; Brill et al 1996). At least IQGAPs are known to be essential for cytokinesis. Thus, in the near future, the detailed mechanism underlying the Ras/Rho family GTPase-dependent cytokinesis shall be revealed.

Both myosins I and II, the intrinsic partners of F-actin, have recently begun to draw much attention of oncologists, as a myosin II-binding protein called l(2)gl was identified as a tumor suppressor (Strand et al 1994), and both actomyosin I and II ATPases are activated by PAK and Rock family kinases (Amano et al 1996; Lee et al 1996).

Based on these basic findings on the oncogenic Ras signaling network, several anti-Ras cancer drugs or toxins have been developed: (i) farnesyltransferase inhibitors and the bacterial toxin LT inactivate Ras or Rho family GTPases directly (Kohl et al 1993; Just et al 1996) and (ii) Azatyrosine, Radicicol and SCH51344 inactivate further downstream effectors such as Raf and Rac (Shindo-Okada et al 1989; Kumar et al 1995; Yoshida et al 1997). Other drugs such as TSA induce anti-Ras tumor suppressors such as gelsolin (Yoshida

et al 1995). Furthermore, anti- sense Ras/Raf oligonucleotides selectively downregulate Ras/Raf gene expression (Monia 1997). These drugs could be potentially useful for the chemotherapy of Ras-associated cancer. Furthermore, several distinct tumor suppressor genes (TSGs) have been identified that suppress Ras transformation (Muellauer et al 1993; Maruta 1996). The TSGs encode F-actin/PIP2-binding proteins such as gelsolin, NF2, tensin, cofilin and HS1 (Tikoo et al 1994), Ras-binding peptides such as NF1 and Raf fragments (Nur-E-Kamal et al 1993), and the large GTPase p190-A (Wang et al 1997). These genes could be potentially useful for the genotherapy of Ras-associated cancer which represent more than 30% of all human carcinomas, notably more than 90% of pancreatic cancers and 50% of colon cancers.

As reviewed by Julian Downward and others, the recent progress in our understanding of Ras signaling network leading to the malignant transformation including the re-organization of actin-cytoskeleton becomes extremely rapid, owing to the combination of our cutting-edge knowledge in molecular biology of G proteins, biochemistry of actin-cytoskeleton, and rational molecular modeling of new anti-cancer drugs. It should be noted that the 3D structure of a Ras-GAP complex determined by Fred Wittinghofer and his colleagues (Scheffzek et al 1997) would probably make it possible for us to design and create a "magic bullet" called "SuperGAP", i.e., our "Holy-Grail" anti-Ras peptide or chemical, that stimulates even the intrinsic GTPase activity of oncogenic Ras mutants, thereby attenuating their malignant activity at last. We hope this unique book will provide us with a great opportunity of educating each other and many other readers who would share the common goal, finding a cure for cancer, and promoting a "quantum" leap in this ever-growing field towards the coming new century... .

Hiroshi Maruta
Ludwig Institute for Cancer Research
Melbourne, Australia
September 1997

References

Abercrombie M. Cold Spring Harbor Symp Quant Biol 1962; 27:427.
Aktories K et al. FEBS Lett 1987; 212:109.
Amano M et al. J Biol Chem 1996; 271:20246.
Amano M et al. Science 1997; 275:1308.
Brill S et al. Mol Cell Biol 1996; 16:4869.
Campbell H et al. Proc Natl Acad Sci USA 1993; 90:11386.
Fernandez JLR et al. J Cell Biol 1992; 119:427.
Fukami K et al. Proc Natl Acad Sci USA 1988; 85:9057.
Glueck U et al. Proc Natl Acad Sci USA 1993; 90:383.
Hartwig J et al. Cell 1995; 82:643.
He H. Jpn J Cancer Chemother 1997; 24:1448.
Just I et al. J Biol Chem. 1996; 271:10149.
Kishi K et al. J Cell Biol 1993; 120:1187.

Kohl N et al. Science 1993; 260:1934.
Kozma R et al. Mol Cell Biol 1996; 16:5069.
Kumar C et al. Cancer Res 1995; 55:5106.
Lebowitz P et al. Mol Cell Biol 1995; 15:6613.
Lee SF et al. J Biol Chem 1996; 271:27044.
Mabuchi I et al. Zygote 1993; 1:325.
Manser E et al. Mol Cell Biol 1997; 17:1129.
Maruta H. In: Maruta H, Burgess AW, eds. Regulation of the RAS Signaling Network. Austin: RG Landes & Springer-Verlag, 1996:139.
Monia B. In: Krieg A, Stein C, eds. Applied Anti-Sense Oligonucleotide Technology. John Wiley & Sons Inc., 1997: (in press).
Muellauer L et al. Oncogene 1993; 8:2531.
Nur-E-Kamal, MSA et al. J Biol Chem 1993; 268:22331.
Ohashi Y et al. J Biol Chem 1987; 262:1430.
Qiu RG et al. Nature 1995; 374:457.
Qui RG et al. Mol. Cell Biol 1997; 17:3449.
Reinhard J et al. EMBO J 1995; 14:697.
Ridley A et al. Cell 1992; 70:401.
Ridley A, Hall A. Cell 1992; 70:389.
Rodriguez-Viciana P et al. Cell 1997; 89:457.
Scheffzek K et al. Science 1997; 277:333.
Shindo-Okada N et al. Mol Carcinog 1989; 2:159.
Strand D et al. J Cell Biol 1994; 127:1361.
Tikoo A et al. J Biol Chem 1994; 269:23387.
Wang D et al. Cancer Res 1997; 57:2478.
Weber K et al. Cold Spring Harbor Symp Quant Biol 1974; 39:363.
Yoshida N et al. BioEssays 1995; 17:423.
Yoshida N. Jpn J Cancer Chemother 1997; 24:1512.

Section I
Actin-Cytoskeleton

CHAPTER 1

Regulation of Actin Assembly and Disassembly

Marie F. Carlier and D. Pantaloni

Actin filaments are dynamic polymers that can assemble and disassemble in a temporally and spatially controlled fashion in living cells, to drive cellular and intracellular movements such as shape changes, cell locomotion, phagocytosis, or particle transport. In recent years, evidence has accumulated showing that actin assembly can produce force (see ref. 1 for a recent review) in response to appropriate stimuli; hence the actin cytoskeleton behaves as a sensory-motor organ of the eukaryotic cell. Noninvasive techniques such as time-resolved observation of motile processes in living cells following injection of fluorescently labeled molecules give a detailed description of actin dynamics in these movements.

One main feature of actin self-assembly is that it is an energy-dissipating reaction, exactly like the assembly of microtubules. These two polymers, which organize intracellular space, are part of a large family of nucleoside triphosphatases involved in energy and signal transduction.[2]

In this chapter we will try to show how the different steps of the ATPase cycle linked to actin filament turnover can be used and modulated by actin binding proteins to generate a variety of actin-based motility processes.

Actin Filament Assembly and Turnover

ATP Hydrolysis in Actin Polymerization: The ATPase Cycle of Actin

In the 1980s, a large number of in vitro experiments have led to a description of actin assembly in terms of a nucleation-elongation process.[3-5] Kinetic data were consistent with the nucleus being a trimer, which was confirmed by the atomic model of the filament derived from the structure of crystallized actin-DNaseI.[6] The self-assembly of actin is accompanied by ATP hydrolysis which takes place on the filament. Figure 1.1 describes the ATPase cycle of actin in which are involved association of monomeric ATP-G-actin to a filament end, ATP hydrolysis on the polymerized subunit, dissociation of ADP-G-actin and regeneration of ATP-G-actin through nucleotide exchange which occurs on G-actin (but not on F-actin), in a medium rich in ATP. The release of P_i is slower than the chemical cleavage of the γ-phosphodiester bond of ATP, and is rate-limited by the isomerization of a central F-ADP-P_i complex, as is generally the case for nucleoside triphosphatases. Hence the F-ADP-P-actin species is a measurable transient in actin assembly. The release of P_i is accompanied by a conformation change of the F-actin subunit which leads to a weakening of its interactions with adjacent subunits in the filament. The main effect of ATP hydrolysis in actin assembly is to increase the rate of monomer dissociation from the filament end: ADP-actin

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

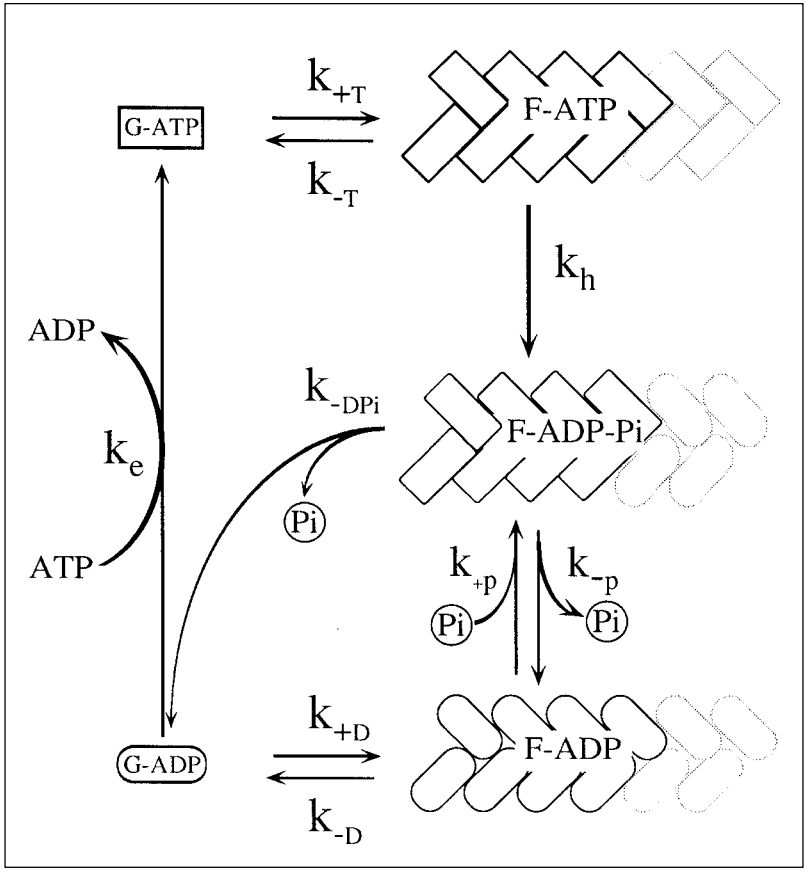


Fig. 1.1. The ATPase cycle of actin. This scheme features the nucleotide-bound states of monomeric G-actin and polymeric F-actin, the monomer-polymer exchange reactions at filament ends in each state and the nucleotide exchange and hydrolysis reactions occurring on G-actin and F-actin. In a medium containing ATP, under physiological ionic conditions, nucleotide exchange occurs on G-actin exclusively whereas ATP hydrolysis occurs on F-actin exclusively. ATP hydrolysis takes place in several consecutive elementary steps. Chemical cleavage of the γ-phosphoester bond leads to a transition-state intermediate F-ADP-P* in which the γ-phosphate group is thought to adopt a pentacovalent configuration, and is mimicked in this configuration by AlF_4^- (14). The transition F-ADP P* → F-ADP-P_i is quasi-irreversible and limits the rate of P_i release which follows (14). Hence this isomerization F-ADP-P* - F-ADP-P_i is linked to the large free energy and structural change of the filament in which the actin-actin bonds are weakened and the rigidity of the filament decreases. The known values of the different rate constants for association-dissociation of ATP- or ADP-G-actin at filament ends, and for nucleotide exchange are listed in Table 1.1.

Table 1.1. Equilibrium and kinetic parameters involved in actin filament steady state and turnover

Parameter		Value	Reference
	C_C^P	0.6 μM	4, 5 (reviews)
	C_C^B	≤ 0.1 μM	4, 5
Barbed end	k_{+T}	5-10 μM^{-1} s^{-1}	4, 5
	k_{-T}, k_{-DPi}	1 s^{-1}	5, 7, 8
	k_{-D}	10 s^{-1}	4, 5
Pointed end	k_{+T}	0.5-1 $\mu M^{-1}s^{-1}$	4, 5
	k_{-D}	0.5-1 s^{-1}	4, 5, 20, 21
	k_e	0.2 s^{-1}	36
	k'_e	24 s^{-1}	36
	k_h	14 s^{-1}	5
	k_{-P}	n.d.	

The notation used for the rate constants is as on Figures 1.1 and 1.2. k_e represents the rate of dissociation of MgADP from ADP-G-actin, k'_e from the profilin-MgADP-G-actin complex. All values are under physiological ionic conditions.

dissociates from filament barbed ends about 10-fold faster than ADP-P_i- or ATP-actin.[7,8] It is of great interest to know which actin-actin contact is destabilized upon P_i release. Conventionally, two main actin-actin bonds, along directions perpendicular and parallel to the filament axis respectively, describe the connectivity of actin subunits in the filament. Electron microscopy studies[9-11] have shown that the structural order of the filament is altered as P_i is released, which can be accounted for by a change in orientation of subdomain 2[11] consistent with a change in the actin-actin contacts along the 2-start helix. Recently, an analysis of the mechanical properties of the actin filament also showed that P_i release is accompanied by an increase in flexibility of the filament,[12] which suggests that ATP hydrolysis in actin assembly might be used to produce force.

The use of fluoroaluminate AlF_4^- and fluorobenyllate BeF_3^-, H_2O as structural analogs of Pi, has helped to probe the role of ATP hydrolysis in F-actin dynamics.[13] Biochemical evidence led us to propose that these complexes bound F-ADP actin and reconstituted the transition state of ATP hydrolysis in which the γ-phosphate is in a pentacovalent configuration.[14] The X-ray structures of a G-protein in complex with GDP and AlF_4^-[15] and of myosin subfragment-1 in complex with ADP and AlF_4^-[16] bring support to this proposal.

Significance of the Critical Concentration for Actin Assembly in the Presence of ATP vs. ADP

In vitro experiments aimed at measuring the sequence of reactions occurring during the self-assembly process of massive amounts of G-actin subunits into an F-actin polymer have been useful to obtain the real values of rate constants for these reactions under ionic physiological conditions. However, in the living cell, the ionic conditions are such that actin is and remains essentially polymerized (F-actin) and at steady-state with G-actin subunits at the critical concentration. Hence the actual processes to be considered as operating in

living cells are monomer-polymer exchange reactions leading to filament turnover, and shifts in steady state of actin assembly. Before commenting on the multiple ways by which these processes can be regulated to drive motility, it is necessary to define the critical concentration at steady-state.

The comparison of the polymerization properties of ATP-actin and ADP-actin (5 for review) has been helpful to understand the effect of ATP hydrolysis on the dynamics of the filament. The polymerization of ADP-actin is a truly reversible process, as described by Oosawa,[17] which means that association of an ADP-actin monomer to a filament end (the elongation site) regenerates an identical elongation site. Hence the rate of filament growth J(c) varies linearly with the concentration of ADP-G-actin(c) as described by the classical equation:

$$J(c) = k_+ c - k_- \quad (1)$$

where k_+ and k_- are the rate constants for ADP-G-actin association to and dissociation from a filament end. The critical concentration $C_c = \frac{k_-}{k_+}$ at which J = 0 is a true monomer-polymer equilibrium dissociation constant, and the ratio $\frac{k_-}{k_+}$ is identical at the two filament ends (barbed and pointed). The polymerization of ATP-actin differs from the above description due to ATP hydrolysis associated to polymerization. Actively growing filaments have terminal ADP-P_i subunits at the barbed end (ATP-subunits in a regime of rapid growth) which dissociate more slowly (k_{-DPi}) from filament ends than ADP-subunits (k_{-D}). As a result the J(c) plot is linear only in a regime of net positive growth, but shows a downward curvature in the region of the critical concentration where J = 0 (Fig. 1.2). The critical concentration at the barbed end no longer has the meaning of an equilibrium dissociation constant because the association flux of ATP-G-actin, $k_{+T}C^B_c$ then is balanced by a dissociation flux of both ADP- and ADP-P_i subunits, which dissociate at different rates k_{-D} and k_{-DPi}. The critical concentration therefore is a steady-state concentration of ATP-G-actin defined as

$$C_c^B = \frac{k_{-D}x + k_{-DPi}(1 - x)}{k_{+T}} \quad (2)$$

where x represents the probability that a terminal subunit has ADP bound, which depends on the rate of P_i release on this subunit. So far x is not known, only an experimental determination of C_c^B is available.

From equation (2) it appears that the value of C_c^B is lower, i.e., filaments are more stable, if x is lower. Hence putative agents that can decrease the rate of P_i release, will increase the proportion of slowly dissociating F-ADP-P_i subunits at filament ends and decrease the value of C_c^B. The converse effect is expected from agents causing an increase in x.

ATP Hydrolysis Allows a Difference in Critical Concentrations at the Two Ends

Wegner[18] showed that the free energy of ATP hydrolysis associated to actin polymerization establishes an energetic difference between the two ends. Steady-state measurements of the concentration of ATP-G-actin coexisting with filaments with versus without blocked barbed ends, as well as measurements of the ATP-G-actin concentration dependence of the rate of filament growth at the two ends separately (Fig. 1.2), have confirmed[19-21] that in the presence of ATP the critical concentration at the barbed end is 5- to 7-fold lower than at the pointed end. When both ends are free, the steady state concentration of monomer C_{ss} (observed critical concentration) is close to the critical concentration of the end where the fluxes of association and dissociation of subunits are more rapid, i.e., the barbed end. As a result the rate of treadmilling, $V_z = k^B_+ (C_{ss} - C_c^B)$ is very slow. It is limited by the rate of subunit dissociation from the pointed end at steady state.

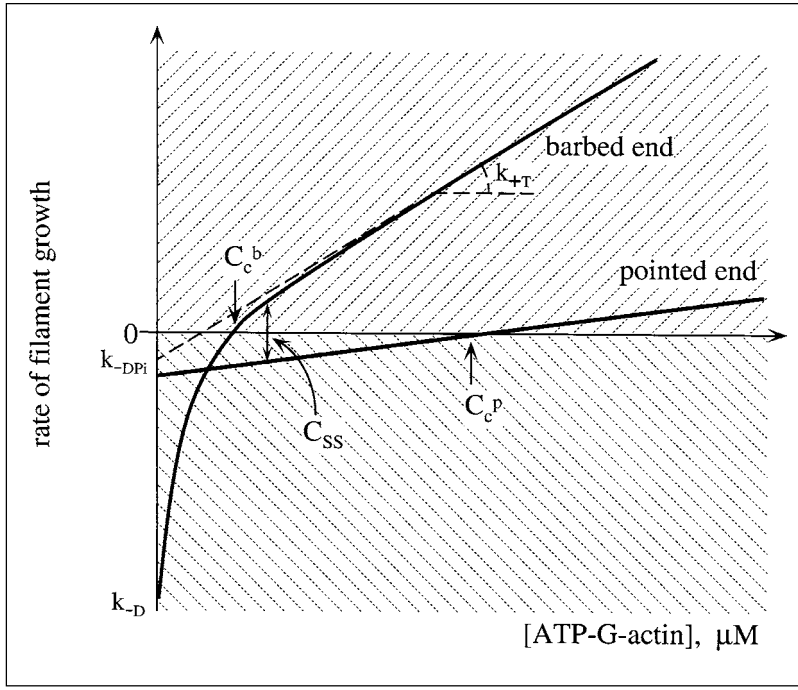


Fig. 1.2. Dynamics of actin assembly at the barbed and at the pointed end in the presence of ATP. The rate of filament growth at the barbed end (B) and at the pointed end (P) is represented as a function of ATP-G-actin concentration.

Filament Turnover in ADP and ATP

In a medium containing ADP, turnover of individual F-ADP-actin filaments results from monomer-polymer exchange at the ends, which occurs as a diffusion-like, $\sqrt{\text{time}}$-dependent penetration of actin subunits from the monomer pool (at the critical concentration) into the polymer. In the presence of ATP, the same reactions take place, however the turnover rate is regulated by ATP hydrolysis, since polymer loss is favored when ADP-subunits, rather than ADP-P_i-subunits, are exposed at the filament ends. Loss of long stretches of F-ADP-actin at steady-state may occur, because the released ADP-G-actin subunits do not rebind with an affinity comparable to the ATP-G-actin subunits present in solution. This process may lead to a fiber-by-fiber renewal in a population of short filaments.

As illustrated in Figure 1.2, because the critical concentrations are different at the two ends, bulk solutions of filaments with both ends free undergo head-to-tail polymerization, or treadmilling, at steady-state, net slow assembly onto the barbed ends being exactly compensated, overall the population, by net slow disassembly from the pointed end. Treadmilling of pure actin in vitro is a very slow process (0.05 μm/min under physiological ionic conditions), which is by itself unlikely to account for actin-based motile processes such as pseudopod extension or the forward movement of the leading edge of locomoting cells, which develop at a rate one order of magnitude higher. Yet FRAP measurements indicate that these processes are mediated by the steady state rate of barbed end growth beneath the plasma membrane[22] in a treadmilling mechanism. These results suggested that treadmilling is regulated in vivo.

Another reaction should be considered as potentially affecting the turnover rate of very crowded solutions of filaments: When filaments are at a high number concentration, which can be obtained, in vitro, by controlled fragmentation of F-actin, evidence has been shown for a steady-state accumulation of ADP-G-actin.[23] This results from the large flux of depolymerizing ADP-subunits (which is proportional to the concentration of filament ends), and of the disappearance of ADP-G-actin via either reassociation to filaments or regeneration of ATP-G-actin through nucleotide exchange (see Fig. 1.1). The amount of ADP-G-actin at steady-state is given by the following equation:

$$[\text{ADP-G-actin}]_{ss} = \frac{k_{-D}[F_D]}{k_{+D}[F] + k_{-A}} \qquad (3)$$

The fact that, in vitro at least, ADP-G-actin accumulates to a steady level in solutions containing a large number of filaments (typically 50 nM or more filament ends), means that ADP dissociation from monomeric actin then becomes rate limiting in the turnover of the F-actin population. The exact local number concentration of filament ends in different regions of living cells is not well known. Whether it falls in a relevant range for nucleotide exchange to limit the filament turnover rate is an open question. Filament-severing proteins such as gelsolin increase the number of filaments, however they remain bound to the newly created barbed ends and blocking monomer-polymer exchange at this end.

In conclusion, the different effects of ATP hydrolysis in actin assembly point to the different crucial steps in the ATPase cycle at which regulation of the steady-state of actin assembly can be effected by actin binding proteins as follows:

1. Proteins binding to monomeric actin preferentially shift the G-actin-F-actin steady state toward the monomer pool and sequester G-actin.
2. Proteins which bind to F-actin specifically can affect monomer dissociation and filament turnover, and modify the stability of the filaments (e.g., tropomyosin, ref. 24).
3. Proteins (P) which bind both F- and G-actin can be considered as making with actin (A) a PA complex that behaves like a polymerizable iso-actin. If the rate parameters for association-dissociation of PA in filaments are different from those of actin, P acts as a modulator of actin dynamics.
4. Proteins which strongly cap the barbed ends slow down filament turnover, but also increase the critical concentration. These proteins therefore govern the steady state of assembly, and control sequestration of G-actin.
5. Proteins which affect the rate of P_i release on terminal F-actin subunits at steady state are expected to change the proportion of rapidly dissociating F-ADP-actin and slowly-dissociating F-ADP-P_i actin at the barbed end. The critical concentration for assembly at the barbed end would be modified by such regulators.
6. Proteins which increase (resp. decrease) the rate of nucleotide exchange on monomeric actin are expected to increase (resp. decrease) the turnover rate of F-actin in highly crowded solutions of filaments.

Some of the above-listed activities are supported by well known actin-binding proteins, and their interplay in actin-based motility processes has recently been unraveled, as will be outlined in the next section.

Shifts in the Steady State of Actin Assembly Are Orchestrated by Capping-Uncapping of Actin Filaments and Promote Changes in the Amount of Assembled Actin in the Presence of Actin-Sequestering Proteins

It has long been recognized that changes in the level of actin assembly are elicited by growth factor stimulation and cell transformation and drive the cell morphological response.[25] This is made possible by the use of a large pool of unassembled actin (10-250 μM in different cell types), which is maintained in the monomer state by interaction with G-actin binding proteins. The components of the signal transduction cascade leading to actin assembly in stimulated cells are not all identified yet, however it is clear that upon stimulation, actin subunits are shifted from the pool of sequestered monomers to the pool of F-actin, by simple dissociation of the profilin-actin or $T\beta_4$ -actin complexes.

Compare $T\beta_4$ and Profilin Activities

Profilin was the first discovered G-actin-binding protein.[26] It is a ubiquitous, abundant (5-50 μM) protein whose structure has recently been elucidated.[27-29] The cellular amount of profilin however is lower than the concentration of unassembled actin. The leading candidate for actin sequestration in most eukaryotic cells was found to be thymosin β_4, a 5 kDa small protein first discovered in platelets.[30,31] Other G-actin-binding proteins such as ADF (actin depolymerizing factor[32]) or cofilin,[33] seem to exist in lower amounts compared to $T\beta_4$ and profilin.

Both $T\beta_4$[34] and profilin[35-37] bind to ATP-G-actin with a much higher affinity than to ADP-G-actin. $T\beta_4$ shows a 50-fold preference, profilin a 20-fold preference for ATP-actin. Hence actin is sequestered in the cells as ATP-G-actin, which is ready to polymerize upon dissociation of the actin-sequestering protein complexes.

Tβ4, ADF and profilin have opposite effects on nucleotide exchange on G-actin: Tβ4 and ADF slow it down, while profilin accelerates it.[32,37,38] Under physiological conditions, the rates of dissociation of MgATP and MgADP are enhanced 40-fold and 120-fold respectively by profilin.[37] The enhancement of nucleotide exchange on G-actin by profilin was initially thought to be important in its function. However, recent results (for a review see ref. 39) showed that plant profilins which do not enhance nucleotide exchange can functionally replace profilin in other cells following the knock out of the endogenous profilin. The sequestering efficiency of G-actin-binding proteins depends both on their affinity for ATP-G-actin, and on the concentration of free ATP-G-actin in solution. Under physiological ionic conditions, the concentration of free G-actin is buffered by the pool of F-actin and is equal to the steady state monomer concentration for actin assembly, Css. The concentration of actin in complex with the G-actin binding protein X can be derived from the law of mass action considering the following equilibrium: $X + A \Leftrightarrow XA$. With [A] = Css, the Gibbs equation becomes:

$$[XA] = [X_0]\frac{C_{SS}}{C_{SS} + K_X} \qquad (4)$$

where Kx is the equilibrium dissociation constant for the XA complex and X_0 the total concentration of protein X. The value of K_X is 1-2 mM for $T\beta_4$[34,40] and 0.1-0.2 μM for profilin.[35,37] The steady state concentration of ATP-G-actin assembly varies, depending on the extent of barbed end capping 42 between 0.1 μM (free barbed ends) and 0.6 μM (100% capped barbed ends). An important implication of equation (4) is that the amount of sequestered actin varies with the extent of capping of the barbed ends. In resting cells, it is believed that most barbed ends are capped, hence C_{ss} = 0.6 μM, and the amount of unassembled actin [Au], assuming total concentrations of 200 μM $T\beta_4$ and 50 μM profilin,

will be $[A_u] = 67 + 36\ \mu M = 103\ \mu M$, a concentration three orders of magnitude higher than that of free G-actin. Another important difference between $T\beta_4$ and profilin is the following. $T\beta_4$ is a simple actin-sequestering protein, which forms a 1:1 complex with G-actin. At steady state, the sequestration of actin by $T\beta_4$ is well described by equation (4), when barbed ends are capped as well as when they are uncapped, which means that the $T\beta_4$-actin complex does not associate significantly to either the barbed or the pointed end.

In contrast to the $T\beta_4$-actin complex, the profilin-actin complex is able to associate productively to the barbed ends, but not to the pointed end. As a result, profilin behaves as a bona fide G-actin sequestering protein when barbed ends are capped, but exhibits a different, opposite behavior when barbed ends are uncapped. The participation of profilin-actin to filament elongation at the barbed end facilitates the assembly, i.e., causes a decrease in the steady-state concentration of free G-actin.[35,42] This happens because in the presence of profilin, both G-actin and profilin-actin can undergo the monomer-polymer exchange reactions at the barbed end which maintain the stability of the filament; the contribution of profilin-actin in this maintenance of steady state reduces the contribution of actin. In other words, the profilin-actin complex can be considered as another species of polymerizable actin. The hydrolysis of ATP provides the free energy necessary for this effect of profilin.[35] As a consequence of the lower value of the concentration of free G-actin at steady state in the presence of profilin, a lower amount of actin is sequestered (equation 4).

In conclusion, the effects of profilin are strongly modulated by the extent of capping of the barbed ends: profilin inhibits actin assembly and is a potent actin sequestering agent when barbed ends are capped, but when barbed ends are uncapped it promotes actin assembly and enhances the actin assembly process induced by uncapping the barbed ends.

In conclusion, G-actin sequestering proteins like $T\beta_4$ amplify the changes in G-actin concentration which are controlled by cappers and profilin. Proteins of the ADF/cofilin family were initially thought of as G-actin sequestering factors. However they bind both F- and G-actin, in their ADP-bound forms preferably, causing partial depolymerization of F-actin. Therefore equation 4 cannot be used to derive the affinity of ADF for G-actin.

Capping Proteins Control the Steady State of Actin Assembly— Strong Cappers vs. Weak Cappers

Most capping proteins known so far cap the barbed end of filaments, except the recently discovered pointed end capper tropomodulin.[43] Barbed end cappers have two essential functions: By blocking the highly dynamic barbed end, they slow down filament turnover, hence reduce the associated ATP consumption; second, they increase the steady state concentration of free G-actin up to the value of the pointed end critical concentration, which, as developed in the previous section, increases the amount of sequestered actin. Hence barbed end cappers exert a direct control of the G/F ratio in living cells, and it is important to understand by which mechanisms the capping/uncapping of actin filaments is regulated in vivo. Many of the capping proteins are Ca^{2+}-dependent and also dissociate from actin upon binding PIP_2.[44] The exact in vivo role of PIP_2, however, as a potential barbed end "uncapper" is elusive, since PIP_2 binds to many other actin binding proteins (profilin, myosin I…), but represents only 0.1% of the cell phospholipids and appears to be synthesized on demand.[45] No correlation can be observed in stimulated neutrophils between the time courses of actin assembly and of the global change in PIP_2 concentration.[46,47] On the other hand, the product of PIP_2 phosphorylation by the stimulus-activated PI-3 kinase,[48] PIP_3, seems to peak at the onset of actin assembly.[49] Thus far nothing is known about the possible cytoskeleton-associated targets of PIP_3; however it has recently been reported[50] that the activity of PI-3 kinase is stimulated by rho, one of the small GTPases of the ras family that regulate actin assembly (for a review see ref. 51). While rho is involved in the pathway lead-

ing to assembly of the focal adhesion complex, from which stress fibers originate, rac appears to regulate actin assembly off membrane-associated complexes more specifically located in ruffles and lamellipodia present at the leading edge of locomoting cells. The nature of the actin-associated proteins in the membrane-bound complexes might be different. Local barbed end elongation of anchored filaments requires uncapping of barbed ends within the membrane-bound complex, while filaments remain capped in the bulk cytoplasm, thus maintaining the difference in potential energy needed for the local actin assembly to occur. Similarly actin assembly which promotes the propulsion of the pathogenic bacterium Listeria, takes place as a result of a local shift in steady state of actin assembly in the cytoplasm of a cell in which barbed ends are capped.[52] It is noteworthy that the higher the extent of barbed end capping in a bulk F-actin solution, the higher the steady state rate of growth of each of the few uncapped barbed ends at steady state, to balance the rate of depolymerization from the pointed ends. Hence capping proteins exert a funneling effect on treadmilling.[53] Although this effect of capping proteins is not easy to test in vivo, genetic studies of capping protein over- and underexpression suggest that capping proteins play a positive role in motility.[54,55]

To understand how uncapping can be effected, it is necessary first to examine the details of the capping mechanism. In addition to the extensively studied strong cappers like proteins of the gelsolin family (including severin, fragmin, brevin, villin) which bind to the barbed ends with an extremely high affinity (10^{11} M^{-1}) more recently discovered weak cappers, like Cap G, capZ, cap32-34 or insertin, a 35 K fragment of tensin that possesses the capping activity of the whole protein,[56,57] exhibit a much lower affinity (10^8-10^9 M^{-1}) for the barbed ends. Strong cappers also sever and nucleate filaments that grow from the pointed end, most likely due to their association to the two actin subunits exposed to the solvent at the barbed ends. In contrast weak cappers generally do not sever, are poor nuleators and interact with a single actin subunit. This last property can generate a complex variety of capping behaviors, when one considers the structure of the filament barbed end, as illustrated on Figure 1.3. At the barbed end, the two terminal subunits, numbered n and n-1 along the genetic (short pitch) helix, are exposed to the solvent. Two situations may occur theoretically, as follows. If the capping protein binds to the n^{th} (terminal) subunit with a higher affinity (K) than to the $(n\text{-}1)^{th}$ (subterminal) subunit (K'), association of G-actin to the filament at the $(n + 1)^{th}$ position may be allowed (Fig. 1.3a); this reaction will switch the conformation of the capper to its low affinity binding for the barbed end, causing its dissociation followed by immediate rebinding to the newly added $(n + 1)^{th}$ subunit. As this cycle repeats, elongation occurs at capped "leaking" barbed ends. This mechanism was proposed by Wegner for insertin,[56] which slows down but does not block the rate of elongation at the barbed ends, hence was identified using an "elongation retardation assay". The rate of growth and the critical concentration at the barbed end both depend on the concentration of free capper [C]. If we call [F], [FC] and [FC'] the concentrations of free barbed ends and of barbed ends capped on the terminal (K) and subterminal (K') subunits respectively, as represented on Figure 1.3, and if we assume the capper to bind in rapid equilibrium to the barbed ends, then the rate of filament elongation in the presence of capper is as follows:

$$Ve = k_+ [F] [A] - k_- [F] + k'_+ [FC].[A] - k'_- [FC'] \quad (5)$$

where k_+ and k_- are the rate constants for G-actin association and dissociation from the free barbed ends, [A] is the concentration of G-actin, k'_+ is the association rate constant of G-actin to barbed ends capped on their terminal subunit (FC), and k'_- is the dissociation rate constant of actin from a filament capped on the subterminal subunit (FC'). It is implicit that actin does not dissociate from an FC filament and does not associate to an FC' filament. In addition, for simplicity, polymerization is assumed to be reversible and the effects of ATP

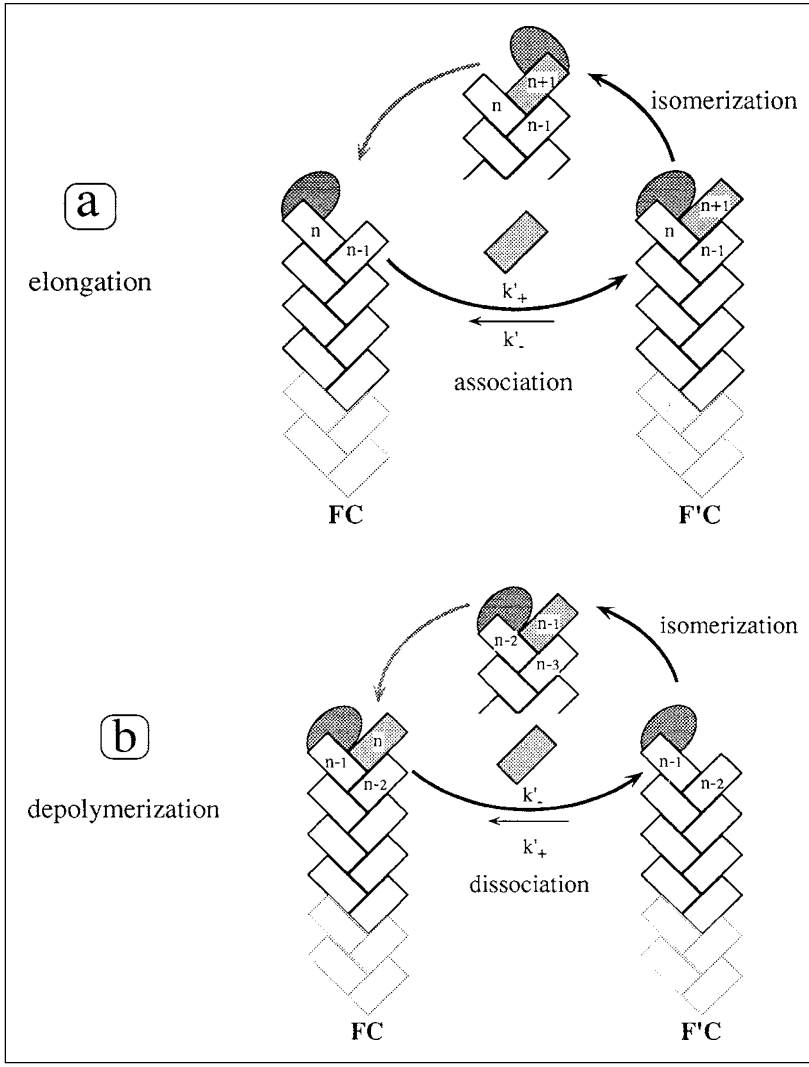


Fig. 1.3. Assembly and disassembly at barbed ends in the presence of weak cappers.

Panel a: The capper binds with a higher affinity to the terminal $(n)^{th}$ than to the subterminal $(n-1)^{th}$ subunit of the filament. Association of a $(n + 1)^{th}$ subunit may occur, followed by isomerization of the capped elongating site into the favored capped $Fn_{+1}C$ conformation. This cycle can repeat with binding of a $(n + 2)^{th}$ subunit.

Panel b: The capper binds with a higher affinity to the $(n-1)^{th}$ subterminal subunit than to the $(n)^{th}$ terminal subunit of the filament. In a regime of depolymerization, dissociation of the $(n)^{th}$ subunit may occur, followed by isomerization of the elongating site toward the favored capped $F_{n-2}C$ configuration, from which again the $(n-1)^{th}$ subunit can dissociate, and so on, leading to endwise leaky disassembly.

hydrolysis, which could result in different affinities of the capper for ADP-P_i and ADP-ends, are not considered.

Equation (5) can be written:

$$Ve = \frac{[F_0]}{1 + [C]/K + [C]/K'} \left[(k_+ + k'_+ [C]/K) \cdot [A] - (k_- + k'_- [C]/K') \right]$$

In the presence of capper, the critical concentration A_c is the value of [A] at which

$V_e = 0 =$

$$A_c = \frac{k_- + k'_- [C]/K'}{k_+ + k'_+ [C]/K]}$$

$$= k_- / k_+ \frac{1 + a[C]/K'}{1 + b[C]/K}$$

where $\alpha = \frac{k'_-}{k_-}$ and $\beta = \frac{k'_+}{k_+}$. If polymerization is strictly reversible, detailed balance illustrated on scheme I imposes that

$$K' \frac{k_-}{k_+} = K \frac{k'_-}{k'_+}$$

which can be written

$$\frac{b}{a} = \frac{K}{K'}$$

and therefore A_c is independent of [C] and remains constant and equal to $\frac{k_-}{k_+}$.

On the other hand, if detailed balance is not respected, which may be the case if ATP hydrolysis is involved in actin assembly, then $\frac{b}{a} = \frac{K}{K'}$, which allows A_c to vary hyperbolically with the concentration of free capper [C], and the critical concentration reached at saturation by C, $A_{c\infty}$, is equal to $\frac{k_-}{k_+} \times \frac{a}{b} \times \frac{K}{K'}$, which may be lower or higher than $\frac{k_-}{k_+}$ depending on the values of α, β, K and K'. If $\frac{a}{K'} < \frac{b}{K}$, A_c decreases with [C], i.e., the capper stabilizes the filaments and favors assembly at the barbed ends. This effect is similar to profilin's. Hence leaky caps are potentially useful to control the steady state of actin assembly at the barbed end, for instance at the leading edge of locomoting cells.

The other interesting case is the one in which the capper is more tightly bound to the penultimate subunit than to the terminal subunit of the filament (Fig. 1.3b). This situation may indicate that the capper actually interacts with both the n^{th} and $(n-1)^{th}$ subunits. In this case ($K' << K$), elongation is eventually totally blocked by C, the FC' filaments being unable to elongate, but the cap may be leaky under depolymerization conditions ([A] = 0), because following the dissociation of the n^{th} (uncapped) subunit at rate k'_-, the cap will switch to position n-2 for which it has a greater affinity, leaving subunit n-1 now free to dissociate, and so on. As this cycle repeats, filaments saturated by C disassemble at rate k'_-. An example of such a leaky cap of the pointed end is DNaseI.[53] These two kinds of leaky caps operate like valves which allow either growth or disassembly of filaments. These mechanisms potentially increase the number of ways in which actin-based motility can be modulated in cells.

ADF/Cofilin Controls Filament Turnover and the Rate of Actin-Based Motility

ADF/cofilin is a family of small actin-binding proteins (16-18 KDa) which control actin dynamics and, like profilin, play an essential role in developmental stages in which actin assembly is involved. These proteins are regulated by reversible phosphorylation in a stimulus-responsive fashion (see ref. 32 for review). ADF is activated by dephosphorylation, but the natures of the kinase and phosphatase which control its activity and their relationship with the signaling pathway are still unknown.

ADF binds both F- and G-actin, which in itself is sufficient to let one expect that it may, by participating in filament assembly, modify the kinetic and thermodynamic parameters of actin polymerization. An additional refinement of ADF function is that it binds ADP-actin preferentially. As a consequence, since at steady state essentially ADP-Pi is bound to the terminal subunits at the barbed ends, while the rest of the filament is made of F-ADP subunits, the binding of ADF to F-ADP-actin affects the dynamics at the pointed ends only. Kinetic data[58] show that ADF-ADP-actin depolymerizes 25-fold faster than ADP-actin from the pointed ends. Therefore at steady state the flux of subunits dissociation from the pointed ends is increased by ADF. The production of ATP-G-actin, as a result, increases and reaches a new steady state value such that the steady state rate of barbed end growth becomes equal to the rate of pointed end depolymerization, in an accelerated treadmilling scheme (Fig. 1.4). Actin-based motility processes, such as the propulsive movement of Listeria, which are powered by barbed end growth, are therefore speeded up by ADF.[58] This regulation of filament treadmilling by ADF appears to operate in vivo as well. Genetic studies have shown that ADF overexpression in Dictyostelium enhances ruffling and motility,[59] and controls the turnover of actin filaments in yeast.[60] The increased rate of depolymerization from the pointed ends is associated to a large increase in the critical concentration at that end. The net consequence is a further increase in the steady state concentration of ATP-G-actin upon capping of the barbed ends. In the presence of sequestering proteins, ADF is therefore expected to cause an increase in G-actin sequestration (equation 4), i.e., in F-actin depolymerization. In this regard, capping proteins and ADF have synergic effects. Similarly, if a large proportion of the barbed ends are capped, the few uncapped barbed ends will grow faster in the presence than in the absence of ADF due to the increase in pointed end critical concentration.

Conclusions

The main points raised in this chapter are the following:

1. The changes in actin assembly which are involved in cell shape changes and locomotion result from shifts in the steady state of actin assembly and changes in the turnover rate of actin filaments.
2. Shifts in the steady state of actin assembly are possible due to ATP hydrolysis which allows the existence of different critical concentrations at the barbed and the pointed ends.
3. The local shifts in steady state of actin assembly responsible for changes in shape are due to capping/uncapping of barbed ends, amplified by G-actin-sequestering agents like $T\beta_4$ and by proteins changing the critical concentration at the pointed end, like ADF. A large variety in the changes in actin assembly is provided by weak, leaky cappers which may allow either filament elongation or filament depolymerization.
4. Profilin is an efficient high affinity (10^7 M^{-1}) G-actin sequestering protein when barbed ends are capped (resting cells) and promoting actin assembly off the pool of $T\beta_4$-actin when barbed ends are uncapped; due to these opposite actions of profilin, a threshold-type regulatory effect of profilin is expected in cell motility reaction.

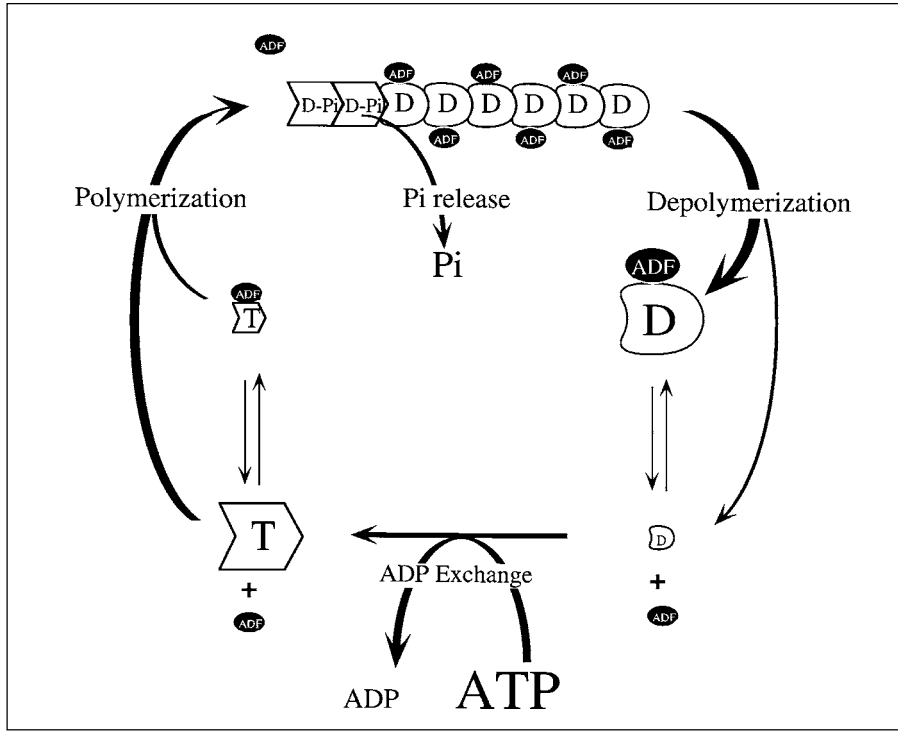


Fig. 1.4. ADF increases the rate of treadmilling of actin filaments. A filament is drawn at steady state in the presence of ATP. Terminal subunits at the barbed end carry ADP-P_i and do not bind ADF. ADP- subunits in the body of the filament and at the pointed end than the unliganded subunits, creating a large flux of ADF-ADP-G-actin. ADF is in rapid association-dissociation equilibrium with ADP-G-actin. Exchange of ATP for ADP on G-actin leads to production of polymerizable ATP-G-actin. ADF has a very weak affinity for ATP-G-actin. Therefore the main polymerizing species remains ATP-G-actin at steady state. The steady-state concentration of ATP-G-actin which is established is such that the on-flux of ATP-G-actin at the barbed end balances the off flux of ADP- and ADF-ADP-G-actin from the pointed end. Reprinted with permission from Carlier MF, J Cell Biol 1997; 136:1307-1322.

5. ADF increases the turnover of actin filaments due to its enhancement of the rate of depolymerization from the pointed ends. In this process, ADF participates in the rapid remodeling of the actin cytoskeleton in regions of high motile activity.
6. In living cells, all these regulatory proteins act in a concerted fashion. The combination of all of them may generate further new features in the regulation of actin dynamics. Reconstitution in vitro assays as well as experiments using cell-free systems or genetically tractable organisms will help to elucidate the many facets of actin polymerization in motility.

Acknowledgments

This work was funded in part by the Association pour la Recherche contre le Cancer (ARC), the Association Française contre les Myopathies (AFM), the EC (grant # CHRX-CT94-0652) and the Ligue Nationale Française contre le Cancer.

References

1. Cramer LP, Mitchison TJ, Theriot JA. Actin-dependent motile forces and cell motility. Curr Opin Cell Biol 1994; 6:82-86.
2. Carlier MF. Nucleotide hydrolysis regulates the dynamics of actin filaments and microtubules. Phil Trans Royal Soc London B 1992; 336:93-97.
3. Carlier MF. Actin: protein structure and filament dynamics. J Biol Chem 1991; 266:1-4.
4. Korn ED. Actin polymerization and its regulation by proteins from nonmuscle cells. Physiological Reviews 1982; 62:672-737.
5. Pollard TD, Cooper JA. Actin and Actin-Binding Proteins. A critical evaluation of mechanisms and functions. Ann Rev Biochem 1986; 55:987-1035.
6. Kabsch W, Mannherz HG, Suck D, Pai EF, Holmes KC. Atomic structure of the actin:DNaseI complex. Nature 1990; 347:37-44.
7. Korn ED, Carlier MF, Pantaloni D. Actin polymerization and ATP hydrolysis. Science 1987; 238:638-644.
8. Carlier MF, Pantaloni D. Binding of phosphate to F-ADP-actin and role of F-ADP-Pi-actin in ATP-actin polymerization. J Biol Chem 1988; 263:817-825.
9. Lepault J, Ranck JL, Erk I, Carlier MF. Small Angle X-Ray Scattering and Electron Cryomicroscopy Study of Actin Filaments: Role of the Bound Nucleotide in the Structure of F-actin. Journal of Structural Biology 1994; 112:79-91.
10. Bremer A, Aebi U. The structure of the F-actin filament and the actin molecule. Current Opinion in Cell Biology 1992; 4:20-26.
11. Orlova A, Egelman EH. Structural basis for the destabilization of F-actin by phosphate release following ATP hydrolysis. J Mol Biol 1992; 227:1043-1053.
12. Isambert H, Venier P, Maggs AC et al. Flexibility of actin filaments derived from thermal fluctuations—Effect of bound nucleotide, phalloidin, and muscle regulatory proteins. J Biol Chem 1995; 270:11437-11444.
13. Combeau C, Carlier MF. Probing the mechanism of ATP hydrolysis on F-actin using vanadate and the structural analogs of phosphate BeF_3^- and the structural analogs of phosphate BeF_3^- and AlF_4^-. J Biol Chem 1988; 263:17429-17436.
14. Combeau C, Carlier MF. Characterization of the aluminum and beryllium fluoride species bound to F-actin and microtubules at the site of the γ-phosphate of the nucleotide. J Biol Chem 1989; 264:19017-19021.
15. Sondek J, Lambright DG, Noel JP, Hamm HE, Sigler PB. GTPase mechanism of Gproteins from the 1.7Å crystal structure of transducin alpha-GDP-AlF4-. Nature 1994; 372:276-279.
16. Rayment I, Smith C, Yount RG. The active site of myosin. Annu Rev Physiol 1996; 58:671-702.
17. Oosawa F, Asakura, T. Thermodynamics of the polymerization of protein. Academic Press, 1975.
18. Wegner A. Head to tail polymerization of actin. J Mol Biol 1976; 108:139-150.
19. Coue M, Korn ED. ATP hydrolysis by the gelsolin-actin complex and at the pointed ends of gelsolin-capped filaments. J Biol Chem 1986; 261:1588-1593.
20. Carlier MF, Criquet P, Pantaloni D, Korn ED. Interaction of Cytochalasin D with Actin Filaments in the Presence of ADP and ATP. J Biol Chem 1986; 261:2041-2050.
21. Weber A, Northrop J, Bishop MF, Ferrone FA, Mooseker MS. Kinetics of Actin Elongation and Depolymerization at the Pointed End. Biochemistry 1987; 26:2537-2543.
22. Wang Y. Exchange of actin subunits at the leading edge of living fibroblasts: possible role of treadmilling. J Cell Biol 1985; 101:597-602.

23. Pantaloni D, Coue MF, Coue M, Lal AA, Brenner SL, Korn ED. The critical concentration of actin in the presence of ATP increases with the number concentration of filaments and approaches the critical concentration of actin-ADP. J Biol Chem 1984; 259:6274-6283.
24. Hitchcock-DeGregori SE, Sampath P, Pollard TD. Tropomyosin inhibits the rate of actin polymerization by stabilizing actin filaments. Biochemistry 1988; 27:9182-9185.
25. Cooper JA. The role of actin polymerization in cell motility. Annu Rev Physiol 1991; 53:585-605.
26. Carlsson L, Nystrom LE, Sundkvist I, Markey F, Lindberg U. Actin polymerizability is influenced by profilin, a low molecular weight protein in non-muscle cells. J Mol Biol 1977; 115:465-483.
27. Schutt CE, Myslick JC, Rozycki MD, Goonesekere NCW, Lindberg U. The structure or crystalline profilin-β-actin. Nature 1993; 365:810-816.
28. Cedergren-Zeppezauer ES, Goonesekere NCW, Rozycki MD et al. Crystallization and Structure Determination of Bovine Profilin at 2,0 A° Resolution. J Mol Biol 1994; 240:459-475.
29. Vinson VK, Archer SJ, Lattman EE, Pollard TD, Torchia DA. Three-Dimensional Solution Structure of Acanthamoeba Profilin-1. J Cell Biol 1993; 122:1277-1283.
30. Safer D, Elzinga M, Nachmias VT. Thymosin beta 4 and Fx, an actin-sequestering peptide, are indistinguishable. J Biol Chem 1991; 266:4029-4032.
31. Nachmias VT. Small actin-binding proteins: the beta-thymosin family [published erratum appears in Curr Opin Cell Biol 1993 Apr;5(2):292]. Curr Opin Cell Biol 1993; 5:56-62.
32. Moon A, Drubin D. The ADF/cofilin proteins: stimulus-responsive modulators of actin dynamics. Mol Biol Cell 1995; 6:1423-1431.
33. Nishida E. Opposite effects of cofilin and profilin from porcine brain on rate of exchange of actin-bound adenosine 5'-triphosphate. Biochemistry 1985; 24:1160-1164.
34. Carlier MF, Jean C, Rieger KJ, Lenfant M, Pantaloni D. Modulation of the interaction between G-actin and Thymosin β4 by the ATP/ADP ratio: Possible implication in the regulation of actin dynamics. Proc Natl Acad Sci USA 1993; 90:5034-5038.
35. Pantaloni D, Carlier MF. How profilin promotes actin filament assembly in the presence of thymosin β4. Cell 1993; 75:1007-1014.
36. Perelroizen I, Marchand JB, Blanchoin L, Didry D, Carlier MF. Interaction of profilin with G-actin and poly(L-proline). Biochemistry 1994; 33:8472-8478.
37. Perelroizen I, Carlier MF, Pantaloni D. Binding of divalent cation and nucleotide to G-actin in the presence of profilin. J Biol Chem 1995; 270:1501-1508.
38. Goldschmidt-Clermont PJ, Furman MI, Wachsstock D, Safer D, Nachmias VT, Pollard TD. The control of actin nucleotide exchange by thymosin beta 4 and profilin. A potential regulatory mechanism for actin polymerization in cells. Mol Biol Cell 1992; 3:1015-1024.
39. Perelroizen I, Didry D, Christensen H, Chua NH, Carlier MF. Role of nucleotide exchange and hydrolysis in the function of profilin in actin assembly. J Biol Chem 1996; 271:12302-12309.
40. Weber A, Nachmias VT, Pennise CR, Pring M, Safer D. Interaction of thymosin beta 4 with muscle and platelet actin: Implications for actin sequestration in resting platelets. Biochemistry 1992; 31:6179-6185.
41. Yu FX, Lin SC, Morrison-Bogorad M, Atkinson MA, Yin HL. Thymosin beta 10 and thymosin beta 4 are both actin monomer sequestering proteins. J Biol Chem 1993; 268:502-509.
42. Carlier MF, Pantaloni D. Actin assembly in response to extracellular signals: role of capping proteins, thymosin beta 4 and profilin. Semin Cell Biol 1994; 5:183-191.
43. Weber A, Pennise CR, Pring M. DNaseI Increases the Rate Constant of Depolymerization at the Pointed (-) End of Actin Filaments. Biochemistry 1994; 33:4780-4786.
44. Hartwig J, Bokoch G, Carpenter C et al. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell 1995; 82:643-653.
45. Wu L, Niemeyer B, Colley N, Socolich M, Zuker CS. Regulation of PLC-mediated signalling in vivo by CDP-diacylglycerol synthase [see comments]. Nature 1995; 373:216-222.

46. Sklar LA, Omann GM, Painter RG. Relationship of actin polymerization and depolymerization to light scattering in human neutrophils: Dependence on receptor occupancy and intracellular Ca++. J Cell Biol 1985; 101:1161-1166.
47. Zigmond SH, Joyce M, Borleis J, Bokoch GM, Devreotes PN. Regulation of actin polymerization in cell-free systems by GTP gamma S and CDC42. J Cell Biol 1997; 138:363-374.
48. Kapeller R, Cantley LC. Phosphatidylinositol 3-kinase. Bioessays 1994; 16:565-576.
49. Eberle M, Traynor-Kaplan AE, Sklar LA, Norgauer J. Is there a relationship between phosphatidylinositol trisphosphate and F-actin polymerization in human neutrophils? J Biol Chem 1990; 265:16725-16728.
50. Zhang J, King WG, Dillon S, Hall A, Feig L, Rittenhouse SE. Activation of platelet phosphatidylinositide 3-kinase requires the small GTP-binding protein Rho. J Biol Chem 1993; 268:22251-22254.
51. Hall A. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu Rev Cell Biol 1994; 10:31-54.
52. Marchand JB, Moreau P, Paoletti A, Cossart P, Carlier MF, Pantaloni D. Actin-based movement of Listeria monocytogenes: actin assembly results from the local maintenance of uncapped filament barbed ends at the bacterium surface. J Cell Biol 1995; 130:331-343.
53. Carlier MF, Pantaloni D. Control of actin dynamics in cell motility. J Mol Biol 1997; 269:459-467.
54. Hug C, Jay P, Reddy I et al. Capping protein levels influence actin assembly and cell motility in dictyostelium. Cell 1995; 81:591-600.
55. Sun H, Kwiatkowska K, Wooten D, Yin H. Effects of CapG overexpression on agonist-induced motility and second messenger generation. J Cell Biol 1995; 129:147-156.
56. Ruhnau K, Gaertner A, Wegner A. Kinetic evidence for insertion of actin monomers between the barbed ends of actin filaments and barbed end-bound insertin, a protein purified from smooth muscle. J Mol Biol 1989; 210:141-148.
57. Weigt C, Gaertner A, Wegner A, Korte H, Meyer HE. Occurrence of an actin-inserting domain in tensin. J Mol Biol 1992; 227:593-595.
58. Carlier MF, Laurent V, Santolini J et al. Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: Implication in actin-based motility. J Cell Biol 1997; 136:1307-1322.
59. Aizawa H, Sutoh K, Yahara I. Overexpression of cofilin stimulates bundling of actin filaments, membrane ruffling, and cell movement in Dictyostelium. J Cell Biol 1996; 132:335-344.
60. Lappalainen P, Drubin DG. Cofilin promotes rapid actin filament turnover in vivo. Nature 1997; 388:78-82.

CHAPTER 2

The Gelsolin Family of Actin Filament Severers and Cappers

Yu-Tsueng Liu, Andrew L. Rozelle and Helen L. Yin

Introduction

The actin cytoskeleton is an important structure. It determines cell shape and empowers cell and organelle translocation. It provides a framework to orchestrate and coordinate multiple critical cell functions. The cytoskeleton responds to extracellular signals by polymerizing, reorganizing and depolymerizing. Therefore, actin remodeling is a major effector of signal transduction pathways. Remodeling can be achieved rapidly by dismantling the cytoskeleton through filament severing and rebuilding of these filaments through nucleated actin assembly. Gelsolin is the first actin filament severing protein to be discovered.[1] It is activated by Ca^{2+} and inhibited by polyphosphoinositides, particularly phosphatidylinositol 4,5, bisphosphate (PIP_2). Since these are critical second messengers in cell activation pathways, gelsolin is potentially a major player in cytoskeletal remodeling.

The Gelsolin Family of Actin Severing and/or Capping Proteins

Many gelsolin-like proteins have been discovered,[1] and these proteins share extensive sequence and functional similarities (Fig. 2.1). Gelsolin, an 80 kDa protein, has two tandem homologous halves, each of which contains a 3-fold repeat of an approximately 15 kDa segment (S1-3 and S4-6).[2] The halves and the individual segments are defined based on proteolytic cleavage[3,4] and correspond roughly (but not precisely) with a more recent definition based on the X-ray structure of full-length gelsolin (see below).[5] The six segments may have evolved independently from an ancestral single segment gene that has duplicated to form a multidomain severing protein. Gelsolin-like proteins are found in vertebrates, as well as invertebrates such as Drosophila and lobster. Lower eukaryotes, including Physarum and Dictyostelium, have a three segment protein most closely resembling the NH_2-terminal half of gelsolin.[6] Previously, all identified gelsolin family members, including the six and three repeat proteins, have been observed to sever and cap filaments. Recently, however, a member that caps but does not sever has been discovered. This protein, originally called gCap39,[8] Macrophage Capping Protein[9] or mbh1, and renamed vertebrate CapG,[10] has three segments resembling the NH_2-terminal half of gelsolin.[8] It coexists with gelsolin in the cytoplasm in many cells.[11] Unlike gelsolin, CapG is also found in the nucleus.[12] Other members of the gelsolin family contain gelsolin-like domains linked to additional motifs. Villin is the first identified member of this group,[13] containing a short COOH-terminal extension ("headpiece") enabling it to crosslink actin filaments in the absence of Ca^{2+}. More recently, members with longer extensions have been discovered. These include flightless I (*fliI*), which

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

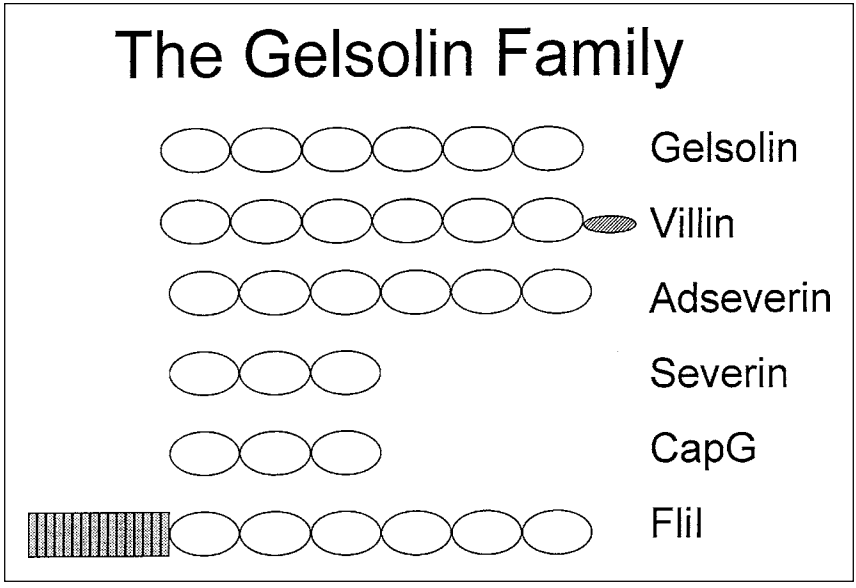


Fig. 2.1. The gelsolin family. Each segment is represented by an oval. Extensions are indicated in shaded areas. Villin, CapG and adseverin (also known as scinderin)[121] were first identified in mammals. Severin and its homolog, fragmin, were identified in Dictyostelium and Physarum, respectively.

was identified in Drosophila as a gene important for actin organization during embryo cellularization[14] and muscle development.[15] FliI homologs are also found in *C. elegans* and mammals. FliI has as its NH_2-terminus a 400 residue leucine-rich repeat extension.[15] This motif is found in a growing number of otherwise unrelated proteins that mediate heterologous protein-protein interactions. Several more novel members of the gelsolin family with unique segmenal organization have also been identified. For example, *C. elegans* has a gelsolin-like protein with the first three segments attached to the S6 segment of gelsolin.[15a]

Gelsolin-Actin Interactions

The interactions of gelsolin with actin are multiple and complex. Gelsolin binds actin monomers and filaments, and has three main effects on actin:[16-18] severing, barbed end capping and filament nucleation. The combined effect of these interactions is to promote the formation of a large number of short actin filaments that are capped at their barbed ends. Of these interactions, the ability to sever is unique to the gelsolin family of proteins. Upon Ca^{2+} addition, gelsolin rapidly produces dramatic changes in filament length distribution and number. Filament shortening reduces the ability of crosslinkers such as filamin, to tie the strands into a gel network, disproportionately decreasing the cytoplasmic viscosity.[17] In addition, the resulting short, barbed end-capped filaments act as sites for actin polymerization after regulated uncapping in response to agonists. Thus, severing, capping and uncapping together constitute an efficient mechanism for precipitously changing filament length and initiating actin filament growth. Gelsolin also promotes actin nucleation by binding two actin monomers to create nuclei. However, this is probably not physiologically relevant, because filaments from these gelsolin nuclei will elongate from the pointed

end while the predominant agonist-induced filament growth in cells is from the barbed end. The other mechanisms involving de novo actin nucleation, by other proteins, filament severing and regulated uncapping by gelsolin-like proteins are therefore more likely to be involved.

More About Severing

Severing is the breaking of the noncovalent bonds between actin subunits within a filament. Severing was a novel idea when first proposed 18 years ago as a mechanism of action of gelsolin.[1] This has now been verified by biochemical and biophysical means, and can be directly visualized under light microscopy. The actin filament bends prior to breakage,[19] suggesting that gelsolin may induce a significant change in the filament structure during severing. Severing involves multiple steps. Gelsolin must first bind to the side of actin filaments.[20] Side binding positions gelsolin to break the initial actin:actin bond. What makes gelsolin particularly effective is that after severing, it caps the filament barbed end. Capping enhances the effectiveness of severing by preventing filament reannealing. As a result, severed filaments remain short. There are also indications that capping generates cooperative conformational changes that are propagated to other actin protomers within the filament.[21] These changes may enhance severing.

Gelsolin is able to sever filaments stabilized with phalloidin. This can be explained by the ability of gelsolin to displace phalloidin from actin filaments.[22] Displacement is probably due to a gelsolin-induced allosteric change in actin, although competition for binding is another possibility.[23] Side binding has an association rate constant of $2x10^7$ M^{-1} s^{-1} and dissociation rate constant of 0.4-1.2 s,[1] and severing has a first order rate constant of 0.25 s^{-1}.[24] This rate of side binding approaches diffusion limits and is much higher than measurements made previously using other methods.[25] One group reports that the rate of gelsolin binding to monomers is also very slow,[26] whereas the rate of capping approaches diffusion limits.[27] Because gelsolin efficiently destroys actin filaments, it is a useful filament-removing reagent. Gelsolin added to permeabilized cells establishes actin involvement during exocytosis[28] and reveals structures obscured by dense actin fibers in muscle and nonmuscle cells. Gelsolin is also currently under clinical trials as a therapeutic agent to reduce sputum viscosity in cystic fibrosis patients.[29]

Besides the gelsolin family, there is another major family of actin binding proteins that may sever filaments. These include actin depolymerizing factor, cofilin, destrin and actophorin.[30] They are reported to sever actin in a Ca^{2+} insensitive manner, although not as effectively as members of the gelsolin family, because they do not cap. However, evidence suggests that they enhance the rate of filament turnover without directly severing filaments.[31]

Domain Analyses of Gelsolin

Ca^{2+} enhances gelsolin's interactions with actin,[1,16] while polyphosphoinositides, particularly PIP_2, inhibit these interactions.[32] Severing, nucleation and barbed end capping have different requirements for Ca^{2+} and different sensitivities to PIP_2. These differences exist because each gelsolin function is mediated by different actin binding domains or combinations thereof.

Limited proteolysis readily cleaves gelsolin into two halves.[3,33] The NH_2-terminal half severs actin filaments. Like intact gelsolin, it is inhibited by PIP_2, but unlike gelsolin, it does not require Ca^{2+} to bind actin.[3] Further cleavage of the NH_2-terminal half generates two actin binding fragments.[34] The fragment encompassing S1 binds actin monomers, while that encompassing S2-3 binds along the side of actin filaments.[20,35] The COOH-terminal half exhibits Ca^{2+}-dependent, EGTA reversible actin binding,[3] and undergoes a conformational change in the presence of Ca^{2+}.[3,36] It does not sever filaments. Since the NH_2-terminal

half does not require Ca^{2+} to bind actin, the COOH-terminal half is the Ca^{2+} regulatory domain for the entire molecule. The precise mechanism for this intramolecular regulation is not well understood.

Deletion analyses have defined the minimal requirements for each actin modifying function.[34,37,38] Expression of gelsolin segments or combinations of segments have further clarified the domain requirements for individual function.

Actin Binding Sites

Gelsolin has three actin binding sites, which are located individually in S1, S2 and S4. The S1 actin binding residues were first identified by mutagenesis and deletion analyses and are predicted to be located in an α helical region.[38] This is subsequently confirmed by the S1:actin crystal structure[39] (see below). In S2, residues 161-172 are implicated in actin binding based on deletion and peptide analog studies.[40-42] In addition, the S2 long helix is a site of interaction as well.[43] In both S1 and S2, the long helix probably is the primary binding site, and the surrounding regions may stabilize binding. The S4 actin binding site has not been mapped but is likely to be similar to that in S1, since these two segments are structurally the most closely related among the six gelsolin segments.[5]

When gelsolin is exposed to actin monomers in Ca^{2+}, two actin molecules are bound through sites attributed to S1 and S4.[42] The two actins in the ternary complex with gelsolin are crosslinked through their COOH-terminal cysteines in an antiparallel fashion.[44] This conformation is unlike that found at the barbed end of a normal actin filament, suggesting that gelsolin alters actin conformation significantly. EGTA dissociates the actin bound to S4, but not that bound to S1. The resulting EGTA-resistant S1:actin complex retains a trapped Ca^{2+}.[45,46] This complex can be dissociated by PIP_2,[47] providing evidence for gelsolin regulation by phosphoinositides (see below).

When exposed to actin filaments, gelsolin and its NH_2-terminal half first sever and then cap the newly-created barbed ends. Since neither S1 nor S2 alone severs, and each segment caps actin filaments with much lower affinity than the gelsolin NH_2-terminal half, efficient severing and capping require the cooperative interaction between these two sites.[42] S2 binds stoichiometrically along the side of the filament and positions S1 in the proper orientation to break actin:actin bonds. The importance of side binding has been demonstrated conclusively. CapG, which does not sever, gains severing function when its S1 is linked to gelsolin S2-3.[48] Likewise, gelsolin S1, when linked to the filament binding domain of the nonsevering protein α-actinin, also severs.[49] The ability to create chimeric severing proteins suggests that side binding and bond breakage are relatively independent functions. Nevertheless, side binding is not completely passive. It may facilitate severing by inducing a conformational change in the filament to promote breakage and formation of a strong barbed end cap.[42]

Ca^{2+} Regulation

Although it has been known for quite some time that Ca^{2+} induces a conformational change in gelsolin and that the COOH-terminal half imposes Ca^{2+} regulation on the NH_2-terminal half, the molecular details of how these events occur are not known. Intrasegmental changes at the COOH terminal half followed by intersegmental changes are likely to be involved. Remarkably, removal of the COOH-terminal 23 amino acid renders gelsolin Ca^{2+}-insensitive,[34] implicating these residues in inter-segmental Ca^{2+} regulation.

Another puzzling aspect is that although S1 itself binds actin in EGTA but does not bind Ca^{2+},[38,50] Ca^{2+} enhances actin binding considerably.[51] The increase in actin affinity is attributed to the formation of a EGTA-resistant complex containing a trapped Ca^{2+}. Deletion of S1's COOH-terminal residues reduces affinity sufficiently to unveil a calcium en-

hancement. Likewise, deletion of equivalent S2 residues from S1-2 allows enhancement of actin affinity by Ca^{2+} to be detected.[34]

Gelsolin binds two Ca^{2+} ions with similar dissociation constants of approximately 1 μM.[16,45,52] The NH_2-terminal half binds one Ca^{2+} with comparable affinity to that of gelsolin.[38] Since S1 does not bind Ca^{2+} in the absence of actin, the Ca^{2+} binding site in the NH_2-half is attributed to S2-3.[37] Surprisingly, the isolated COOH-terminal half binds two Ca^{2+}, with k_d of 0.2 and 2 μM.[50] The higher affinity site is in S5-6, while the lower affinity site is in S4-5. It is not known whether the latter site is important for gelsolin function and why it is not detected in full-length gelsolin.

There is still some uncertainty as to how much Ca^{2+} is required to activate gelsolin. One group reports that Ca^{2+} induces half-maximal activation of actin binding and nucleation at around 10 μM Ca^{2+},[53] while others found that 1 μM Ca^{2+}[4,16,52] is sufficient. While this issue is not resolved, there is ample evidence that gelsolin is activated in cells. Interestingly, acidic pH reduces the calcium requirement,[53] providing an alternative mechanism for activating gelsolin.

PIP_2 Regulation

Polyphosphoinositides are important in signal transduction, functioning as precursors to signaling molecules, as physical anchors and as regulators of proteins.[54] Their role as regulators of the cytoskeleton was first described in 1985, when Lassing and Lindberg[55] showed that PIP_2 inhibits profilin:actin interactions. Subsequently, gelsolin was also identified as a PIP_2-regulated protein.[32] The list of PIP_2-regulated cytoskeletal proteins has grown to include Capping Protein (CP, also known as CapZ),[56] cofilin/actin depolymerizing factor/destrin,[57] α-actinin[58,59] and vinculin.[60] It has been hypothesized that PIP_2 induces explosive actin assembly by dissociating capping proteins from the barbed ends of filaments and releasing actin monomers bound to profilin. PIP_2 involvement in actin polymerization is supported by the finding that Rac1 and RhoA, small GTPases that have well-defined effects on the cytoskeleton,[61] stimulate PIP_2 synthesis.[62-64] Furthermore, manipulations that alter the availability of PIP_2 in cells have profound effects on agonist- and/or Rac1-induced filament end uncapping, actin polymerization and cell motility.[64,65]

Gelsolin binds PIP_2 with μM affinity.[66] Binding involves electrostatic as well as hydrophobic interactions.[47] Binding requires the lipids to be clustered so that multiple headgroups are contacted. Thus, the physical state and geometry of PIP_2 packing in the plasma membrane may be important parameters.[67] Gelsolin and CapG affinity for PIP_2 are increased 8- and 4-fold respectively by μM Ca^{2+}, and less Ca^{2+} is required to increase this affinity when pH is reduced from 7.5 to 7.0.[66] Ca^{2+} does not enhance gelsolin NH_2-terminal half binding to PIP_2, and the COOH-terminal half has a much lower affinity for PIP_2.[66] Therefore, the pronounced Ca^{2+} enhancement of PIP_2 binding to full-length gelsolin most likely reflects a Ca^{2+}-dependent exposure of the NH_2-terminal half PIP_2 binding sites. This is consistent with the current model of how gelsolin is activated by Ca^{2+} to bind actin. Gelsolin also binds PI(4)P, PI(3,4,5)P_3, and PI(3,4)P_2.[68] Additional studies will be required to determine whether these phosphoinositides are physiological regulators of gelsolin. A recent study comparing profilin binding to D3 and D4 phosphoinositides shows that profilin binds D3 lipids preferentially.[69]

The gelsolin NH_2-terminal half has at least two PIP_2 binding sites, as defined by deletion analyses and peptide analog studies.[41,42,70] PIP_2 inhibits actin monomer binding by S1 and filament side binding by S2-3, respectively. One PIP_2 binding site is located between residues 135-142 at the COOH-terminus of the proteolytically defined S1[70] and another is located between residues 161-169,[41,42] close to the beginning of the proteolytically defined S2. The PIP_2-binding sequences are rich in positively charged amino acids and have a

K/RxxxKxK/RK/R consensus.[70] PIP_2 may inhibit actin binding simply by blocking access to actin. Alternatively, it may induce a conformational change to disrupt actin binding or directly compete with actin for binding sites. In the case of S2, the PIP_2 binding sequence is in a region that has also been implicated in actin binding, so steric interference and/or competition are possible. In addition, there are evidence for conformational changes in gelsolin. PIP_2 quenches the intrinsic tryptophan fluorescence of gelsolin and CapG[66] and NMR study shows that PIP_2 induces a gelsolin S2 PIP_2 binding peptide (residues 150-169) to undergo a coil-to-helix transformation.[71]

Post-Translational Modifications

Although there is no evidence for gelsolin phosphorylation in vivo so far, it can be phosphorylated by protein kinase C and $pp60^{c\text{-}src}$ in vitro.[72] Phosphorylation is enhanced by PIP_2. Additional studies will be required to determine whether gelsolin's functions are modulated by phosphorylation and whether phosphorylation has physiological significance. CapG is constitutively phosphorylated on serine and threonine residues in a variety of cells and phosphorylation is enhanced by inhibiting phosphatases.[11,12] Phospho-CapG is found preferentially in nuclei, suggesting that phosphorylation may promote its entry into the nucleus.[12] It is not known how CapG phosphorylation may affect its other functions. Fragmin is phosphorylated in Physarum, and it is a substrate for casein kinase II.[73]

X-Ray Crystallographic Studies of Gelsolin S1:Actin Complex

In 1993, the structure of the gelsolin S1:actin crystal was solved at atomic resolution,[39] providing valuable insight into how gelsolin binds and severs actin. Gelsolin S1 is organized as a three-layer structure with a central stack of β-sheets sandwiched between a long and a short α-helix that are oriented approximately parallel and perpendicular to the hydrophobic core, respectively. The gelsolin:actin contacts involve a number of residues that are centered around Ile 103 in the long helix and the surrounding regions. The long α-helix inserts tangentially into a cleft at the interface between actin subdomains 1 and 3, disrupting the packing of this actin against subdomain 2 of an actin in the same strand. Since the bond within a strand is stronger than that between strands, this may be sufficient to destabilize the remaining bonds to completely sever the filament. S1 itself cannot sever because it does not bind to the side of filaments, and it does not cap them with high affinity.

X-ray analysis of the gelsolin S1:actin complex identified two calcium binding sites, one that is coordinated by residues within S1 (intramolecular binding site), and another coordinated by residues contributed by S1 and by actin (intermolecular binding site). Subsequent studies show that the Ca^{2+} trapped in the actin:S1 complex is located at the intramolecular site, while the intermolecular Ca^{2+} is probably an artifact of the crystallization conditions used.[46] The creation of the intramolecular Ca^{2+} binding site suggests that actin binding induces a conformational change in S1 to trap Ca^{2+}. This change is likely to be subtle however, because only minor differences are found between the gelsolin S1 structure with and without a bound actin (see below).[5]

The actin structure in the gelsolin S1:actin complex is very similar to that of actin complexed with DNaseI, which binds the "pointed" end of actin. Thus, neither ligand appear to produce dramatic changes in actin. Nevertheless, S1 does induce some unique changes which, though subtle, may be relevant to bond breakage. The other gelsolin segments may generate additional rearrangements to account for the biochemical evidence for significant changes in actin conformation.

The crystal structure of gelsolin S1 is remarkably similar to the solution structure of villin S1[74] and severin S2,[75] confirming sequence based predictions. Unexpectedly, destrin, a member of the cofilin/ADF family that has no sequence homology to gelsolin, has a strik-

ingly similar folding pattern.[76] Profilin also has a similar structure.[77] These results suggest that many actin binding proteins use a common scaffold to present their binding sites to actin and this core structure is dictated by the architecture of actin.

The elucidation of how S1 binds actin sparked renewed interest in modeling how gelsolin severs filaments. Several models are proposed,[78,79] but information on how the gelsolin segments are arranged on a filament has been lacking until recently.

The X-Ray Structure of Full-Length Gelsolin in EGTA

The gap in our knowledge has been partially filled in with the solution of the X-ray crystal structure of full-length gelsolin by Burtnick et al[5] (Fig. 2.2). This breakthrough provides important new information and refocuses our attention on several aspects suggested by previous biochemical studies. First, the crystal structure redefines some of the boundaries between segments that were previously assigned based on susceptibility to proteolysis. This highlights similarities as well as differences between segments. It is now clear that the conserved residues in each segment maintain the basic folds of the molecule, while actin binding per se involves residues customized for each domain.

Second, the gelsolin crystal suggests how the unique COOH-terminal extension of S6 can impart Ca^{2+} regulation to the NH_2-terminal half. This extension contains a random coil capped with a 10 residue helix which is in intimate contact with the S2 actin binding helix[5] (Fig. 2.2). Thus, it imposes a structural constraint on the relative orientation of the two halves of gelsolin to block actin binding.

Third, the EGTA/gelsolin structure suggests that Ca^{2+} must generate large shifts in the relative orientation of the segments. When the EGTA/gelsolin structure is superimposed on the model for an actin filament as dictated by the S1:actin crystal, it is clear why gelsolin does not bind actin in EGTA. In EGTA, S3 clashes with S1, blocking access to actin, and neither S2 nor S4 contacts the appropriate sites on actin. Since there is no evidence for extensive rearrangement of S1 after binding actin or Ca^{2+}, it is unlikely that any of the other segments will change its basic folding pattern either. Changes in the linker regions between segments therefore seem more plausible. The EGTA structure hints at how this might be achieved. The two halves of gelsolin are organized similarly and are linked together by a highly convoluted 50 residue tether. This tether can unwind to allow the halves to straddle two actin strands. Within each half, the first and third segments (for example, S1 and S3 for the NH_2-terminal half) are tightly linked together to form a continuous 10 strand β-sheet. The second segment (S2) is relatively isolated and is connected to the first segment (S1) through a small β-strand and a short linker, and to the third segment (S3) through a longer loop. Therefore, it is easy to envision how S1 and S3 pivot against S2 as a rigid unit to relieve some of the structural constraints observed in EGTA.

Fourth, the crystal structure defines the PIP_2 binding region. It shows that the PIP_2 binding sequence identified previously at the COOH-terminus of S1 is actually part of S2. This, together with the PIP_2 binding site of proteolytically defined S2, map to a common flat, solvent exposed surface centered around the first and third β-sheet strands and their associated linkers (Fig. 2.2). The arginines and lysines cluster to form potential phosphate binding sites and are near the actin binding face of the S2 long α-helix. Therefore, PIP_2 may block actin binding simply by steric interference. Alternatively, gelsolin upon PIP_2 binding may undergo the coil-to-helix transformation inferred from a previous peptide study.[71] This change will significantly disrupt the S2 core structure. Actin binding residues may be displaced and the distance between S1 and S2 may be shortened sufficiently to interfere with their coordination for severing. However, the behavior of the short peptide may not be predictive of that of gelsolin, because it contains only part of the newly-defined PIP_2 binding region[5] and it is not stabilized by neighboring β-strands as in the intact molecule.

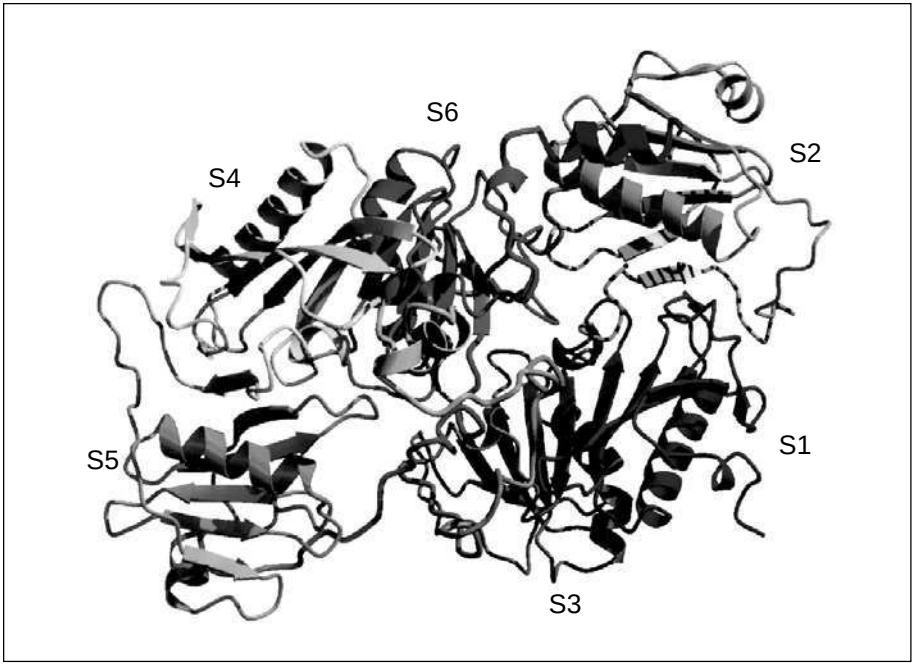


Fig. 2.2. Schematic representation of the structure of horse plasma gelsolin crystallized in the presence of EGTA. PIP_2 binding sequences are stripped. Crystal structure is published in ref. 5, and this figure is supplied by R.C. Robinson (The Salk Institute, San Diego, California).

Based on the structural and functional data, the following model for how gelsolin works is proposed.[5] In EGTA, the COOH-terminal helical tail prevents actin binding. Ca^{2+} relieves the constraint of the COOH-terminal half on S2, allowing S2 to bind to the side of the filament. S2 binding induces the S1 and S3 structural unit to pivot around the S1-S2 junction, inserting S1 between actin subunits. As the filament contacts break, S2-3 caps the actin in one strand. S4-6 flips over to cap actin at the opposite strand.

Changes in Gelsolin Level in Cells Are Correlated with Motility

Gelsolin has been widely implicated in cell motility. Low level gelsolin overexpression in stably transfected fibroblasts increases the rate of chemotaxis.[80] However, increased severing does not appear to be the primary cause because cells overexpressing nonsevering capping proteins such as CapG[10] and CP[81] also move faster. Thus, capping alone can alter actin dynamics.[82] Other experiments support the importance of severing for cell migration. A comparison of motile and stationary gingival fibroblasts shows that the former population has higher Ca^{2+}-dependent severing activity.[83]

The importance of gelsolin for cell motility has been established conclusively with transgenic gelsolin null mice.[84] These mice are developmentally normal and viable, at least in the mixed strain background used. However, they have reduced leukocyte and fibroblast motility and increased clotting time reflecting abnormal platelet cytoskeletal responses. Dermal fibroblasts from the gelsolin null mice have robust stress fibers that are more resistant to serum starvation or cytochalasin B treatment. Thus, gelsolin is important for maximal motile response and rapid restructuring of the cytoskeleton in certain cells. In other cells, capping and uncapping by nonsevering proteins may be sufficient for actin remodeling.

Gelsolin:Actin Interactions are Modified by Agonists

Gelsolin:actin interaction in cells can be assayed readily by capitalizing on the inability of EGTA to dissociate gelsolin:actin complexes once they are formed.[85] This method has been used to characterize the effects of agonist stimulation on many types of cells, including macrophages,[85] neutrophils,[86] endothelial cells,[87] A431,[88] osteoclasts[89] and platelets.[90] In resting cells, some gelsolin is complexed with actin, and agonist stimulation induces complex association and/or dissociation. The platelet system is particularly well characterized. Platelet activation is accompanied by dramatic shape change, followed by extension of lamellae and filopodia.[64,91] These changes are powered by rearrangement of the actin cytoskeleton through Ca^{2+}-dependent filament severing, followed by barbed end filament uncapping and explosive polymerization. In quiescent platelets, gelsolin is predominantly cytosolic, although a fraction is associated with the plasma membrane or actin.[92,93] After thrombin stimulation, there is an increase in membrane association and decrease in cytoskeletal association. Gelsolin is required for severing, because platelets from transgenic gelsolin null mice do not produce short filaments in response to thrombin, compromising the clotting cascade.[84] These platelets however have residual capping activity and expose capped ends during agonist stimulation because they have an additional capping protein, CP.[93] Unlike gelsolin, CP does not sever and does not require Ca^{2+} to cap. Like gelsolin, it is inhibited by PIP_2.[56] The picture that emerges from this system is that the thrombin-induced rise in Ca^{2+} activates cytosolic gelsolin to sever filaments, and PIP_2 dissociates gelsolin and CP from filament ends. PIP_2 promotes actin uncapping when added to permeabilized platelets and stimulates uncapping in a PIP_2-dependent manner.[64] These results suggest that gelsolin and CP are possible downstream components of cytoskeletal effector pathways involving PIP_2. These include the Rac, Rho and/or phosphoinositide-3 kinase cascades.

A Model

As the possibility that PIP_2 regulates the cytoskeleton gains wider acceptance, several issues remain to be resolved. The most baffling problem is that although the time course of PIP_2 hydrolysis and recovery correlate with actin filament growth in some cells, they do not in the majority of cells examined. Particularly puzzling is the finding that in many cells, actin polymerizes at a time when PIP_2 level is reduced, rather than increased, as would be expected if uncapping and monomer desequestration are initiated by PIP_2. To explain this discrepancy, it is often hypothesized that local PIP_2 availability can be enhanced by compartmentalization or differential turnover[94,95] even as the bulk PIP_2 mass is reduced. Our finding that PIP_2 binding to gelsolin and CapG is enhanced by Ca^{2+} and mild acidification[66] suggests another mechanism to explain how PIP_2 uncaps gelsolin and CapG even as the plasma membrane PIP_2 content decreases following agonist stimulation.

Taking into consideration currently available data on the behavior of gelsolin in cells, we propose the following model. In resting cells, some gelsolin caps actin filaments (10-30%),[90,92,93] some is attached to the plasma membrane (< 5%),[65,92] while the bulk is cytosolic.[96,97] Gelsolin is able to cap filament ends in spite of low ambient Ca^{2+} and high PIP_2 concentrations because once gelsolin caps filament in the presence of Ca^{2+} it is not dissociated by lowering Ca^{2+}.[85] Furthermore it has a poor affinity for PIP_2 at low cytosolic Ca^{2+}.[66] When cells are stimulated, Ca^{2+} level rises rapidly, followed by a drop in PIP_2. Ca^{2+} promotes gelsolin binding to PIP_2 by increasing affinity to an extent that overcomes the negative effect of a modest fall in PIP_2. Filaments are uncapped and can therefore elongate. Thus, there is an increase in membrane associated gelsolin[92] and a decrease in gelsolin:actin complexes during cell activation.[90] Meanwhile, cytosolic gelsolin that is not directly in contact with PIP_2 is activated by Ca^{2+} to sever filaments, creating short filaments with capped barbed ends that are then uncapped as gelsolin binds PIP_2. Actin monomers are transferred

sequentially from β-thymosins, the major monomer sequestering proteins in cells (30), to profilin, and then to the uncapped filament ends.[98] Since Ca^{2+}-activated gelsolin has a higher affinity for PIP_2 than profilin,[66] it will compete more effectively with profilin for PIP_2, particularly when PIP_2 is decreased. This displaces profilin from the plasma membrane, allowing it to bind actin monomers to catalyze polymerization.[98] Multiple rounds of severing, uncapping and facilitated actin addition at the barbed ends fuel explosive amplification of filament growth.

During the recovery phase, filament elongation is arrested as the barbed ends become capped. Although gelsolin has a higher affinity for filament ends than CP and is present in comparable amounts as CP in platelets, more CP is associated with the cytoskeleton of resting platelets.[93,99] A model for the coordinated roles of gelsolin and CP in activated cells can be proposed by postulating that CP, which is Ca^{2+}-insensitive in actin binding, is Ca^{2+}-insensitive in PIP_2 binding as well, and its affinity for PIP_2 is comparable to that of gelsolin in EGTA. When cytosolic Ca^{2+} concentration rises, CP can cap filament ends because PIP_2 concentration is too low to inhibit, while gelsolin cannot cap because of its increased PIP_2 affinity. Furthermore, Ca^{2+}-activated gelsolin displaces membrane-bound CP to the cytosol to cap filaments. The novel feature of this hypothesis is that although gelsolin caps only a small fraction of the actin filaments in resting cells, this population is the first to be uncapped during stimulation. Gelsolin further increases the number of nuclei by severing and uncapping. CP contributes by remaining active (not bound to PIP_2) during activation and is poised to cap filament ends after elongation from gelsolin-uncapped nuclei. This will explain why there is an increase in CP associated with the cytoskeleton during platelet activation.[93]

The effect of Ca^{2+} can be potentiated or minimized by changes in intracellular pH. Changes in intracellular pH, in addition Ca^{2+} and PIP_2, may explain why under some circumstances, agonist stimulation causes a decrease in cytoskeleton-associated CP[99] and membrane-associated gelsolin.[65] Dissociation of gelsolin from the membrane may occur when the PIP_2 concentration drops too much to be compensated for by the Ca^{2+}-induced increase in binding affinity. Thus, the finding that gelsolin binding to PIP_2 is modulated by Ca^{2+} and pH can overcome the major conceptual hurdle toward accepting a link between PIP_2 and actin polymerization.

Since only a handful of the currently identified PIP_2-binding proteins are known to be both Ca^{2+} and pH-sensitive, there is a selective regulation of the gelsolin family. Nevertheless, changes in gelsolin and CapG binding affinity will impact many other PIP_2-dependent processes indirectly, by altering PIP_2 availability to other binding proteins. Significantly, some pleckstrin homology domain proteins that also bind PIP_2[100,101] have PIP_2 affinity similar to that of the gelsolin class. Therefore, gelsolin and CapG can potentially compete with these proteins for PIP_2, especially when the Ca^{2+} concentration is increased and the PIP_2 concentration is decreased. This possibility is supported by in vitro and in vivo experiments.

Crosstalk Among PIP_2 Binding Proteins

There is emerging evidence to suggest that PIP_2 binding to actin modulating proteins may have implications beyond a direct effect on the cytoskeleton. This was first demonstrated for profilin.[102] Profilin inhibits phosphoinositide-specific phospholipase C_γ (PLC_γ), but not PLC_β. Phosphorylation of PLC_γ by growth factor receptor kinase reduces inhibition, so profilin does not interfere with PLC_γ activity following agonist stimulation. Gelsolin and CapG inhibit a wider spectrum of PLCs, including PLC_γ, PLC_β and PLC_δ. Inhibition is most likely due to competition for PIP_2, although steric hindrance may also be a contributing factor. There is also evidence that gelsolin binds to PLC,[103,104] raising the possibility that this

direct interaction alters PLC activity. Curiously, low concentrations of gelsolin and CapG stimulate rather than inhibit PLC_γ and PLC_β.[10,105] Since only gelsolin domains with PIP_2 binding sites stimulate, this effect depends on PIP_2 binding. The simplest model is that gelsolin and CapG bind multiple PIP_2 molecules and that PIP_2 clustering improves their presentation to PLC.

The existence of this type of crosstalk within cells is demonstrated by overexpression. Moderate overexpression of gelsolin or CapG dramatically decreases cell responsiveness to braydykinin by suppressing PLC_β activity.[105] Inhibition occurs at a step downstream of heterotrimeric G-protein activation, presumably at the level of PIP_2 hydrolysis. Washout and addback of gelsolin to semi-intact cells clearly establish that excess gelsolin is the primary cause of PLC inhibition in the gelsolin-overexpressing cells. Gelsolin and CapG also have biphasic effects on platelet-derived growth factor activation of PLC_γ, but with different dose-response characteristics.[10,105] Thus, at certain low levels of CapG overexpression, cells are more responsive to platelet-derived growth factor even though they have reduced braydykinin responses. CapG and gelsolin can therefore provide positive and negative inputs on PLC signaling, and these pathways are modulated selectively.

There is also evidence that gelsolin modulates phosphoinositide 3-kinase in vivo and in vitro. Phosphoinositide 3-kinase phosphorylates the D3 position of phosphoinositides to generate important lipid second messengers including $PI(3,4,5)P_3$ and $PI(3,4)P_2$.[106] Gelsolin has been shown to stimulate[107] as well as inhibit[108] phosphoinositide 3-kinase in vitro. Gelsolin is associated with this lipid kinase and $PI(3,4,5)P_3$ during osteoclast stimulation.[89]

In summary, gelsolin may restructure the cytoskeleton as a downstream effector of the signaling cascade and may also participate further upstream by altering the availability of PIP_2 to other PIP_2-requiring signaling enzymes.

Tumor Transformation

Many tumors have decreased gelsolin expression[109,110] and a disorganized actin cytoskeleton. Gelsolin may contribute to the transformed phenotype by acting directly on the cytoskeleton. Cancer cells have less of the high molecular weight forms of tropomyosin and caldesmon, which are protective against severing by gelsolin.[111,112] This increased susceptibility to severing could offset a decrease in gelsolin content to create a disorganized actin structure. Another possibility is that decreased gelsolin expression creates an imbalance in phosphoinositide metabolism that contributes to loss of growth control. This possibility is supported by the finding that gelsolin suppresses Ras-induced transformation in foci assays, and a gelsolin point mutation that increases PIP_2 binding is particularly effective in this suppression.[113,114]

Plasma Gelsolin and Amyloidosis

Besides existing as a cytosolic protein, a slightly larger form of gelsolin (83 kDa in human) is found in plasma at 0.2 mg/ml.[115] It has a 25 amino acid extension at its NH_2-terminus compared with cytoplasmic gelsolin and a signal sequence to direct secretion.[2] Cytoplasmic and plasma gelsolins are derived by alternative transcriptional initiation and message processing from a single gene.[115] Plasma gelsolin may be part of an extracellular actin scavenger system that clears actin filaments released by injured tissues. These filaments could otherwise cause microcirculatory obstruction and disseminated intravascular coagulation.[116,117]

Patients with familial amyloidosis, Finnish type, have a mutation in a single gelsolin S2 residue. The mutated gelsolin is proteolyzed to generate fragments that aggregate into amyloid

fibrils.[118-120] The gelsolin crystal structure[5] shows how this change can facilitate polymerization of the gelsolin β-sheet cores to form the fibril characteristics of many amyloid proteins.

Acknowledgment

This work is supported in part by grants from the United States National Institute of Health (RO1 GM51112) and the Robert A. Welch Foundation.

References

1. Yin HL, Stossel TP. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent protein. Nature 1980; 218:583-586.
2. Kwiatkowski DP, Stossel TP, Orkin SH, Mole JE, Colten HR, Yin HL. Plasma and cytoplasmic gelsolins contain a duplicated actin-binding domain. Nature 1986; 323:455-458.
3. Chaponnier C, Janmey PA, Yin HL. The actin filament severing domain of plasma gelsolin. J Cell Biol 1986; 103:1473-1481.
4. Kwiatkowski D, Janmey PA, Mole J, Yin HL. Isolation and properties of two actin-binding domains in gelsolin. J Biol Chem 1985; 260:15232-15238.
5. Burtnick LD, Koepf EK, Grimes J, Jones EY, Stuart DI, McLaughlin PJ, Robinson RC. The crystal structure of plasma gelsolin: Implications for actin severing, capping and nucleation. Cell 1997; 90:661-670.
6. Ampe C, Vandekerckhove J. The F-actin capping proteins of Physarum polycephalurn: Cap42(a) is very similar, if not identical, to fragmin and is structurally and functionally very homologous to gelsolin; Cap42(b) is Physarum actin. EMBO J 1987; 6:4149-4157.
7. Andre E, Brink M, Gerisch G, Isenberg G, Noegel A, Schleicher J, Segall E, Wallraff E. A Dictyostelium mutant deficient in severin, and F-actin fragmenting protein, shows normal motility and chemotaxis. J Cell Biol 1989; 108:985-995.
8. Yu F-X, Johnston PA, Sudhof TC, Yin HL. gCap39, a calcium ion- and polyphosphoinositide-regulated actin capping protein. Science 1990; 250:1413-1415.
9. Dabiri GA, Young CL, Rosenbloom J, Southwick FS. Molecular cloning of human macrophage capping protein cDNA. J Biol Chem 1992; 267:16545-16552.
10. Sun H-Q, Kwiatkowska K, Wooten DC, Yin HL. Effects of CapG overexpression on agonist-induced motility and second messenger generation. J Cell Biol 1995; 129:147-156.
11. Onoda K, Yu F-X, Yin HL. gCap39 is a nuclear and cytoplasmic protein. Cell Motil Cytoskel 1993; 26:227-238.
12. Onoda K, Yin HL. gCap39 is phosphorylated. Stimulation by okadaic acid and preferential association with nuclei. J Biol Chem 1993; 268:4106-4112.
13. Pringault E, Arpin M, Garcia A, Finidori J, Louvard D. A human villin cDNA clone to investigate the differentiation of intestinal and kidney cells in vivo and in culture. EMBO J 1986; 5:3119-3124.
14. Straub KL, Stella MC, Leptin M. The gelsolin-related *flightless I* protein is required for actin distribution during cellularisation in *Drosophila*. J Cell Sci 1996; 109:263-270.
15. Campbell HD, Schimansky T, Claudianos C, Ozsarac N, Kasprzak AB, Cotsell JN, Young IG, de Couet HG, Miklos GLG. The *Drosophila melgnogaster flightless-I* gene involved in gastrulation and muscle degeneration encodes gelsolin-like and leucine-rich-repeat domains, and is conserved in *C. elegans* and human. Proc Natl Acad Sci USA 1993; 90:11386-11390.
15a. Sulton J, Du Z, Thomas K et al. The *C. elegans* genome sequencing project: A beginning. Nature 1992; 356:37-41.
16. Yin HL, Stossel TP. Purification and structural properties of gelsolin, a Ca^{2+}-activated regulatory protein of macrophages. J Biol Chem 1980; 255:9490-9493.
17. Yin HL, Zaner KS, Stossel TP. Ca^{2+} control of actin gelation. Interaction of gelsolin with actin filaments and regulation of actin gelsolin. J Biol Chem 1980; 255:9494-9500.
18. Janmey PA, Chaponnier C, Lind SE, Zaner KS, Stossel TP, Yin HL. Interactions of gelsolin and gelsolin-actin complexes with actin. Effects of calcium on actin nucleation, severing and end blocking. Biochemistry 1985; 24:3714-3723.

19. Bearer EL. Direct observation of actin filament severing by gelsolin and binding by gCap39 and CapZ. J Cell Biol 1991; 115:1629-1638.
20. Yin HL, Iida K, Janmey PA. Identification of a polyphosphoinositide-modulated domain in gelsolin which binds to the side of actin filaments. J Cell Biol 1988; 106:805-812.
21. Prochniewicz E, Zhang Q, Janmey PA, Thomas DD. Cooperativity in F-actin: binding of gelsolin at the barbed end affects structure and dynamics of the whole filament. J Mol Biol 1996; 260:756-766.
22. Allen PG, Janmey PA. Gelsolin displaces phalloidin from actin filaments. J Biol Chem 1994; 269:32916-32923.
23. Feinberg J, Lebart M, Benyamin Y, Roustan C. Localization of a calcium sensitive binding site for gelsolin on actin subdomain I: Implication for severing process. Biochem Biophys Res Commun 1997; 233:61-65.
24. Kinosian HJ, Selden LA, Estes JE, Gershman LC. Kinetics of gelsolin interaction with phalloidin-stabilized F-actin. Rate constants for binding and severing. Biochemistry 1996; 35:16550-16556.
25. Ditsch A, Wegner A. Two low-affinity Ca^{2+}-binding sites of gelsolin that regulate association with actin. Eur J Biochem 1995; 229:512-516.
26. Schoepper B, Wegner A. Gelsolin binds to polymeric actin at a low rate. J Biol Chem 1992; 267:13924-13927.
27. Selve N, Wegner A. Rate constant for capping of the barbed ends of actin filaments by the gelsolin-actin complex. Eur J Biochem 1986; 155:397-401.
28. Muallem S, Kwiatkowska K, Xu X, Yin HL. Actin filament disassembly is a sufficient final trigger for exocytosis in non-excitable cells. J Cell Biol 1995; 128:589-598.
29. Vasconcellos CA, Allen PG, Wohl ME, Drazen JM, Janmey PA, Stossel TP. Reduction in viscosity of cystic fibrosis sputum in vitro by gelsolin. Science 1994; 263:969-971.
30. Sun H-Q, Kwiatkowska K, Yin HL. Actin monomer binding proteins. Curr Opin Cell Biol 1995; 7:102-110.
31. Carlier MF, Laurent V, Santolini J, Melki R, Didry D, Xia G, Hong Y, Chua N, Pantaloni D. Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: Implication in actin-based motility. J Cell Biol 1997; 136:1307-1322.
32. Janmey PA, Stossel TP. Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. Nature 1987; 325:362-364.
33. Bryan J, Hwo S. Definition of an N-terminal actin-binding domain and a C-terminal Ca^{2+} regulatory domain in human brevin. J Cell Biol 1986; 102:1439-1446.
34. Kwiatkowski D, Janmey PA, Yin HL. Identification of critical functional and regulatory domains in gelsolin. J Cell Biol 1989; 108:1717-1726.
35. Bryan J. Gelsolin has three actin binding sites. J Cell Biol 1988; 106:1553-1562.
36. Patkowski A, Seils J, Hinssen H, Dorfmuller T. Size, shape parameters, and Ca^{2+}-induced conformational change of the gelsolin molecule: A dynamic light scattering study. Biopolymers 1990; 30:427-435.
37. Way M, Gooch J, Pope B, Weeds AG. Expression of human plasma gelsolin in *Escherichia coli* and dissection of actin binding sites by segmental deletion mutagenesis. J Cell Biol 1989; 109:593-605.
38. Way M, Pope B, Gooch J, Hawkins M, Weeds AG. Identification of a region in segment 1 of gelsolin critical for actin binding. EMBO J 1990; 9:4103-4109.
39. McLaughlin PJ, Gooch JT, Mannherz H-G, Weeds AGA. Structure of gelsolin segment 1-actin complex and the mechanism of filament severing. Nature 1993; 364:685-692.
40. de Arruda MV, Bazari H, Wallek M, Matsudaira P. An actin footprint on villin. Single site substitutions in a cluster of basic residues inhibit the actin severing but not capping activity of villin. J Biol Chem 1992; 267:13079-13085.
41. Janmey PA, Lamb J, Allen PG, Matsudaira PT. Phosphoinositide-binding peptides derived from the sequences of gelsolin and villin. J Biol Chem 1992; 267:11818-11823.
42. Sun H-Q, Wooten DC, Janmey PA, Yin HL. The actin side-binding domain of gelsolin also caps actin filaments. Implications for actin filament severing. J Biol Chem 1994; 269:9473-9479.

43. Van Troys M, Dewitte D, Goethals M, Vandekerckhove J, Ampe C. Evidence for an actin binding helix in gelsolin segment 2; have homologous sequences in segments 1 and 2 of gelsolin evolved to divergent actin binding functions? FEBS Lett 1996; 397:191-196.
44. Hesterkamp T, Weeds AG, Mannherz HG. The actin monomers in the ternary gelsolin: 2 actin complex are in an antiparallel orientation. Eur J Biochem 1993; 218:507-513.
45. Bryan J, Kurth MC. Actin-gelsolin interactions. J Biol Chem 1984; 259:7480-7487.
46. Weeds AG, Gooch J, McLaughlin P, Pope B, Bengtsdotter M, Karlsson R. Identification of the trapped calcium in the gelsolin segment 1-actin complex: Implications for the role of calcium in the control of gelsolin activity. FEBS Lett 1995; 360:227-230.
47. Janmey PA, Iida K, Yin HL, Stossel TP. Polyphosphoinositide micelles and polphosphoinositide-containing vesicles dissociate endogenous gelsolin-actin complexes and promote actin assembly from the fast-growing end of actin filaments blocked by gelsolin. J Biol Chem 1987; 262:12228-12236.
48. Yu F, Zhou D, Yin HL. Chimeric and truncated gCap39 elucidate the requirements for actin filament severing and end capping by the gelsolin family of proteins. J Biol Chem 1991; 266:19269-19275.
49. Way M, Pope B, Weeds A. Molecular biology of actin binding proteins: Evidence for a common structural domain in the F-actin binding sites of gelsolin and α-actinin. J Cell Sci 1991; 14(Suppl): 91-94.
50. Pope B, Maciver S, Weeds A. Localization of the calcium-sensitive actin monomer binding site in gelsolin to segment 4 and identification of calcium binding sites. Biochemistry 1995; 34:1583-1588.
51. Weber A, Pring M, Lin S-L, Bryan J. Role of the N- and C-terminal actin-binding domains of gelsolin in barbed filament end capping. Biochemistry 1991; 30:9327-9334.
52. Weeds AG, Gooch J, Pope B, Harris HE. Preparation and characterization of pig plasma and platelet gelsolins. Eur J Biochem 1986; 161:69-76.
53. Lamb JA, Allen PG, Tuan BY, Janmey PA. Modulation of gelsolin functiion. Activation of low pH overrides Ca^{2+} requirement. J Biol Chem 1993; 268:8999-9004.
54. De Camilli P, Emr SD, McPherson PS, Novick P. Phosphoinositides as regulators in membrane traffic. [Review]. Science 1996; 271:1533-1539.
55. Lassing I, Lindberg U. Specific interaction between phosphatidylinositol 4,5 bisphosphate and profilactin. Nature 1985; 314:604-606.
56. Schafer DA, Jennings PB, Cooper JA. Dynamics of capping protein and actin assembly in vitro: uncapping barbed ends by polyphosphoinositides. J Cell Biol 1996; 135:169-179.
57. Yonezawa N, Nishida E, Iida K, Yahara I, Sakai H. Inhibition of the interactions of cofilin, destrin, and deoxyribonuclease I with actin by phosphoinositides. J Biol Chem 1990; 265:8382-8386.
58. Fukami K, Furuhashi K, Inagaki M, Endo T, Hatano S, Takenawa T. Requirement of phosphatidylinositol 4,5-bisphosphate for α-actinin function. Nature 1992; 359:150-152.
59. Fukami K, Endo T, Imamura M, Takenawa T. α-Actinin and vinculin are PIP_2-binding proteins involved in signaling by tyrosine kinase*. J Biol Chem 1994; 269:1518-1522.
60. Gilmore AP, Burridge K. Regulation of vinculin binding to talin and actin by phosphatidylinositol-4-5-bisphosphate. Nature 1996; 381:531-535.
61. Hall A. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Ann Rev Cell Biol 1994; 10:31-54.
62. Tolias KF, Cantley LC, Carpenter CL. Rho family GTPases bind to phosphoinositide kinases. J Biol Chem 1995; 270:17656-17659.
63. Ren X-D, Bokoch GM, Traynor-Kaplan A, Jenkins GH, Anderson RA, Schwartz MA. Physical association of the small GTPase Rho with a 68 kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. Mol Biol Cell 1996; 7:435-442.
64. Hartwig JH, Bokoch GM, Carpenter CL, Janmey PA, Taylor LA, Toker A, Stossel TP. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell 1995; 82:643-653.
65. Chen P, Murphy-Ullrich JE, Wells A. A role for gelsolin in actuating epidermal growth factor receptor-mediated cell motility. J Cell Biol 1996; 134:689-698.

66. Lin K, Wenegieme EF, Lu P, Chen C, Yin HL. Gelsolin binding to phosphatidylinositol 4,5 bisphosphate is modulated by calcium and pH. J Biol Chem 1997; 272:20443-20450.
67. Janmey PA. Protein regulation by phosphatidylinositol lipids. Chem Biol 1995; 2:61-65.
68. Hartwig JH, Kung S, Kovacsovics T, Janmey PA, Cantley LC, Stossel TP, Toker A. D3 phosphoinositides and outside-in integrin signaling by glycoprotein IIb-IIIa mediate platelet actin assembly and filopodial extension induced by phorbol 12-myristate 13-acetate. J Biol Chem 1996; 271:32986-32993.
69. Lu P, Shieh W, Rhee SG, Yin HL, Chen C. Lipid products of phosphoinositide 3-kinase bind human profilin with high affinity. Biochemistry 1996; 35:14027-14034.
70. Yu F-X, Sun H-Q, Janmey PA, Yin HL. Identification of a polyphosphoinositide-binding sequence in an actin monomer-binding domain of gelsolin. J Biol Chem 1992; 267:14616-14621.
71. Xian W, Vegners R, Janmey PA, Braunlin WH. Spectroscopic studies of a phosphoinositide-binding peptide from gelsolin: behavior in solutions of mixed solvent and anionic micelles. Biophys J 1995; 69:2695-2702.
72. De Corte V, Gettemans J, Vandekerckhove J. Phosphatidylinositol 4,5-bisphosphate specifically stimulates $PP60^{c\text{-}src}$ catalyzed phosphorylation of gelsolin and related actin-binding proteins. FEBS Lett 1997; 491:191-196.
73. De Corte V, Gettemans J, Waelkens E, Vandekerchove J. In vivo phosphorylation of actin in Physarum polycephalum. Study of the substrate specificity of the actin-fragmin kinase. Eur J Biochem 1996; 241:901-908.
74. Markus MA, Matsudaira P, Wagner G. Refined structure of villin 14T and a detailed comparison with other actin-serving domains. Protein Science 1997; 6:1197-1209.
75. Schnuchel A, Wiltscheck R, Eichinger L, Schleicher M, Holak TA. Structure of severin domain 2 in solution. J Mol Biol 1995; 247:21-27.
76. Hatanaka H, Ogura K, Moriyama K, Ichikawa S, Yahara I, Inagaki F. Tertiary structure of destrin and structural similarity between two actin-regulating protein families. Cell 1996; 85:1047-1055.
77. Vinson VK, Archer SJ, Lattman EE, Pollard TD, Torchia DA. Three-dimensional solution structure of Acanthamoeba profilin-I. J Cell Biol 1993; 122:1277-1283.
78. McGough A, Way M. Molecular model of an actin filament capped by a severing protein. J Struct Biol 1995; 115:144-150.
79. Downey GP, Grinstein S. Receptor-mediated actin assembly in electropermeabilized neutrophils: role of intracellular pH. Biochem & Biophys Res Comm 1989; 160:18-24.
80. Cunningham CC, Stossel TP, Kwiatkowski DJ. Enhanced motility in NIH 3T3 fibroblasts that overexpress gelsolin. Science 1991; 251:1233-1236.
81. Hug C, Jay PY, Reddy I, McNally JG, Bridgman PC, Elson EL, Cooper JA. Capping protein levels influence actin assembly and cell motility in Dictyostelium. Cell 1995; 81:591-600.
82. Carlier MF, Pantaloni D. Control of actin dynamics in cell motility. J Mol Biol 1997; 269:459-467.
83. Arora PD, McCulloch CAG. Dependence of fibroblast migration on actin severing activity of gelsolin. J Biol Chem 1996; 271:20516-20523.
84. Witke W, Sharpe AH, Hartwig JH, Azuma T, Stossel TP, Kwiatkowski DJ. Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. Cell 1995; 81:41-51.
85. Chaponnier C, Yin HL, Stossel TP. Reversibility of gelsolin/actin interaction in macrophages. Evidence of Ca^{2+}-dependent and Ca^{2+}-independent pathways. J Exp Med 1987; 165:97-106.
86. Deaton JD, Guerrero T, Howard TH. Role of gelsolin interaction with actin in regulation and creation of actin nuclei in chemotactic peptide activated polymorphonuclear neutrophils. Mol Biol Cell 1992; 3:1427-1435.
87. Carson MR, Shasby SS, Lind SE, Shasby DM. Histamine, actin-gelsolin binding and polyphosphoinositides in human umbilical vein endothelial cells. Am J Physiol 1992; 263: L664-669.

88. Dadabay CY, Patton E, Cooper JA, Pike LJ. Lack of correlation between changes in polyphosphoinositide levels and actin/gelsolin complexes in A431 cells treated with epidermal growth factor. J Cell Biol 1991; 112:1151-1156.
89. Chellaiah M, Hruska K. Osteopontin stimulates gelsolin-associated phosphoinositide levels and phosphatidylinositol triphosphate-hydroxyl kinase. Mol Biol Cell 1996; 7:743-753.
90. Lind SE, Janmey PA, Chaponnier C, Herbert T-J, Stossel TP. Reversible binding of actin to gelsolin and profilin in human platelet extracts. J Cell Biol 1987; 105:833-842.
91. Hartwig JH. Mechanisms of actin rearrangements mediating platelet activation. J Cell Biol 1992; 118:1421-1442.
92. Hartwig JH, Chambers KA, Stossel TP. Association of gelsolin with actin filaments and cell membranes of macrophages and platelets. J Cell Biol 1989; 108:467-479.
93. Barkalow K, Witke W, Kwiatkowski DJ, Hartwig JH. Coordinated regulation of platelet actin filament barbed ends by gelsolin and capping protein. J Cell Biol 1996; 134:389-399.
94. Hope HR, Pike LJ. Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains. Mol Biol of Cell 1996; 7:843-851.
95. Glaser M, Wanaski S, Buser CA, Boguslavsky V, Rashidzada W, Morris A, Rebecchi M, Scarlata SF, Runnels LW, Prestwich GD et al. Myristoylated alanine-rich C kinase substrate (MARCKS) produces reversible inhibition of phospholipase C by sequestering phosphatidylinositol 4,5-bisphosphate in lateral domains. J Biol Chem 1996; 271: 26187-26193.
96. Cooper JA, Bryan J, Schwab III B, Loftus DJ. Microinjection of gelsolin into living cells. J Cell Biol 1987; 104:491-501.
97. Cooper JA, Loftus DJ, Frieden C, Bryan J, Elson EL. Localization and mobility of gelsolin in cells. J Cell Biol 1988; 106:1229-1240.
98. Pantaloni D, Carlier M-F. How profilin promotes actin filament assembly in the presence of thymosin β4. Cell 1993; 75:1007-1014.
99. Nachmias VT, Golla R, Casella JF, Barron-Casella E. Cap Z, a calcium insensitive capping protein in resting and activated platelets. FEBS Lett 1996; 378:258-262.
100. Shaw G. The pleckstrin homology proteins: An intriguing multifunctional protein module. BioEssays 1996; 18:35-46.
101. Zheng J, Cahill SM, Lemmon MA et al. Identification of the binding site for acidic phospholipids on the PH domain of dynamin: Implications for stimulation of GTPase activity. J. Mol. Biol. 1996; 22:14-21.
102. Goldschmidt-Clermont PJ, Machesky LM, Rhee SG, Pollard TD. Regulation of phospholipase C-$_{\gamma 1}$ by profilin and tyrosine phosphorylation. Science 1991; 251:1231-1233.
103. Banno Y, Nakahima T, Kumada T, Ebisawa K, Nonomura Y, Nozawa Y. Effects of gelsolin on human pletelet cytosolic phosphoinositide-phospholipase C isozymes. J Biol Chem 1992; 267:6488-6494.
104. Baldassare JJ, Henderson PA, Tarver A, Fisher GJ. Thrombin activation of human platelets dissociates a complex containing gelsolin and actin from phosphatidylinositide-specific phospholipase $C_{\gamma 1}$. Biochemistry 1997; 324:283-287.
105. Sun H, Lin K, Yin HL. Gelsolin modulates phospholipase C activity in vivo through phospholipid binding. J Cell Biol 1997; 138:811-820.
106. Toker A, Cantley LC. Signaling through the lipid products of phosphoinositide-3-OH kinase. Nature 1997; 387:676-676.
107. Singh SS, Chauhan A, Murakami N, Chauhan VP. Profilin and gelsolin stimulate phosphatidylinositol 3-kinase activity. Biochemistry 1996; 35:16544-16549.
108. Lu PJ, Hsu AL, Wang DS, Yan HY, Yin HL, and Chen CS. Phosphoinositide 3-kinase signaling in rat liver nuclei. Biochemistry 1998; 37:5738-5745.
109. Asch HL, Head K, Dong Y, Natoli F, Winston JS, Connolly JL, Asch BB. Widespread loss of gelsolin in breast cancers of humans, mice, and rats. Cancer Research 1996; 56:4841-4845.
110. Chaponnier C, Gabbiani G. Gelsolin modulation in epithelial and stromal cells of mammary carcinoma. Am J Pathol 1989; 134:597-603.

111. Ishikawa R, Yamashiro S, Matsumura F. Differential modulation of actin-severing activity of gelsolin by multiple isoforms of cultured rat cell tropomyosin. J Biol Chem 1989; 264:7490-7497.
112. Dabrowska R, Hinssen H, Galazkiewicz B, Nowak E. Modulation of gelsolin-induced actin-filament severing by caldesmon and tropomyosin and the effect of these proteins on the actin activation of myosin Mg(2+)-ATPase activity. Biochem J 1996; 315(Pt 3): 753-759.
113. Fujita H, Suzuki H, Kuzumaki N, Müllauer L, Ogiso Y, Oda A, Ebisawa K, Sakurai T, Nonomura Y, Kijimoto-Ochia S. A specific protein, p92, detected in flat revertants derived from NIH/3T3 transformed by human activated c-Ha-*ras* oncogene. Exp Cell Res 1990; 186:115-121.
114. Fujita H, Laham LE, Janmey PA, Kwiatkowski DJ, Stossel TP, Banno Y, Nozawa Y, Mllauer L, Ishizaki A, Kuzumaki N. Functions of [His321]gelsolin isolated from a flat revertant of ras-transformed cells. Eur J Biochem 1995; 229:615-620.
115. Kwiatkowski DP, Mehl RA, Izumo S, Nadal-Ginard B, Yin HL. Muscle is the primary source of gelsolin found in plasma. J Biol Chem 1988; 263:8239-8243.
116. Haddad JG, Harper KD, Guoth M, Pietra GG, Sanger JW. Angiopathic consequences of saturating the plasma scavenger system for actin. Proc Natl Acad Sci 1990; 87:1381-1385.
117. Lee WM, Galbraith RM. The extracellular actin-scavenger system and actin toxicity. New Eng J Med 1992; 326:1335-1341.
118. Gorevic PD, Munoz PC, Gorgone G, Purcell JJ, Jr., Rodrigues M, Ghiso J, Levy E, Haltia M, Frangione B. Amyloidosis due to a mutation of the gelsolin gene in an American family with lattice corneal dystrophy type II. New England J Med 1991; 325:1780-1785.
119. Maury CP, Nurmiaho-Lassila EL. Creation of amyloid fibrils from mutant Asn187 gelsolin peptides. Biochem & Biophys Res Comm 1992; 183:227-231.
120. Kangas H, Paunio T, Kalkkinen N, Jalanko A, Peltonen L. In vitro expression analysis shows that the secretory form of gelsolin is the sole source of amyloid in gelsolin-related amyloidosis. Human Molecular Genetics 1996; 5:1237-1243.
121. Nakamura S, Sakurai T, Nonomura Y. Differential expression of bovine adseverin in adrenal gland revealed by in situ hybridization. J Biol Chem 1994; 269:5890-5896.

CHAPTER 3

Molecular Links Between Rho Family GTPases and Myosins

John A. Hammer, III and Graham P. Côté

Introduction

The demonstration in 1992 that constitutively active forms of Rho, Rac, and CDC42, when microinjected into fibroblasts, cause dramatic and specific rearrangements of the actin cytoskeleton,[1,2] spawned an enormous interest in the role that these GTPases play in determining the organization of F-actin within cells (for recent reviews see refs. 3-6). This landmark study, together with numerous subsequent studies, have firmly established that these three GTPases, all of which are members of the Rho family of G proteins, are indeed key mediators in signaling pathways that control, amongst other things, the organization of the actin cytoskeleton. Given this, and given the fact that myosins are the molecules that use F-actin to generate movement and force, it is very exciting to see the flurry of recent reports that link these same GTPases to the regulation of several members of the myosin superfamily. In this review, we will focus on these reports, which together suggest that two groups of serine/threonine protein kinases, both of which are regulated by Rho family GTPases, serve to regulate myosins I and II, two ubiquitous members of the myosin superfamily. These studies provide insight into the mechanism by which Rho family GTPases could alter the organization of the actin cytoskeleton and point to a number of other cellular processes that, because they are dependent on myosins I and II, may also be regulated by these GTPases. We will also summarize the available information regarding possible interactions between Rho-dependent signaling pathways and other members of the myosin superfamily. These studies include at least one instance (class IX myosins) where the "tables are turned" such that the myosin may control the activity of Rho family GTPases.

The Myosin Superfamily

The general notion of what constitutes a myosin, gleaned by most biologists from textbook diagrams of the structure of the sarcomere in striated muscle, has changed dramatically in the last several years. This change began innocuously enough with the demonstration over thirty years ago that nonmuscle cells contain a form of myosin very reminiscent of the myosin in muscle (for review see refs. 7-9). Like muscle myosins, this nonmuscle form (now referred to as class II myosin) is composed of two ~200 kDa heavy chains that fold into a highly asymmetric molecule possessing a pair of N-terminal globular head domains joined to a single, long rod-like tail. The globular head domains (also called subfragment 1 or S1 for the proteolytic fragment of muscle myosin that they correspond to) contain the binding sites for ATP, F-actin and light chains, a Mg^{2+} ATPase activity that is highly stimulated by

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

F-actin, and all of the machinery necessary to move actin filaments and generate force. The rod-like tail, which is formed by the intertwining of the α helical C-terminal halves of the heavy chains into a coiled-coil structure, mediates the self-association of myosin II molecules into small bipolar filaments, the functional form of the protein in cells. These myosins, which are expressed ubiquitously, have been shown to play important roles in cytokinesis, cell locomotion, maintenance of tension within the actin-rich cortical cytoskeleton, and capping of cell surface receptors.

The change in our perception of what constitutes a myosin became more dramatic with the discovery in 1971 of type I myosins (for review see refs. 10-13). These founding members of the so-called unconventional myosins (to distinguish them from conventional type II nonmuscle myosins) were originally identified in the soil ameba *Acanthameba castellani* and have since been identified in yeast, fungi, fruit flies, nematodes, plants, and mammals. Structurally, these myosins differ dramatically from type II myosins in being single-headed (i.e., they contain only one ~110-130 kDa heavy chain), roughly globular, and incapable of self-assembly into filaments. Despite these differences, type I myosins are actin-activated Mg^{2+} ATPases and can support contractile and motile activities in vitro. The monomeric and nonfilamentous nature of these proteins, as well as their enzymatic and mechanochemical properties, are reflected in the primary structure of their heavy chain, which in every case is composed of an S1-like domain fused to a C-terminal domain that shows no similarity to conventional myosin sequences and clearly cannot participate in forming a coiled-coil structure. These tail domains, which vary considerably in length and sequence, have been found in every case where examined to bind to phospholipid membranes, and in certain cases to also bind to actin filaments. The ability of the tail domain to anchor myosins I to membranes should allow these motor proteins to move membranes (e.g., plasma membrane, organelle membranes) relative to actin and vice versa. This type of interaction could support motile events such as cell locomotion and shape change, as well as the movement of intracellular vesicles/organelles on actin filaments. The presence of an actin binding site in the tail, together with the actin binding site common to all myosin head domains, allows those type I myosins containing this second actin binding site to crosslink actin filaments, slide actin filaments relative to each other, and generate a contractile tension within isotopic actin meshworks, such as those found in lamellipodia and ruffles. This type of interaction could allow type I myosins to power cell migration and changes in cell shape through its effects on the physical properties of F-actin networks. Consistent with these ideas, biochemical studies, in vitro motility assays, and cellular localization studies, together with the characterization of the behavioral defects exhibited by mutant cells engineered to lack myosins I, have together implicated these proteins in a large number of actin-dependent membrane-based motile processes, including cell locomotion, the extension, maintenance, and retraction of actin-rich cell surface projections (e.g., microvilli, lamellipodia, pseudopodia), various forms of endocytosis (fluid phase and receptor-mediated endocytosis, phagocytosis), and the transport of intracellular vesicles/organelles on actin filaments.

Finally, conservative estimates regarding the complexity of the myosin superfamily were laid to rest in recent years by the identification of ten additional classes of unconventional myosins (classes III-XII; for recent reviews see refs. 14-17). Like the myosins I, these proteins all share the ~80 kDa mechanochemical domain corresponding to S1. They differ dramatically, however, in the sequence of their nonmotor domains. These differences are not trivial, as no significant sequence similarity exists between classes. Moreover, some of the sequence motifs present in these nonmotor domains are quite surprising. For example, depending on the particular class of unconventional myosin, one can find zinc fingers, pleckstrin homology domains, calmodulin binding domains, src homology domains, protein kinase

domains, membrane binding domains, and GAP domains for Rho family GTPases. These unique nonmotor domain sequences are thought to confer functional specificity on a more-or-less generic motor domain by mediating specific interactions with different cellular structures, proteins, or membranes, i.e., by defining the "cargo". While some of these unconventional myosins may be organism-specific, at least two classes appear to be expressed across all phyla (type I and V myosins). With regard to the in vivo functions of these unconventional myosins, a great deal is yet to be learned. Indeed, knowledge regarding many of them is currently limited largely to sequence information. Nevertheless, the fact that all of them possess a myosin head domain suggests that they are all involved in some form of actin-based motility. Furthermore, recent studies of class I and V myosins suggest that many of them may support some form of membrane-associated motility (i.e., organelle motility).

Regulation of Myosin I by PAK Family Kinases

Shortly after their discovery, the myosins I from *Acanthameba* were found to require phosphorylation of a single site in their heavy chain to display actin-activated ATPase activity[17a] and to produce movement in in vitro motility assays (for review see ref. 18). The ~97 kDa myosin I heavy chain kinase (AMIHCK) responsible for phosphorylating this site was subsequently purified to homogeneity and shown to phosphorylate all three known isoforms of *Acanthameba* myosin I (AMIA, AMIB, AMIC) at a conserved serine residue (or threonine, in the case of AMIA). This residue resides within the motor domain in a surface loop that forms part of the actomyosin interface. In all three ameba myosin I isoforms, there is a tyrosine two residues C-terminal, and one or more basic residues two or three residues N-terminal, of the phosphorylated residue, and studies using synthetic peptides as substrates have confirmed the importance of these flanking residues in determining the specificity of the kinase (yielding the sequence RX^{1-3} S/T XY where X is any amino acid – as the consensus phosphorylation site for AMIHCK).[19,20] Recent studies using baculovirus-expressed *Acanthameba* myosin IC in which the phosphorylatable serine has been changed to aspartate, glutamate or alanine have confirmed the importance of this phosphorylation site in regulating the ATPase activity of myosin I (Z. Wang, E.D. Korn, and J.A. Hammer, III, unpublished observations).

Like the myosins I from *Acanthameba*, two closely-related myosin I isoforms isolated from the cellular slime mold *Dictyostelium discoideum* (DMIB, DMID) have also been shown to be regulated by heavy chain phosphorylation in vitro. This was demonstrated first using the AMIHCK,[21] and subsequently using a ~110 kDa myosin I heavy chain kinase isolated from *Dictyostelium* (DMIHCK), which is specific for the DMID isoform.[22] Sequence alignments show that these two *Dictyostelium* myosin I isoforms, as well as several other isoforms, contain a serine or threonine at the conserved site identified in the ameba proteins, and that the sequence context for these sites matches the consensus phosphorylation site sequence defined for AMIHCK. Furthermore, evidence has been presented that phosphorylation of this site is required for the function of DMIB in vivo.[23]

While the studies outlined above span some 15 years of research, the identification of these myosin I kinases as members of the family of p21-activated protein kinases (PAKs) was made only recently. The PAK kinase family, whose first member was identified in rat brain extracts on the basis of its ability to bind the Rho family GTPases Rac and CDC42 in gel overlay assays, includes numerous vertebrate PAKs, as well as PAKs from *C. elegans, Drosophila* and yeast (Ste20, Cla4, Skm1) (for a recent review see ref. 24). The kinase activity of PAK kinases towards exogenous substrates increases dramatically (~50-fold) following the binding of GTP-bound forms of Rac or CDC42, which induce autophosphorylation of the kinase at several sites. In terms of their function, these serine/threonine kinases are thought to play critical roles as activators of mitogen-activated (MAP) protein kinase cascades and

stress-activated (SAPK) protein kinase cascades through their ability to be activated by Rac and/or CDC42, and their ability to phosphorylate the next protein kinase in the cascade. These kinase cascades result in the transcription of numerous genes and effect a variety of complex cellular processes. In addition to their roles in stimulating MAP and SAPK kinase cascades, there is widespread speculation that PAK kinases may also serve as mediators of the cytoskeletal changes induced by Rac and CDC42, given that PAKs are activated by these same GTPases. The localization of a PAK kinase in actin-rich structures present within *Drosophila* and mammalian cells,[25-27] the demonstration that PAK kinases induce cytoskeletal rearrangements when microinjected into cells,[25,26] and the fact that the yeast PAK kinase Ste20 is concentrated in cortical actin patches and binds to BEM1, an actin binding protein,[28] are all consistent with this idea.

The identification of the MIHCKs described above as PAK family kinases was made first through sequence analyses of the ~110 kDa DMIHCK[29] and the ~97 kDa AMIHCK,[30] which revealed a very high degree of sequence similarity between their catalytic domains and that of PAK kinases. Like PAKs, DMIHCK was also found to contain the conserved ~60-residue sequence that specifically binds activated (i.e., GTP-bound) CDC42 or Rac1 (known alternatively as the CRIB domain (for CDC42/Rac interactive binding) or GBD (for GTPase binding domain)). The assignment of DMIHCK as a PAK kinase was confirmed when it was shown that DMIHCK binds GTP-CDC42 and GTP-Rac1 (but not GTP-Rho) in gel overlay assays and in solution, that GTP-Rac1 stimulates the kinase activity of DMIHCK towards DMID 10-fold, and that this stimulation is associated with the enhanced autophosphorylation of DMIHCK.[29] Recent work on the AMIHCK has revealed the presence of a CRIB domain in this kinase and shown that it is also activated by CDC42 and Rac1 (but not Rho) in GTP-dependent manner (H. Brzeska, R. Young, U. Knause, and E.D. Korn, personnel communication). Finally, the overall domain structures of both DMIHCK[29] and AMIHCK[31] closely resemble that of PAKs, based on the presence of proline-rich and acidic sequences, and on the C-terminal localization of the catalytic domain (Fig. 3.1). Together these results argue strongly that these two MIHCKs are indeed PAK family members.

Further evidence that AMIHCK and DMIHCK are PAK kinases have come from the recent demonstration that rat brain PAK, as well as two yeast PAKs (Ste20 and Cla4), phosphorylate the heavy chain of DMID,[36] while a human recombinant PAK phosphorylates the heavy chain of AMIC.[37] In all four cases, the actin-activated ATPase of the myosin I was stimulated to the same extent as with the authentic MIHCKs. This fact, together with phosphopeptide mapping and the measurement of the stoichiometry of phosphorylation in cophosphorylation experiments,[36] all suggest that the vertebrate and yeast PAKs phosphorylate the same regulatory serine/threonine in the myosin I heavy chain as is phosphorylated by the MIHCKs. Consistent with this, a synthetic peptide corresponding to the phosphorylation site of AMIC is a good substrate for PAK, and PAK, like AMIHCK, shows a preference for peptides with a tyrosine two residues C-terminal of the phosphorylated serine.[37]

What are the implications if, as it now seems, MIHCKs are PAK family members? First, this finding allows us for the first time to link the regulation of these myosins to a particular signal transduction pathway. This link provides the framework for future efforts directed at connecting the regulation of myosin I-dependent cellular functions to various types of extracellular signals (it should be noted in this regard that the regulation of PAK kinases is complex and involves multiple interactions in addition to those with Rac/CDC42; see Fig. 3.1). Second, this finding adds to the growing evidence that PAK kinases may be important mediators in the formation of the actin-rich lamellopodia and filopodia that are induced by CDC42 and Rac1, respectively. Third, these results suggest that type I myosins may be important effectors in producing these cytoskeletal rearrangements.

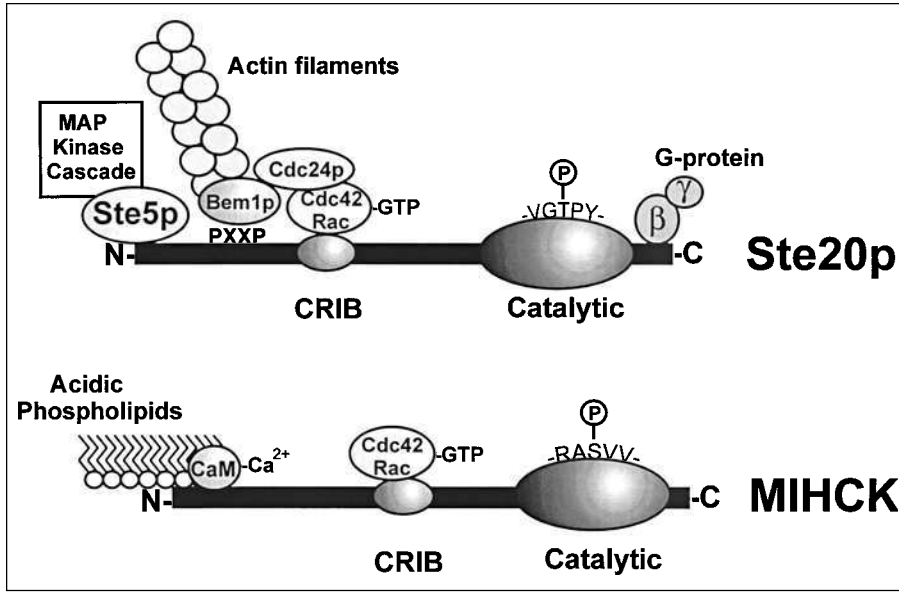


Fig. 3.1. Schematic representation of the domain structure of yeast Ste20p and the amoeboid MIHCKs. Ste20p and MIHCK both contain a conserved CDC42/Rac-binding domain (CRIB domain) and a C-terminal protein kinase catalytic domain, but share little sequence identity outside of these regions. In addition to binding CDC42/Rac in a GTP-dependent manner, Ste20p binds G-protein βγ subunits, the MAP kinase scaffold protein Ste5p, and the SH3 domain-containing protein Bemlp.[32] Ste5p associates with downstream members of the MAP kinase cascade, while Bemlp binds actin filaments and Cdc24p, a guanine nucleotide exchange factor for CDC42. Note that the regions of Ste20p which bind Ste5p and Bemlp have not yet been mapped. Phosphorylation of Thr-777 in the catalytic domain "activation segment" is required for Ste20p to display kinase activity in vitro and in vivo.[33] The interactions shown for MIHCK combine data for both the *Dictyostelium* and *Acanthameba* MIHCKs (for review see ref. 18). The *Dictyostelium* kinase binds CDC42/Rac in a GTP-dependent manner, Ca^{2+}-calmodulin and acidic phospholipids. Studies on the *Acanthameba* kinase have shown that Ca^{2+}-calmodulin and phospholipids compete for a site close to the N-terminus[34] and have identified a Ser in the activation segment as a site of autophosphorylation.[35]

While the results described above are quite exciting, they also raise several important questions. First, besides the myosins I from *Acanthameba* and *Dictyostelium*, what other type I myosins might be regulated by PAK family kinases? Alignments of myosin I heavy chain sequences have shown that the conserved serine/threonine residue which serves as the regulatory phosphorylation site in the *Acanthameba* and *Dictyostelium* myosins I is also present in myosins I from *Saccharomyces cerevisiae* and *Aspergillus nidulans*, suggesting that PAK kinases may regulate type I myosins in yeast and fungi, as well as in ameba and slime molds.[18,38] While this is quite exciting, it is nevertheless the case that all of the metazoan myosins I, including numerous isoforms sequenced from vertebrates, *Drosophila*, and *C. elegans*, do not possess this conserved phosphorylation site.[38] Moreover, analyses of all the other myosin classes indicated that only class VI unconventional myosins from rat and *Drosophila* contained a serine at this position. In almost all cases, this position was found to be occupied by either a glutamate or an aspartate residue (the TEDS rule).[38] Given that acidic amino acid residues are known to mimic phosphorylated residues both in vitro and

in vivo, it has been hypothesized that the majority of myosins, including all metazoan myosins I identified to date, have relinquished the requirement for phosphorylation-dependent activation by replacing the hydroxyl amino acid with a fixed negative charge.[18,38] Indeed, where studied vertebrate myosins I have been shown to be highly active in the absence of heavy chain phosphorylation (see for example 39). These results suggest that PAK family kinases are unlikely to be involved in the regulation of vertebrate myosins, with the possible exception of type VI myosins. It is important to keep in mind, however, that (i) slight shifts in the sequence alignments described above can in certain myosins place an hydroxyl amino acid in the "correct" position, (ii) that PAK kinase phosphorylation sites may exist elsewhere in myosins, and (iii) that these alternate sites need not fit the consensus sequence as defined for the *Acanthameba* myosins I (see, for example, the PAK-mediated phosphorylation of myosin II regulatory light chains below). Whether such cases exist can only be established through biochemical efforts to identify phosphorylation-dependent regulation of vertebrate myosin I isoforms, as well as members of other classes of unconventional myosins.

The second major question raised by the identification of MIHCKs as PAK family members is to what extent are the cytoskeletal rearrangements induced by Rac1 and CDC42 mediated by PAK-dependent activation of myosins I in lower eukaryotes? Recent studies on type I myosins and PAK kinases in budding yeast shed considerable light on this issue. Efforts to sequence myosins in *Saccharomyces cerevisiae*, together with the recent completion of efforts to sequence the genome of this organism, make it clear that budding yeast contain two type I myosin heavy chain genes (*myo3* and *myo5*) (for review see ref. 40). The encoded proteins, which closely resemble in primary structure the myosins I from *Acanthameba*, appear to have largely redundant functions in that single mutants exhibit no obvious behavioral defects. Double mutants, on the other hand, exhibit striking defects in the organization of the actin cytoskeleton, cell shape, polarized growth, fluid phase and receptor-mediated endocytosis, and secretion, although the extent to which each of these defects is a primary response to the elimination of class I myosins from the cell, as opposed to being downstream of generalized defects in the actin cytoskeleton, remains to be seen.[41,42] In terms of their regulation, both myo3p and myo5p possess the consensus phosphorylation site defined for the *Acanthameba* myosins I, and are, therefore, candidates for PAK-dependent phosphorylation. While in vitro studies demonstrating that these yeast myosins I are activated by heavy chain phosphorylation have yet to be performed, in vivo studies are completely consistent with this idea. Specifically, the ability of myo3p to rescue the phenotype of a *myo3/myo5* double mutant is lost when the serine at the putative heavy chain phosphorylation site is mutated to an alanine, but not when it is mutated to an aspartate, indicating that a negative charge at this site is needed for normal function (unpublished data referred to in ref. 36).

In terms of candidate PAK kinases (i.e., MIHCKs) in yeast, *Saccharomyces* expresses at least three PAKs: Ste20, Cla4, and Skm1 (for review see refs. 24,32,43,44) (Fig. 3.1). As in vertebrates, these yeast kinases have been linked to the activation of a variety of MAP kinase cascades. Furthermore, all three kinases have been linked to signaling pathways that result in dramatic changes in cell morphology. These changes, which involve major rearrangements of the actin cytoskeleton, include budding (where Ste20 and Cla4 play essential roles in actin deposition at the incipient bud site and in septin deposition at cytokinesis[45]), the formation of the actin-rich cell surface projections (schmoos) associated with mating (where Ste20 activates the pheromone-responsive MAP kinase cascade that triggers cell cycle arrest, the transcription of mating-specific genes, and schmoo formation[46]), and filamentous growth during nitrogen starvation (where Ste20 probably activates a separate MAP kinase cascade associated with this response[47]). Given that yeast CDC42 has also been shown to play an essential role in budding and the formation of mating-specific projections and given the

likelihood that Ste20 and Cla4 function in vivo as activators of myosin I (which, while not proven, is likely based on their ability to activate a *Dictyostelium* myosin *I* in vitro[36]), it seems reasonable to suspect that activated myosins I play significant roles as effectors of the cytoskeletal rearrangements induced by CDC42. While the fact that *myo3/myo5* double mutants exhibit dramatic defects in the actin cytoskeleton[42] is consistent with this, using targeted gene disruptions of PAK kinases to get a firm handle on the extent to which myosins I are responsible for the PAK-dependent morphological changes outlined above will be very difficult. This is due in large part to the fact that each yeast PAK kinase is probably multifunctional. For example, Ste20 has already been linked to the activation of three different MAP kinase cascades, each of which has important effects on different complex cellular processes.[24] It is not surprising, therefore, that the phenotypes in yeast of myosin I knockouts and of PAK kinase knockouts are not the same, and it very unlikely that a myosin I containing an aspartate in place of the regulatory serine would rescue all of the defects exhibited by Ste20/Cla4 knockouts (although it might rescue some of the defects). Clearly, much needs to be learned. It is probably safe to say, however, that yeast lend themselves best to resolving these complex interactions because of the powerful genetic approaches that can be applied.

In summary, therefore, a picture is emerging in which myosins I, by virtue of their ability to be activated by PAK kinases, serve as important effectors of the cytoskeletal rearrangements induced by Rac and CDC42. The fact that myosins I have been localized in a variety of cell types to actin-rich regions, such as lammelopodia, ruffles and phagocytic cups (reviewed in refs. 10-13, 15), and that they have been implicated through the analysis of mutants in a variety of actin-dependent cellular processes, such as endocytosis, phagocytosis, cell locomotion, pseudopod extension and polarized cell growth,[41,42,48-51] are all consistent with this idea. Nevertheless, it remains to be seen how widespread this type of regulation is, given that PAK-dependent phosphorylation of vertebrate myosins I appears unlikely.[38]

Regulation of Myosin II by PAK Family Kinases

The actin-activated ATPase activity, self-assembly properties and in vitro motility of smooth muscle and vertebrate nonmuscle myosins II are all stimulated by phosphorylation of the myosin's 20 kDa regulatory light chain (MLC_{20}) (reviewed in ref. 52). The principal player in this phosphorylation is calcium/calmodulin-dependent myosin light chain kinase (MLCK). This kinase, which has been identified in many vertebrate cell types, phosphorylates MLC_{20} on serine-19 and, at a much slower rate, on threonine 18. MLC_{20} can also be phosphorylated in vitro by protein kinase C, the cell cycle dependent protein kinase Cdc2, and calmodulin-dependent protein kinase II, all of which effect the ATPase activity of the myosin. The dephosphorylation of MLC_{20} is catalyzed primarily by a trimeric smooth muscle myosin phosphatase, which is composed of a 37 kDa type-1 protein phosphatase catalytic subunit, a 130 kDa myosin binding subunit, and a 20 kDa subunit.[53]

In addition to MLCK and the other kinases mentioned above, there is growing evidence that PAK kinases also activate smooth muscle and vertebrate nonmuscle myosins II by phosphorylating MLC_{20}. First, AMIHCK has been shown to phosphorylate MLC_{20} in intact turkey gizzard smooth muscle myosin with a specific activity that is close to that measured for authentic calcium/calmodulin-dependent MLCK.[54] Moreover, this phosphorylation, which now appears to be on threonine-18,[37] fully stimulates the actin-activated ATPase activity of turkey gizzard myosin II.[54] Second, a protease-activated kinase isolated from rabbit reticulocytes,[55] and later identified by sequence analysis as a PAK,[56] also activates smooth muscle myosin by phosphorylation of its regulatory light chain. Third, a PAK kinase (S6/H4 kinase) isolated from human placenta has been shown to phosphorylate the

regulatory light chain of myosin II from bovine arterial endothelium in a GTP-CDC42-dependent manner.[57] Through the use of phosphopeptide maps, phosphoamino acid analyses and recombinant MLC_{20} mutants, it was shown that this PAK phosphorylates serine-19 only, even after extensive incubation.

Two detergent-permeabilized cell systems have recently been used to investigate the ability of PAKs to stimulate myosin II-mediated contractility in situ. First, permeabilized bovine pulmonary artery endothelial cells, which contract upon exposure to calcium/calmodulin-dependent MLCK,[58] were shown to contract under conditions where MLCK is inactive (in the absence of free calcium and in the presence of a MLCK inhibitor) by the addition of PAK.[57] Second, addition of PAK kinase to permeabilized guinea pig taenia coli smooth muscle fibers produced a calcium-independent contraction that correlated with the increase in the level of MLC_{20} phosphorylation.[59] These studies suggest that PAK-dependent phosphorylations of smooth and nonmuscle type II myosins could provide cells with an additional (and calcium-independent) mechanism by which to control myosin II-driven cellular functions. Furthermore, these results suggest that in certain cases PAK kinases may activate both type I and type II myosins coordinately (Fig. 3.2). These results also argue that PAK phosphorylation sites in myosins need not fit the consensus *Acanthameba* myosin I phosphorylation site sequence (while there are basic residues N-terminal of serine-19 in MLC_{20}, the sequence C-terminal of serine-19 does not fit the myosin I consensus, and synthetic peptides with a similar sequence C-terminal of the serine are poor substrates for AMIHCK[37]). Future efforts need to be directed at determining whether PAK kinases play a significant role in regulating type II myosins in vivo. If this is the case, then these myosins may also be important effectors of the cytoskeletal changes induced by Rac1/CDC42. It should be noted, however, that the role of PAK kinases (and, therefore, PAK-dependent activation of type II myosins) in mediating the cytoskeletal changes induced by Rac1 and CDC42 is still very controversial. For example, mutant forms of CDC42 and Rac1, which are thought to no longer interact with PAK, are still able to induce their characteristic changes in the actin cytoskeleton of fibroblasts.[60,61] Furthermore, a constitutively active form of PAK causes disruption of actin stress fibers in fibroblasts without inducing either membrane ruffling or micro spikes.[25] These results suggest that proteins other than PAK, such as WASP,[62] POR1,[63] or N-chimerin,[64] mediate the cytoskeletal rearrangements induced by Rac1/CDC42, although there are alternate explanations which still implicate PAKs as the mediators.[24]

Regulation of Myosin II by Rho-Associated Kinases

In addition to their possible regulation by Rac/CDC42-dependent PAK kinases, several recent studies have implicated Rho-dependent kinases in the regulation of smooth muscle and nonmuscle type II myosins. These serine/threonine kinases, which include p164 Rho-associated kinase (Rho-K; also known as ROK α)[65,66] and p160 Rho-associated coiled coil containing protein kinase ($p160^{ROCK}$),[67] are composed of an N-terminal kinase domain, a central domain that is predicted to form a coiled-coil structure, and a C-terminal domain containing a cysteine-rich zinc finger motif and a pleckstrin homology domain. These kinases, which we will refer to here simply as Rho-associated kinases, are activated 2- to 15-fold by GTP-Rho.

The link between Rho-associated kinases and the regulation of type II myosins grew out of the initial observation that the addition of GTP-Rho to permeabilized smooth muscle fibers enhances both the calcium sensitivity of contraction[68] and MLC_{20} phosphorylation.[69] Consistent with this, the addition of a constitutively-active Rho-kinase catalytic domain fusion protein to permeabilized rabbit portal vein smooth muscle was recently shown to induce contraction under conditions where MLCK would be inactive (in the absence of cytosolic free calcium and in the presence of Wortmanin, an inhibitor of MLCK).[70] Insight

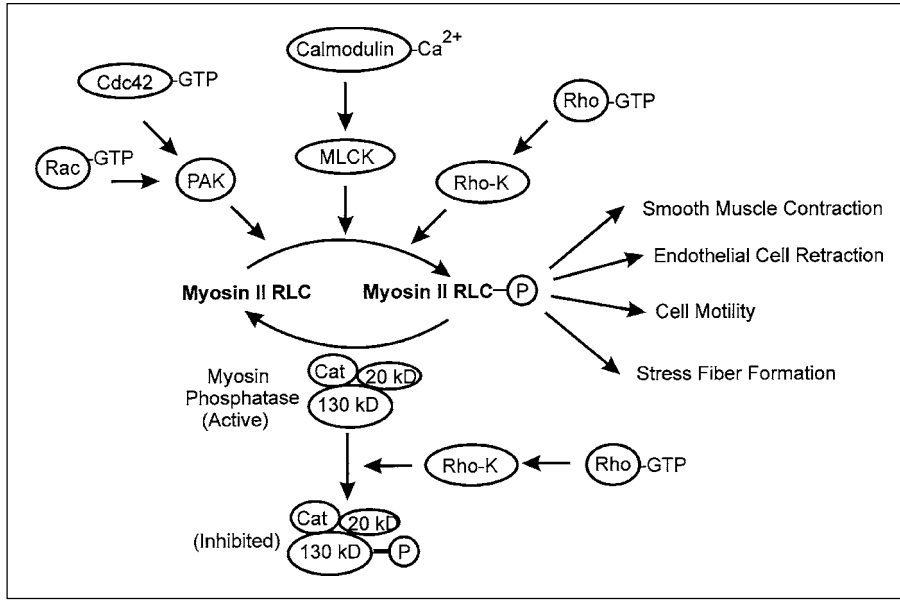


Fig. 3.2. Schematic depicting the possible coordinate regulation of type I and II myosins by kinases that are regulated by Rho-family GTPases. As indicated in the text, Rho-kinase can influence the level of P-MLC_{20} both by directly phosphorylating the light chain and by inhibiting the light chain phosphatase.

into the mechanism of this regulation has come from the recent finding that the 130 kDa myosin-binding subunit of smooth muscle myosin light chain phosphatase binds Rho-GTP.[71] While the significance of this interaction remains unclear, it was also shown that Rho-kinase phosphorylates this subunit of the phosphatase, resulting in an ~80% reduction in the enzymatic activity of the holoenzyme in vitro.[71] Shortly after publication of this work, it was reported that Rho-kinase can also phosphorylate MLC_{20} directly in intact smooth muscle myosin.[72] Furthermore, Rho kinase was shown to phosphorylate serine-19, the regulatory site phosphorylated by authentic MLCK, and to activate the ATPase activity of the myosin. These results suggest, therefore, that Rho-kinase would cause an increase in the level of phosphorylated MLC_{20} in vivo by (i) inhibiting the activity of the light chain phosphatase, and (ii) by directly phosphorylating MLC_{20}. If true, these results would provide an explanation for the fact that excitory agonists increase the calcium sensitivity of MLC_{20} phosphorylation, and hence smooth muscle contraction, a process that is dependent on one or more G proteins (reviewed in ref. 73).

With regard to nonmuscle myosin II, overexpression of a constitutively active form of RhoA in NIH 3T3 fibroblasts has recently been shown to result in an increase in the level of phosphorylated MLC_{20}.[71] Furthermore, two recent studies suggest that this Rho-induced elevation in P-MLC_{20} levels is in fact mediated by Rho-kinase. First, Wodnicka and Burridge[74] reported that protein kinase inhibitors like KT5926 block the Rho-induced contraction of fibroblasts and that this block is accompanied by a reduction in the level of P-MLC_{20} and a loss of both stress fibers and focal adhesions. Second, overexpression of wild type Rho-associated kinases (but not mutant forms that lack kinase activity) in fibroblasts has been shown to result in the formation of stress fibers and focal adhesions.[66,75] Given these

observations, and given the fact that GTP-Rho induces the assembly of stress fibers and focal adhesions in cultured cells, it is tempting to speculate that myosin II, by virtue of its ability to be activated by Rho-dependent kinases, is a major effector of these Rho-induced rearrangements of F-actin. Indeed, it is reasonable to propose a model in which active bipolar filaments of myosin II, generated following phosphorylation of MLC_{20} by Rho-associated kinases, participate in the formation of actin stress fibers, which are then recruited to adhesion complexes, leading to the formation of typical focal adhesion plaques.[6] Future efforts should be directed at testing such a model and at determining the relative contributions of light chain phosphatase inactivation versus direct phosphorylation of MLC_{20} in determining the level of P-MLC_{20} (in the permeabilized smooth muscle system, at least, the Rho-kinase-induced contraction can be largely attributed to the direct phosphorylation of MLC_{20}, since in the absence of active MLCK the inhibition of the phosphatase would not, by itself, promote contraction[70]).

Links Between Signal Transduction Pathways and Other Members of the Myosin Superfamily

In contrast to type I and II myosins, very little is known about the regulation of the unconventional myosins representing classes III through XII (see ref. 15). Having said this, it is the case that many of these myosins appear to bind authentic calmodulin as a light chain and so may be regulated by signaling pathways that influence the concentration of free calcium in the cytosol.[15] Furthermore, the sole class IV unconventional myosin identified to date, as well as certain class I myosins from both lower and higher eukaryotes, contain the ~50-residue sequence corresponding to src-homology-region 3 (SH3) in nonreceptor tyrosine kinases.[15] The presence of this domain, which is also found in a large number of proteins involved in signal transduction (for review see ref. 76), including adapter proteins that link membrane tyrosine kinases to the ras signaling pathway, may link type I and IV myosins to important signaling molecules (although the only protein identified to date that binds to an SH3 domain in a myosin (Acanth 125) does not appear to be a signaling molecule[77]). Finally, the class III unconventional myosin *ninaC*,[78] which is expressed within photoreceptor cells in the *Drosophila* eye, possesses a protein kinase domain as an N-terminal extension of the myosin head (for review see ref. 15). The regulation of this kinase domain, whose sequence is most closely related to that of Ste20, will probably play a critical role in the control of signal transduction in these photoreceptor cells.

Against this backdrop of relatively fragmentary data, there is one striking example of linkage between a recently identified unconventional myosin and a specific signal transduction pathway. This case involves the type IX myosins, which were originally identified in rat (myr5),[79] and subsequently cloned from human.[80] The ~225 kDa heavy chain of this myosin contains a number of novel features, the most striking of which is the presence within the tail domain of a ~140-residue region that exhibits striking similarity to GTPase activating proteins (GAPs) for the Rho family GTPases. These proteins, which include p190, N-chimerin, 3BP-1 and Rho-GAP, serve to accelerate the hydrolysis of the GTP bound to Rho family GTPases, thereby catalyzing the conversion of these "signaling switches" from their active GTP-bound form to their inactive GDP-bound form. Together with GDIs (GDP dissociation inhibitors, which prevent GDP release) and GDSs (GDP dissociation stimulators, which promote exchange of GDP for GTP), GAPs serve to modulate the activity of Rho family GTPases.

Evidence that the putative GAP domain in the type IX myosin from rat (myr5) actually functions as a GAP was obtained by in vitro analysis of a GST fusion protein containing this domain, which was shown to stimulate the GTPase activities of RhoA and to a much lower extent CDC42 and Rac1.[79,81] Furthermore, the introduction into the myr5 GAP domain of

a mutation that abolishes the ability of bonafide GAPs to stimulate GTP hydrolysis also abolished the GAP activity of the myr5 GAP domain. Results similar to these have also been obtained in lysates of insect cells expressing myr5 or just its GAP domain.[81]

The results described above not only draw a link between type IX unconventional myosins and Rho-dependent signaling, they do so in a way that is the opposite of the link drawn between PAKs/Rho-associated kinases and type I and II myosins, since in this case it is the myosin that is potentially regulating the activity of the GTPase, and not vice versa. One implication of this finding is that type IX myosins could serve in vivo to downregulate the formation of the stress fibers/focal adhesions that are induced by Rho. Preliminary results are in fact consistent with this idea.[81] Specifically, overexpression in Hela cells of myr5, or just its GAP domain, results in the loss of actin stress fibers and focal adhesions. This result, and the finding that the loss of these actin-rich structures is prevented in cells cotransfected with a constitutively active form of Rho, together indicate that myr5 is a Rho-GAP in vivo, and support the idea that the loss of actin stress fibers and focal adhesions induced by myr5 is due to the myr5 GAP domain-mediated acceleration in the hydrolysis of GTP-Rho. One clue to the mechanism by which this activity could be regulated comes from the presence in the myr5 heavy chain of a zinc coordination domain immediately adjacent to the GAP domain.[79,80] This zinc binding domain, which is homologous to the regulatory domain of protein kinase C and to a region immediately N-terminal of the GAP domain in the candidate Rac GAP proteins N- and B-chimerin, has been shown to bind two zinc atoms in vitro, but not diacylglycerol (as in PKC).[79] Regardless of how the regulation occurs, what is clear is the broader implication of these studies, which is that myosin IX (and perhaps other myosins) may actually regulate the organization of the cytoskeletal element on which it moves.

Summary

The studies summarized above suggest that two families of serine/threonine kinases, the Rac1/CDC42-dependent PAK kinases and the Rho-dependent kinases typified by Rho-K and P160^{ROCK}, serve to regulate the enzymatic and mechanochemical properties of a number of different myosins. Furthermore, in the case of the PAK kinases, it appears that these enzymes may simultaneously activate (i) the transcription of genes essential for a particular cellular function (through activation of a MAP kinase cascade) and (ii) effector proteins (including myosins) that are involved in supporting this same cellular function (through direct phosphorylation)[24,32,36] (Fig. 3.3). While these studies provide the foundation for future efforts to define in detail the regulation of myosin-dependent cellular functions via extracellular signals, many parts of this story are still unclear. For example, the idea that PAK kinases mediate the cytoskeletal changes induced in mammalian cells by Rac/CDC42 has recently been called into question.[60,61] In addition, some PAK-dependent functions do not appear to require the expression of kinase activity by PAK in vivo.[26] Moreover, direct proof that these kinases phosphorylate myosins in vivo is missing (even in the case of the protozoan MIHCKs, which have been studied for many years). Furthermore, while the studies summarized herein point to myosins as one potential effector of the rearrangements in the actin cytoskeleton induced by Rho family GTPases, a direct link between these kinases and other proteins that are likely to be involved in these rearrangements (e.g., proteins which sequester monomeric actin, proteins which nucleate, cap, sever and bundle filamentous actin) is tenuous (for review see ref. 5). Indeed, it is still quite possible that these kinases actually regulate other pathways (e.g., PI metabolism), which in turn lead to changes in the actin cytoskeleton.[5,6] On top of these issues is the issue of specificity. For example, *Acanthameba* and *Dictyostelium* contain at least four and seven myosin I heavy chain isoforms, respectively.[14,17] If each isoform supports a different function, as many think, then it makes sense that each would be independently regulated by an isoform-specific PAK kinase. The

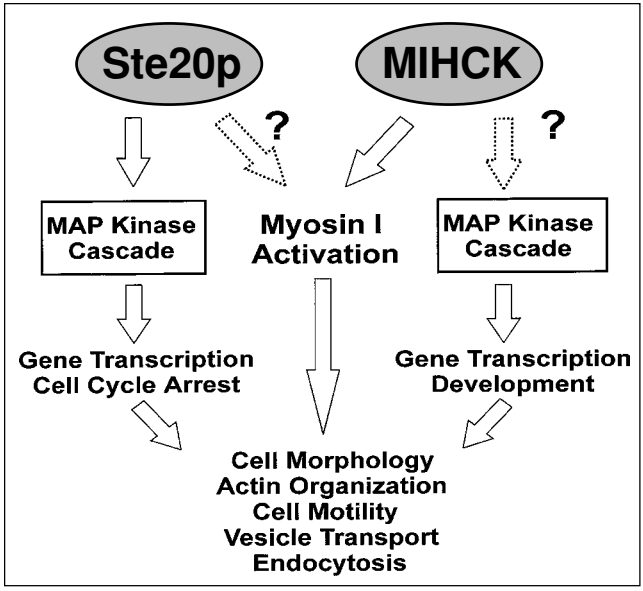


Fig. 3.3. Yeast Ste20p and the amoeboid MIHCKs may couple the activation of MAP kinase signaling pathways to motile events driven by myosin I. Ste20p is an essential component of the yeast pheromone response pathway, where it serves to activate upstream elements of a MAP kinase cascade. As described in the text, Ste20p may also activate the two yeast myosins I (myo3p and myo5p) by heavy chain phosphorylation. Therefore, Ste20 appears to be positioned in such a way as to coordinate processes that might be dependent on myosins I, such as the rearrangement of the actin cytoskeleton and the directed transport of vesicles, with the stimulation of signaling pathways that modulate gene transcription. In the case of the *Dictyostelium* and *Acanthameba* MIHCKs, their activation, which may be triggered by stimulation of chemotactic receptors, may also lead to the simultaneous activation of both myosin I-dependent motile events and gene transcription.

potential exists, therefore, for tremendous complexity in the regulation of myosins by the members of these two kinase families. Finally, the recent results with the class IX unconventional myosin from rat indicates that the regulatory interactions between Rho family GTPases and myosins can go both ways.

Acknowledgment

We thank Dr. Hanna Brzeska and Dr. Edward D. Korn for their comments on the manuscript.

References

1. Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell, 1992; 70:389-399.
2. Ridley AJ, Paterson HF, Johnston CL, Diekman P, Hall A. The small GTP binding protein rac regulates growth factor-induced membrane ruffling. Cell, 1992; 70:401-410.
3. Hall A. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Ann Rev Cell Biol 1994; 10:31-54.
4. Ridley AJ. Rho: Theme and variations. Curr Biol 1996; 6:1256-1264.
5. Zigmond SH. Signal transduction and actin filament organization. Curr Opin Cell Biol 1996; 8:66-73.
6. Tapon N, Hall A. Rho, Rac and CDC42 GTPases regulate the organization of the actin cytoskeleton. Curr Opin Cell Biol 1997; 9:86-92.
7. Korn ED, Hammer JA III. Myosins of nonmuscle cells. Annual Review of Biophysics and Biophysical Chemistry, 1988; 17:23-45.
8. Warrick HM, Spudich JA. Myosin structure and function in cell motility. Ann Rev Cell Biol 3:379-421. In pursuit of myosin function. Cell Regulation 1987; 1:1-11.

9. Spudich JA. In pursuit of myosin function. Cell Regulation, 1989; 1:1-11.
10. Korn ED, Hammer JA III. Myosin I. Curr Opin Cell Biol 1990; 2:57-61.
11. Pollard TD, Doberstein SK, Zot HG. Myosin-I. Ann Rev Physiol 1991; 53:653-681.
12. Hammer JA III. Novel myosins. Trends in Cell Biology 1991; 1:50-56.
13. Ostap EM, Pollard TD. Overlapping functions of myosin-I isoforms? J Cell Biol 1996; 133:221-224.
14. Hammer JA III. The structure and function of unconventional myosins: A review. J Muscle Res and Cell Motility 1994; 15:1-10.
15. Mooseker MS, Cheney RE. 1995; Unconventional myosins. Ann Rev Cell and Dev Biol 11:633-675.
16. Sellers JR, Goodson HV, Wang F. A myosin family reunion. J Muscle Res and Cell Motility 1996; 17:7-22.
17. Titus MA. Unconventional myosins:new frontiers in actin-based motors. Trends Cell Biol 1997; 7:119-123.
17a. Maruta H, Korn ED. Acanthamoeba cofactor protein is a heavy chain kinase required for actin-activation of the Mg^{2+}-ATPase activity of Acanthamoeba myosin I. J Biol Chem 1977; 252:8329-8332.
18. Brzeska H, Korn ED. Regulation of class I and class II myosins by heavy chain phosphorylation. J Biol Chem 1996; 271:16983-16986.
19. Brzeska H, Lynch TJ, Martin B, Korn ED. The localization and sequence of the phosphorylation sites of Acanthamoeba myosin I. J Biol Chem 1989; 264:19340-19348.
20. Brzeska H, Lynch TJ, Martin B, Corigliano-Murphy A, Korn ED. Substrate specificity of *Acanthamoeba* myosin I heavy chain kinase as determined with synthetic peptides. J Biol Chem1990; 265:16138-16144.
21. Côté GP, Albanesi JP, Ueno T, Hammer JA III, Korn ED. Purification from *Dictyostelium* of a low-molecular weight myosin that resembles myosin I from *Acanthamoeba castellanii*. J Biol Chem 1985; 260:4543-4546.
22. Lee S-F, Côté GP. Purification and characterization of a *Dictyostelium* protein kinase required for the actin-activated MgATPase activity of *Dictyostelium* myosin ID. J Biol Chem 1995; 270:11776-11782.
23. Novak KD, Titus MA. Myosin I overexpression impairs cell migration. J Cell Biol 1997; 136:633-647.
24. Sells MA, Chernoff J. Emerging from the PAK: The p21-activated protein kinase family. Trends Cell Biol1997; 7:162-167.
25. Manser E, Huang HY, Loo TH, Chen XQ, Dong JM, Leung T, Lim L. Expression of constitutively active α-PAK reveals effects of the kinase on actin and focal complexes. Mol Cell Biol 1997; 17:1129-1143.
26. Sells MA, Knaus UG, Bagrodia S, Ambrose DM, Bokoch GM Chernoff J. Human p21-activated kinase (PAK1) regulates actin organization in mammalian cells. Curr Biol 1997; 7:202-210.
27. Harden N, Lee J, Loh H-Y, Ong Y-M, Tan I, Leung T, Manser E, Lim L. A *Drosophila* homolog of the Rac- and CDC42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures. Mol Cell Biol 1996; 16:1896-1908.
28. Leeuw T, Fourest-Lieuvin A, Wu CL, Chenevert J, Clark K, Whiteway W, Thomas DY, Leberer E. Pheromone response in yeast: Association of Bem1p with proteins of the MAP kinase cascade and actin. Science 1995; 270:1210-1213.
29. Lee S-F, Egelhoff TT, Mahasneh A, Côté GP. Cloning and characterization of a *Dictyostelium* myosin I heavy chain kinase activated by CDC42 and Rac. J Biol Chem 1996; 271: 27044-27048.
30. Brzeska H, Szczepanowska J, Hoey J, Korn ED. The catalytic domain of *Acanthamoeba* myosin I heavy chain kinase. II. Expression of active catalytic domain and sequence homology to p21-activated kinase (PAK). J Biol Chem 1996; 271:27056-27062.

31. Brzeska H, Martin BM, Korn ED. The catalytic domain of *Acanthamoeba* myosin I heavy chain kinase. I. Identification and characterization following tryptic cleavage of the native enzyme. J Biol Chem 1996; 271:27049-27055.
32. Leberer E, Thomas DY, Whiteway M. Pheromone signalling and polarized morphogenesis in yeast. Curr Opin Gen and Dev 1997; 7:59-66.
33. Wu C, Whiteway M, Thomas DY, Leberer E. Molecular characterization of Ste20p, a potential mitogen-activated protein or extracellular signal-regulated kinase kinase (MEK) kinase kinase from *Saccharomyces cerevisiae*. J Biol Chem 1995; 270:15984-15992.
34. Brzeska H, Kulesza-Lipka D, Korn ED. Inhibition of *Acanthamoeba* myosin I heavy chain kinase by Ca^{2+}-calmodulin. J Biol Chem 1992; 267:23870-23875.
35. Szczepanowska J, Zhang X, Herring CJ, Qin J, Korn ED, Brzeska H. Identification by mass spectrometry of the phosphorylated residue responsible for activation of the catalytic domain of myosin I heavy chain kinase, a member of the PAK/Ste20 family. Proc Natl Acad Sci USA 1997; 94:8503-8508.
36. Wu C, Lee S-F, Furmaniak-Kazmierczak E, Côté GP, Thomas DY, Leberer E. Activation of myosin-I by members of the Ste20p protein kinase family. J Biol Chem 1996; 271: 31787-31790.
37. Brzeska H, Knaus UG, Wang ZY, Bokoch GM, Korn ED. p21-activated kinase has substrate specificity similar to *Acanthamoeba* myosin I heavy chain kinase and activates *Acanthamoeba* myosin I. Proc Natl Acad Sci USA 1997; 94:1092-1095.
38. Bement WM, Mooseker MS. TEDS rule: A molecular rationale for differential regulation of myosins by phosphorylation of the heavy chain head. Cell Motility & the Cytoskeleton 1995; 31:87-92.
39. Collins K, Sellers JR, Matsudaira P. Calmodulin dissociation regulates brush border myosin I (110-kDa-calmodulin) mechanochemical activity in vitro. J Cell Biol 1990; 110:1137-1147.
40. Brown SS. Myosins in yeast. Curr Opin Cell Biol 1997; 9:44-48.
41. Geli MI, Riezman H. Role of type I myosins in receptor-mediated endocytosis in yeast. Science 1996; 272:533-535.
42. Goodson HV, Anderson BL, Warrick HM, Pon LA, Spudich JA. Synthetic lethality screen identifies a novel yeast myosin I gene (MYO5): Myosin I proteins are required for polarization of the actin cytoskeleton. J Cell Biol 1996; 133:1277-1291.
43. Simon M-N, DeVirgillio C, Souza B, Pringle JR, Abo A, Reed SI. Role for the Rho-family GTPase CDC42 in yeast mating pheromone signal pathway. Nature 1995; 376:702-706.
44. Kyriakis JM, Avruch J. Protein kinase cascades activated by stress and inflammatory cytokines. Bioessays 1996; 18:567-577.
45. Cvrcková F, De Virgilio C, Manser E, Pringle JR, Nasmyth K. Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. Genes & Development 1995; 9:1817-1830.
46. Leberer E, Dignard D, Harcus D, Thomas DY, Whiteway M. The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein beta and gamma subunits to downstream signalling components. EMBO Journal 1992; 11:4815-4824.
47. Herskowitz I. MAP kinase pathways in yeast: for mating and more. Cell 1995; 80:187-197.
48. Novak KD, Peterson MD, Reedy MC, Titus MA. *Dictyostelium* myosin I double mutants exhibit conditional defects in pinocytosis. J Cell Biol 1995; 131:1205-1221.
49. Jung G, Wu XF, Hammer JA III. Dictyostelium mutants lacking multiple classic myosin I isoforms reveal combinations of shared and distinct functions. J Cell Biol 1996; 133:305-323.
50. Doberstein SK, Baines IC, Wiegand G, Korn ED, Pollard TD. Inhibition of contractile vacuole function in vivo by antibodies against myosin I. Nature (London) 1993; 365:841-843.
51. McGoldrick CA, Gruver C, May GS. *MyoA* of *Aspergillus nidulans* encodes an essential myosin-I required for secretion and polarized growth. J Cell Biol 1995; 128:577-587.
52. Sellers JR, Adelstein RS. Regulation of contractile activity. In: Boyer P, Krebs EG, ed. The Enzymes. San Diego, CA: Academic Press, 1987; 18:381-418.

53. Alessi D, MacDougall LK, Sola MM, Ikebe M, Cohen P. The control of protein phosphatase-1 by targeting subunits—The major myosin phosphatase in avian smooth muscle is a novel form of protein phosphatase-1. Eur J Biochem 1992; 210:1023-1035.
54. Hammer JA, III, Sellers JR, Korn ED. Phosphorylation and activation of smooth muscle myosin by *Acanthamoeba* myosin I heavy chain kinase. J Biol Chem 1984; 259:3224-3229.
55. Tuazon PT, Traugh JA. Activation of actin-activated ATPase in smooth muscle by phosphorylation of myosin light chain with protease-activated kinase I. J Biol Chem 1984; 259:541-546.
56. Jakobi R, Chen C-J, Tuazon PT, Traugh JA. Molecular cloning and sequencing of the cytostatic G protein-activated protein kinase PAKI. J Biol Chem 1996; 271:6206-6211.
57. Chew T-L, Masaracchia RA, Wysolmerski RB. Phosphorylation of myosin regulatory light chain by a member of the p21-activated kinase (PAK) family, S6/H4 kinase. J Biol Chem 1997; 272: (in press).
58. Wysolmerski RB, Lagunoff D. Involvement of myosin light-chain kinase in endothelial cell retraction. Proc Natl Acad Sci USA 1990; 87:16-20.
59. Van Eyk Je, Heinenon T, Arrel DK, Foster BD, Strauss JD, Furmaniak-Kazmierczak E, Côtè GP, Mak AS. Ca^{2+}-independent contraction of smooth muscle induced by the p21-activated kinase (PAK). J Biol Chem 1997; (submitted).
60. Lamarche N, Tapon N, Stowers L, Burbelo PD, Aspenstrom P, Bridges T, Chant J, Hall A. Rac and CDC42 induce actin polymerization and G1 cell cycle progression independently of $p65^{PAK}$ and the JNK/SAPK MAP kinase cascade. Cell 1997; 87:519-529.
61. Joneson T, McDonough M, Bar-Sagi D, Van Aelst L. Rac regulation of actin polymerization by a pathway distinct from Jun kinase. Science 1996; 274:1374-1376.
62. Symons M, Derry J, Karlak B, Jiang S, Lemahieu V, McCormick F, Francke U, Abo A. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. Cell 1996; 4:723-734.
63. Kozma R, Ahmed S, Best A, Lim L. The GAP N-chimerin cooperates with Rac and CDC42 to induce the formation of lamellipodia and filopodia. Mol Cell Biol 1996; 16:5069-5080.
64. Van Aelst L, Joneson T, Bar-Sagi D. Identification of a novel Rac-interacting protein involved in membrane ruffing. EMBO J 1996; 15:3778-3786.
65. Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Rho-associated kinase, a novel serine threonine kinase, as a putative target for the small GTP binding protein Rho. EMBO J 1996; 15:2208-2216.
66. Leung T, Chen XQ, Manser E, Lim L. The p160 RhoA-binding kinase ROKα is a member of a kinase family is involved in the reorganization of the cytoskeleton. Mol Cell Biol 1996; 16:5313-5327.
67. Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, Morii N, Narumiya S. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J 1996; 15:1885-1893.
68. Hirata K, Kikuchi A, Sasaki T, Kuroda S, Kaibuchi K, Matsuura Y, Seki H, Saida K, Takai Y. Involvement of rho p21 in the GTP enhanced calcium ion sensitivity of smooth muscle contraction. J Biol Chem 1992; 267:8718-8722.
69. Gong MC, Iizuka K, Nixon G, Browne JP, Hall A, Eccleston JF, Suigai M, Kobayashi S, Somlyo AV, Somlyo AP. Role of guanine nucleotide-binding proteins-ras-family or trimeric proteins or both—in Ca^{2+} sensitization of smooth muscle. Proc Natl Acad Sci USA 1996; 93:1340-1345.
70. Kureisha Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, Ito M. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. J Biol Chem 1997; 272:12257-12260.
71. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng JH, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-Associated kinase (Rho-kinase). Science 1996; 273:245-248.

72. Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 1996; 271:20246-20249.
73. Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. Nature (London) 1994; 372:231-236.
74. Chrzanowska-Wodnicka M, Burridge K. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J Cell Biol 1996; 133:1403-1415.
75. Ishizaki T, Naito M, Fujisawa K, Maekawa M, Watanabe N, Saito Y, Narumiya S. p160^{ROCK}, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. FEBS Letters 1997; 404:118-124.
76. Pawson T, Schlessinger J. SH2 and SH3 domains. Curr Biol 1993; 3:434-442.
77. Xu P, Zot AS, Zot HG. Identification of Acan125 as a myosin-I-binding protein present with myosin-I on cellular organelles of Acanthamoeba. J Biol Chem 1995; 270:25316-25319.
78. Montell C, Rubin GM. The *Drosphila* ninaC locus encodes two photoreceptor cell specific proteins with domains homologous to protein kinases and the myosin heavy chain head. Cell 1988; 52:757-772.
79. Reinhard J, Scheel AA, Diekmann P, Hall A, Ruppert C, Bahler M. A novel type of myosin implicated in signallig by rho family GTPases. EMBO J 1995; 14:697-704.
80. Wirth JA, Jensen KA, Post PL, Bement WM, Mooseker MS. Human myosin-IXb, an unconventional myosin with a chimerin-like rho/rac GTPase-activating protein domain in its tail. J Cell Sci 1996; 109:653-661.
81. Muller RT, Honnert U, Reinhard J, Bahler M. The unconventional myosin myr5 functions as a RhoGAP in vitro and in vivo. Mol Biol Cell 1996; 373a.

CHAPTER 4

The Light-Chain-Linked Regulation of Muscle Myosin II

Ryoki Ishikawa and Kazuhiro Kohama

Myosins are the group of motor proteins which generate mechanical force by hydrolyzing ATP with interaction with actin filaments and which are thought to be responsible for a wide variety of cell motilities including muscle contraction, morphogenesis, cytokinesis, and cell migration. Myosin II, a conventional double-headed myosin, is the first-discovered and most abundant family among them. It is classified into three major types, striated type including skeletal and cardiac myosin II, smooth muscle type, and nonmuscle type. The activity and regulatory systems of these three types of myosins are quite different. This review will focus on the structure and function of these striated and smooth muscle myosin II (referred to as myosin throughout this review).

The Organization of Myosins and Their Genes

Each myosin consists of two identical heavy chains (HCs) (200-240 kDa) and two distinct pairs of light chains (LCs) (14-20 kDa) (Fig. 4.1). The N-terminal-half of HC termed head region (or subfragment 1, S1) is sufficient to hydrolyze ATP and produce mechanical force. This region is further divided into globular and α-helical neck domains. The former contains both the actin-binding and ATP-binding sites and the latter the LC-binding sites. The C-terminal-half that is termed tail region is rich in α–helix to form a rod-like shape, and essential for the self-assembly of myosin molecules to form a filament. The tail region is further divided into subfragment 2 (S2) and light meromyosin (LMM) by trypsin digestion. S1 plus S2 is called heavy meromyosin (HMM) (Fig. 4.1).

In vertebrate, at least eight striated muscle HC genes, two smooth muscle HC genes, and three nonmuscle HC genes have been cloned.[1] All the myosin heads contain a consensus GESGAGKT sequence, which is known as a phosphate-binding motif (P-loop), and is thought to be an ATP binding site. In a remaining portion of the myosin head, amino acid identity between three types of myosins are 40-50%, and the tail region is much less conserved.

Among striated type myosins, however, amino acid sequences are highly conserved from head to tail, ranging from 78-98% identity. The isoforms of skeletal and cardiac muscle myosins are differentially expressed temporally and spatially. The motor activity of the isoforms seems to be diverse. For example, the actin activated ATPase activity of α-cardiac myosin was 2-fold higher than that of β-cardiac myosin.[2]

Each head of HC is associated with "essential light chain" (ELC) and "regulatory light chain" (RLC). The former is a group of LCs named after their difficulty in dissociation from HC. Alkali- or urea-treatments to denature HC are necessary for the dissociation. "Regulatory" originated from smooth muscle myosin RLC that regulates the myosin motor activity

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

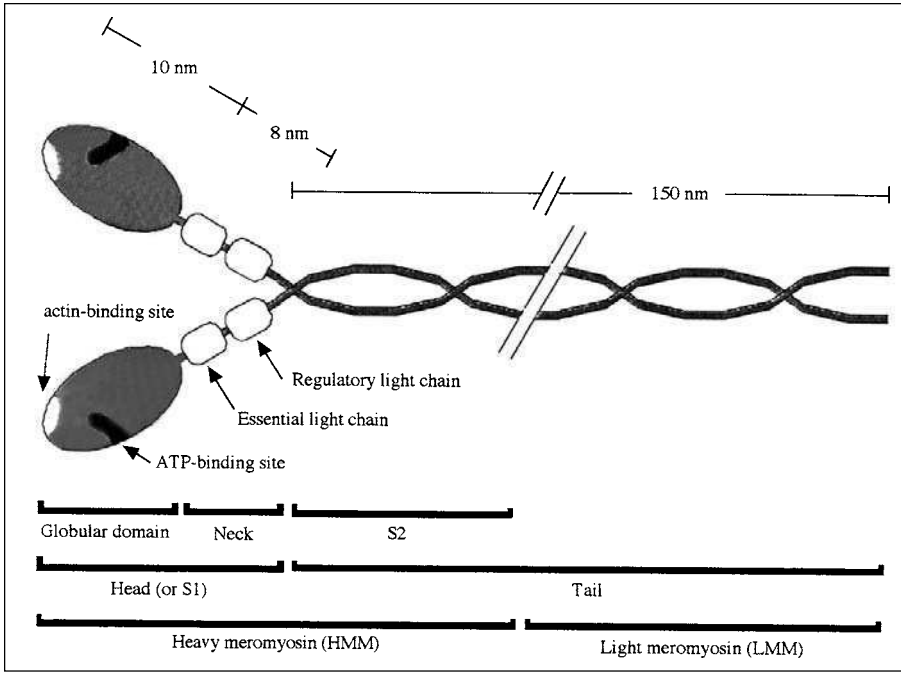


Fig. 4.1. Schematic illustration of myosin molecule. Myosin consists of two heavy chains, two essential light chains, and two regulatory light chains. Catalytic site for ATP hydrolysis and actin-binding site localize in globular domain.

by phosphorylation, although the corresponding RLC of unregulated myosin from skeletal and cardiac muscles plays no regulatory role as discussed later. All the LCs contain EF-hand structure of helix-loop-helix, a putative Ca^{2+} binding motif. But only scallop and Physarum myosins can actually bind Ca^{2+} through ELC.

X-ray crystallography of myosin head from chicken skeletal muscle revealed that P-loop (Gly^{177}-Thn^{184}) and following helix (Lys^{185}-Ile^{199}) constructs the putative ATP-binding site, the topology of which is the outside position of globular heads (Fig. 4.1).[3] The actin-binding site (Thy^{626}-Gln^{647}) localizes in the tip of the globular heads opposite the ATP-binding site. ELC and RLC tandemly bind to the neck position (Leu^{783}-Met^{806} and Glu^{808}-Leu^{842}, respectively).

Recombinant myosins displaying motor activity have been expressed in Sf-9 cells[4-6] and Dictyostelium cells.[7,8] Mutant myosin replaceing Gly^{182} with Glu, or Lys^{185} with Arg of HC loses the ability to bind ATP and thus the motor activity.[7] Recombinant Dictyostelium HC deleted from His^{788} to Ala^{817} (corresponding to from Arg^{809} to Ile^{838} of chicken) failed to associate with RLC, and that deleted from Arg^{761} to Ala^{817} (corresponding to from Lys^{782} to Ile^{838} of chicken) failed to associate both ELC and RLC.[8] These results are consistent with the predicted function of the corresponding domains deduced from X-ray crystallography.

Assembly of Myosins

In skeletal muscle, myosin molecules assemble to form bipolar bundles called thick filaments (Fig. 4.2A). In both ends of the thick filament, two pairs of myosin heads rotate by

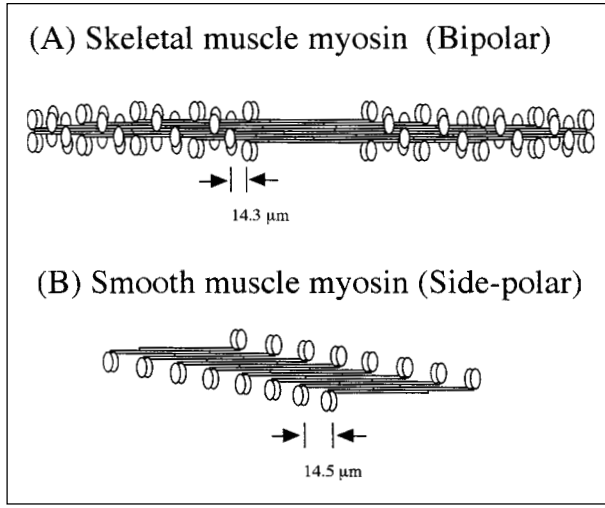


Fig. 4.2. Assembly of skeletal muscle myosin (A) and smooth muscle myosin (B). Skeletal muscle myosin forms bipolar helical bundles while smooth muscle myosin form flat, side-polar bundles.

120 degrees relative to the next pairs with a 14.3 μm interval, resulting in a helical arrangement with a pitch of 42.9 (14.3 x 3) μm.[9] This structure is thought to be ideal to interact with highly organized actin filaments in skeletal muscle cells. The central region of thick filament without myosin heads is called the bare zone. The typical length of thick filament in higher organism is about 1 μm. However, purified myosin also forms thick filaments in physiological salt concentration, but the length is much longer, suggesting that the length of thick filaments in vivo is determined by another factor, probably myosin-binding proteins. In higher salt concentration (e.g., 0.5 M NaCl), myosin loses the ability to assemble.

In smooth muscle cells, the bundles of thick filaments are tiny and less organized as examined by the electron microscopy. Optical diffraction analysis reveals that the tails of smooth muscle myosin assemble side-to-side to form a flat sheet in which two neighboring myosins are antiparallelly directed (Fig. 4.2B).[10] Assembly of smooth muscle myosin in vitro is regulated by the phosphorylation of its RLC. When the RLC is unphosphorylated, the tail of myosin is folded and no longer forms a bundle. Only when RLC is phosphorylated, does the tail become straight and is able to assemble. It must be noted, however, that myosin forms bundles in smooth muscle cells regardless of whether myosin is phosphorylated or not. This apparent discrepancy might be due to the possible contribution of smooth muscle myosin-binding protein(s) such as telokin.[11]

The Function of Myosins

Myosin converts chemical energy to a mechanical energy by hydrolyzing ATP. This process is called the "crossbridge cycle" in which four distinct chemical states are detected (Fig. 4.3). In the absence of nucleotides, the myosin head strongly binds to actin filaments (state 1 in Fig. 4.3A). This state is called the "rigor" state. The binding of ATP to myosin head causes a slight conformational change in the actin-binding site of the myosin head, resulting in dissociation of the myosin head from actin filaments (state 2 in Fig. 4.3A). Then ATP is hydrolyzed to ADP plus Pi in the cleft of the ATP-binding site (state 3 in Fig. 4.3A). Pi is released from myosin heads, the binding of myosin head to actin filament becomes strong, and the force generating process (power stroke) is initiated (state 4 in Fig. 4.3A). Finally, myosin head releases ADP to go back to the state 1.

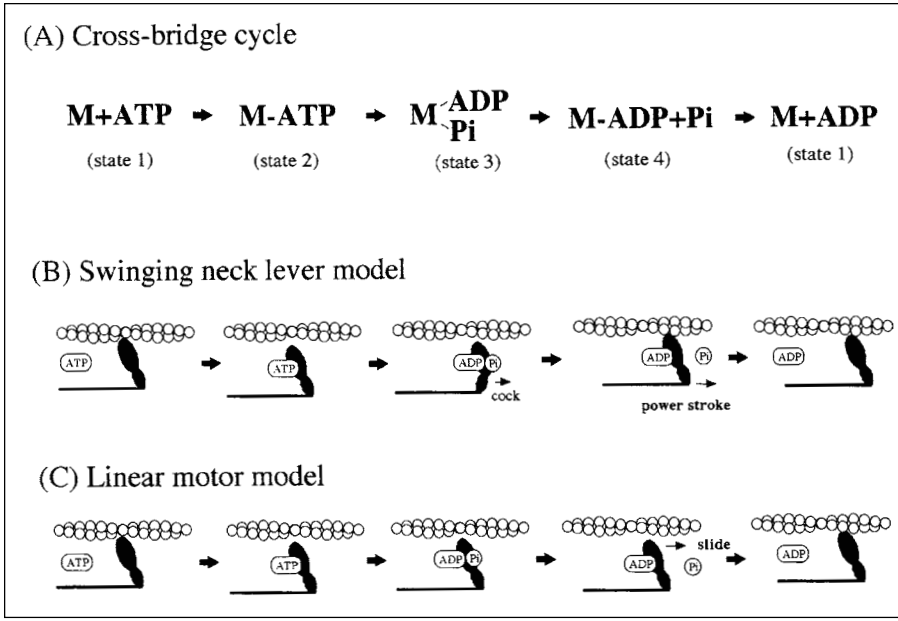


Fig. 4.3. Crossbridge cycle (A) and two models for power generation, swinging neck lever model (B) and linear motor model (C). M indicates myosin head, and Pi indicates phosphate. Affinity for actin is strongest in state 1, weak in states 2 and 3, and strong in state 4.

How does myosin II generates such a mechanical force? Two major hypotheses, "swinging neck lever model" and "linear motor model", are proposed (Fig. 4.3B,C). In the first model, intramolecular the conformational change of myosin head is important for the force generation.[12] When ATP is hydrolyzed to ADP + Pi, the neck domain cocks (Fig. 4.3B, state 3). When Pi is released, the globular domain attaches actin filament and the neck domain swings back to the original state to pull the myosin rod (Fig. 4.3B, state 4). If this model is correct, the neck length should affect the sliding speed. Indeed, modification of the neck length revealed that the sliding velocity of actin filaments on recombinant myosin mutants was proportional to neck length.[8]

Yanagida's group showed that actin filament could slide on myosin by 100 nm during the hydrolysis of one ATP molecule, which was far longer than expected derived from the "swinging neck lever model".[13] Therefore, they proposed a new concept for the force generation, "linear motor model" (Fig. 4.3C). In their model, the myosin head slides along the actin filament without coupling conformational changes during the power stroke. However, the precise mechanism for force generation is still unclear.

The Regulation of Smooth Muscle Myosin

Regulatory modes of myosin activity are quite different among tissues and species. Cardiac and skeletal muscle myosins of vertebrates show motor activities when monitored by measuring actin-activated ATPase activity and by monitoring its in vitro motility in the presence of ATP. Thus, the activity of the myosin molecule itself is always active or in the "on" state. In the relaxed form of skeletal muscle, the troponin-tropomyosin complex, that periodically binds to actin filaments, inhibits the interaction between actin and myosin.

When muscle is stimulated, intracellular Ca^{2+} increases and binds to troponin resulting in a conformational change of troponin to release the inhibition.[14]

Unlike these unregulated myosins from skeletal and cardiac muscles, the activity of smooth muscle myosin is inactive or in the "off" state under when unphosphorylated. Phosporylation of RLC is necessary for it to become active form, i.e., "on" state to interact with actin. When smooth muscle cells are stimulated, intracellular Ca^{2+} increases, calmodulin (CaM) binds Ca^{2+}, and activates myosin light chain kinase (MLCK). Then MLCK phosphorylates Ser^{19} of RLC so that myosin is in the "on" state to interact with actin filaments.[15]

RLC can be removed from HC by treating smooth muscle myosin with trifluoperazine, an antagonist to CaM, without denaturing myosin HC. To the myosin thus treated, chimeric RLC can be hybridized. Myosin containing chimeric smooth/skeletal RLC, in which the N-terminal half of smooth muscle RLC is fused to the C-terminal half of skeletal muscle RLC, is not activatable by phosphorylation. However, the myosin containing a reverse (N-skeletal/C-smooth) chimeric RLC is activated by phosphorylation.[16] Therefore, the C-terminal half of the RLC and Ser^{19} at the N-terminal are required for the activation.

HMM of smooth muscle was produced in Sf-9 cells by cotransfection of cDNAs encoding ELC, RLC, and truncated HC.[5] The recombinant HMM showed motor activity when RLC was phosphorylated. The activity was preserved even when the cDNA of the ELC was not coinfected, indicating that the ELC of smooth muscle is not essential for motor activity. Unlike scallop myosin (see below), the interface between ELC and RLC does not play a key role in regulating motor activity of smooth muscle myosin.

Cremo et al produced partially digested smooth muscle myosin to form a single-headed myosin, i.e., myosin containing one head attached to an intact tail.[17] Whether RLC of this single head myosin was phosphorylated or not, its actin-activated ATPase activity was as high as that of the phosphorylated form of intact smooth muscle myosin. Further, actin filaments slide on the unphosphorylated single head myosin as fast as on the phosphorylated normal myosin. Similar results were obtained by expressing truncated myosins utilizing a Sf-9-baculovirus expression system.[18] The activity of myosin with double-heads was regulated by LC phosphorylation, but the activity of myosin with a single head was always in the "on" state independent of light chain phosphorylation. These results suggest that the intrinsic nature of smooth muscle myosin head itself is the "on" state. The double-head structure produces the "off" state, which is then turned "on" by the RLC phosphorylation.

The Regulation of Myosin Through Ca^{2+}-Binding

The myosin that binds Ca^{2+} with a high affinity is not from vertebrates, but molluscan. Scallop myosin is one of the best characterized myosins. The motor activity of scallop myosin is low in the absence of Ca^{2+}. Upon elevation of Ca^{2+} concentration, scallop myosin binds Ca^{2+}, which then enhances the activity (Fig. 4.4A,B). The site for Ca^{2+} is in ELC. Ca^{2+} sequestered by ELC is stabilized by RLC that binds Mg^{2+}. Such a model is shown by producing hybrid myosins and 10kDa "regulatory domains" (RD) of the hybrid myosin as follows.[19] RLC of scallop myosin is removed by EDTA treatment, and then RLC from other myosin can be rebound to it. ELC of scallop myosin is not removable but is exchangeable with ELC from other myosin to some extent. The complete exchange of ELC is possible with RD, which is reconstituted from ELC, RLC and the LC-binding domain of myosin HC.

RLC and ELC are composed of four helix-loop-helix domainsdesignated by domains I-IV. The purified ELC alone is unable to bind Ca^{2+} both HC and RLC are required for the ELC-Ca^{2+} interaction. Szent-Gyorgyi and his colleagues produced chimeras between scallop ELC (regulated myosin) and cardiac ELC (unregulated myosin) as recombinant proteins.[20] They showed that the N-terminal domain I of scallop ELC was of primary importance for Ca^{2+}-binding to RD and, hence, myosin. Similar domain analysis of RLC was carried

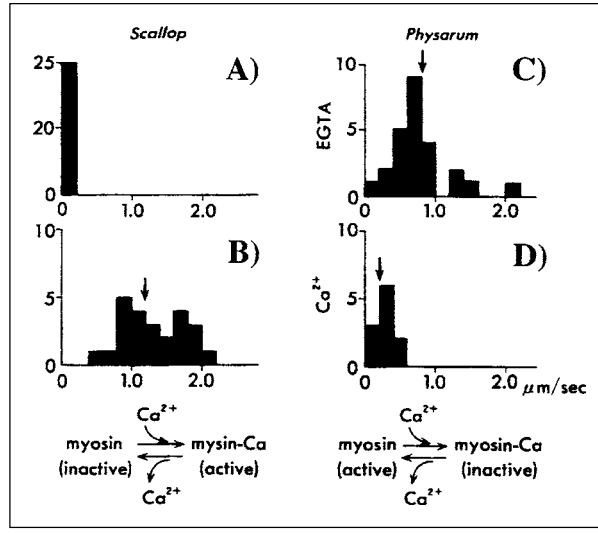


Fig. 4.4. Stimulatory (A,B) and inhibitory (C,D) effects of Ca^{2+}. Actin was labeled with rhodamine-phalloidine and mounted on a coverslip coated with scallop myosin (A,B) or Physarum myosin (C,D).[21] ATP-dependent movement of actin was observed in the presence of 0.1 mM EGTA (A,C) or 0.1 mM Ca^{2+} (B,D) with a fluorescent microscope equipped with a videocamera. Ordinate, number of moving actin; abscissa, velocities (μm/sec). Arrows indicate average velocity of the movement.

out with chimeras of scallop RLC (regulated myosin) and skeletal RLC (unregulated myosin).[21] The chimera conferred Ca^{2+}-binding and Ca^{2+}-sensitivity on hybrid myosin only if it contains domain III of scallop RLC. When Gly^{117} of domain III in scallop RLC was substituted with Ala or Cys, the hybrid myosin containing the mutated RLC lost ability to restore Ca^{2+}-binding and to confer Ca^{2+}-sensitivity on the ATPase activity. Gly^{117} is strictly conserved among regulated myosins from molluscan and vertebrate smooth muscles. When Cys^{126} of skeletal muscle RLC which corresponds to Gly^{117} of scallop myosin was replaced by Gly, the mutated RLC conferred Ca^{2+}-binding and Ca^{2+}-sensitivity. The crystal structure of RD of scallop myosin showed Gly^{117} of RLC is in close contact with Gly^{23} of domain I of ELC and is stabilized by hydrogen bonds.[22]

Myosin from a lower eukaryote, *Physarum polycephalum*, also binds Ca^{2+} with a high affinity. Unlike scallop myosin, however, it is in the active form when it loses Ca^{2+}.[23] Upon binding Ca^{2+}, its motor activity is inhibited (Fig. 4.4C,D). The physiological implication of this calcium inhibition is as follows: the myosin supports cytoplasmic streaming observed in plants, a phenomenon that occurs under the resting state, i.e., in low Ca^{2+} concentration. The streaming ceases only when the plant cell is excited to increase intracellular Ca^{2+}.[24]

The subunit that binds Ca^{2+} is calcium binding LC (CaLC), which belongs to the ELC class with helix-loop-helix structures.[25] The LC of RLC class is a phosphorylatable LC (PLC), and has been cloned and sequenced. It has a Gly residue at the position that corresponds to Gly^{117} of scallop RLC. To test whether Ca^{2+} bound to CaLC is stabilized by PLC, we need LC-binding domain of physarum HC, and its cloning is now under way.

Acknowledgment

This work was supported in part by grants from the Mitsubishi Foundation, and by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

1. Weiss A, Leinwand LA. The mammalian myosin heavy chain gene family. Annu Rev Cell Dev Biol 1996; 12:417-439.
2. Pope B, Hoh JF, Weeds A. The ATPase activities of rat cardiac myosin isoenzymes. FEBS Lett 1980; 118:205-209.
3. Rayment I, Rypniewski R, Schmidt-Base K et al. Three-dimensional structure of myosin subfragment-1: a molecular motor. Science 1993; 261:50-58.
4. Sweeney HL, Straceski AJ, Leinwand LA et al. Heterologous expression of a cardiomyopathic myosin that is defective in its actin interaction. J Biol Chem 1994; 269:1603-1605.
5. Trybus KM. Regulation of expressed truncated smooth muscle myosins. J Biol Chem 1994; 269:20819-20822.
6. Onishi H, Maeda K, Maeda Y et al. Functional chicken gizzard heavy meromyosin expression in and purification from baculovirus-infected insect cells. Proc Natl Acad Sci USA 1995; 92:704-708.
7. Ruppel KM, Spudich JA. Structure-function analysis of the motor domain of myosin. Annu Rev Cell Dev Biol 1996; 12:543-573.
8. Uyeda TQP, Abramson PD, Spudich JA. The neck region of the myosin motor domain acts as a lever arm to generate movement. Proc Natl Acad Sci USA 1996; 93:4459-4464.
9. Huxley HE. The mechanism of muscular contraction. Science 1969; 164:1356-1366.
10. Xu JQ, Harder B, Uman P et al. Myosin filament structure in vertebrate smooth muscle. J Cell Biol 1996; 134:53-66.
11. Shirinsky VP, Vorotnikov AV, Birukov KG et al. A kinase-related protein stabilizes unphosphorylated smooth muscle myosin minifilament in the presence of ATP. J Biol Chem 1993; 268:16578-16583.
12. Rayment I, Holden HM, Whittaker M. et al. Structure of the actin-myosin complex and its implications for muscle contraction. Science 1993; 261:58-65.
13. Harada Y, Sakuraba K, Aoki T et al. Mechanochemical coupling in actomyosin energy transduction studies by in vitro movement assay. J Mol Biol 1990; 216:49-68.
14. Ebashi S, Endo M. Calcium ion and muscle contraction. Prog Biophys Mol Biol 1968; 18:123-183.
15. Horowitz A, Menice CB, Laporte R et al. Mechanisms of smooth muscle contraction. Physiol Rev 1996; 76:967-1003.
16. Trybus KM, Chatman TA. Chimeric regulatory light chains as probes of smooth muscle myosin function. J Biol Chem 1993; 268:4412-4419.
17. Cremo CR, Sellers JR, Facemyer KC. Two heads are required for phosphorylation-dependent regulation of smooth muscle myosin. J Biol Chem 1995; 270:2171-2175.
18. Sata M, Matsuura M, Ikebe M. Characterization of the motor and enzymatic properties of smooth muscle long S1 and short HMM: Role of the two headed structure on the activity and regulation of the myosin motor. Biochemistry 1996; 35:11113-11118.
19. Szent-Gyorgyi AG, Fromherz S, Jancso A et al. Regulation of muscle contracton by a calcium-binding myosin: structure and mutational studies. In: Maruyama K, Nonomura Y, Kohama K, eds. Calcium as Cell Signal. Tokyo: Igaku-shoin, 1995:65-72.
20. Fromherz S, Szent-Gyorgyi AG. Role of essential light chain EF hand domains in calcium binding and regulating of scallop myosin. Proc Natl Acad Sci USA 1995; 92:7652-7656.
21. Jancso A, Szent-Gyorgyi AG. Regulationof scallop myosin by the regulatory light chain depends on a single glycine residue. Proc Natl Acad Sci USA 1994; 91:8762-8766.
22. Xie S, Harrison I, Schlichting RM et al. Structure of the regulatory domain of scallop myosin at 2.8 A resolution. Nature 1994; 368:304-312.
23. Okagaki T, Higashi-Fumime S, Kohama K. Ca^{2+} activates actin-filament sliding on scallop myosin but inhibits that on Physarum myosin. J Biochem 1989; 106:955-957.
24. Kohama K. Inhibitory mode for Ca^{2+} regulation. Trends Pharmacol Sci 1990; 11:433-435.
25. Kobayashi T, Takagi T, Konishi K et al. Amino acid sequence of the calcium-binding light chain of myosin from the lower eukaryote, Physarum polycephalum. J Biol Chem 1988; 263:305-313.

Chapter 5

The Tumor Suppressor *l(2)gl*: A Myosin-Binding Protein Family

Dennis Strand

Summary

The prototype of the *l(2)gl* protein family is the *lethal(2)giant larvae* tumor suppressor of *Drosophila melanogaster,* designated as *l(2)gl* according to the drosophilist nomenclature or more recently as *D-lgl.* The *l(2)gl* protein family consisted first of only dipteran members but has become wider in the recent years including members in species as diverse as man, mouse, insect, worm, slime mold and yeast. Biochemical and genetic analyses of the *l(2)gl* gene and its human homolog *hugl-1*, showed that the encoded proteins are components of the cytoskeleton and interact physically with a domain located near the carboxyl extremity of nonmuscle myosin II heavy chain. Further studies have also revealed that the *l(2)gl* protein may also interact with the Nucleosome Assembly Protein-1, or NAP-1, a component of the cyclin B-p34^{cdc2} kinase complex. Our investigations showed that NAP-1 is intimately associated with the cytoskeletal matrix during interphase and accumulates in the nucleus during prophase where it becomes associated with the spindle apparatus. Through the different interactions established by the *l(2)gl* protein we have begun to understand the key roles played by this tumor suppressor in the maintenance of cell shape and tissue organization and in the regulation of the cell cycle.

Introduction

Our knowledge on the function of the *l(2)gl* protein is essentially based on genetic and biochemical investigations which have been performed in the fruitfly *Drosophila.* Among the numerous effects caused by mutations in the *l(2)gl* gene, the neoplastic transformation of the brain hemispheres and the imaginal discs is certainly the most striking feature which was recognized by Elisabeth Gateff[1-3] more than 30 years after the initial discovery of the first *l(2)gl* mutant by Calvin Bridges in 1933. With the recent cloning of *hugl-1*, a human homolog to the *Drosophila l(2)gl* gene,[4] the question arises as to whether the human gene may be specifically altered in human diseases and, if this is the case, whether these diseases are related to proliferative disorders. Our investigations revealed that the *hugl-1* gene is intensively expressed in brain and maps on chromosome 17p11.2-12. Finer mapping showed that *hugl-1* is constantly uncovered by a microdeletion affecting one chromosome 17 of patients displaying the Smith-Magenis syndrome, a neurological disorder affecting young children. Furthermore, *hugl-1* was found to be located in the near vicinity of one translocation breakpoint occurring in chromosome 17 of patients with primitive neuroectodermal tumors, or PNETs. These preliminary data indicate that the homolog to the *Drosophila l(2)gl*

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

gene may be causally related to proliferative disorders striking humans and emphasize the notion that evolutionarily conserved sequences may not only retain similar cellular functions but can also be involved in comparable pathogenesis, although insects are separated from humans by more than 600 millions of divergent evolution. If the causation of PNETs by mutations in *hugl-1* can be confirmed, this finding would represent the rewards of decades of basic research of the genetic maneuvers of *Drosophila,* much as has been the case for the *Escherichia coli* DNA repair genes which have been used to isolate human mismatch repair genes and show that human genes are frequently defective in inherited forms of colon cancer.

The l(2)gl *Gene: A Brief History*

In 1933 Calvin Bridges discovered a spontaneous recessive lethal mutation in *Drosophila melanogaster* giving rise to abnormally large and bloated larvae.[5] This lethal factor was mapped on the left arm of the second chromosome and the locus was designated as *lethal(2)giant larvae*, or *l(2)gl*. From the mid-thirties up to the beginning of the 1950s this gene was intensively studied by Ernst Hadorn and his collaborators and played a pioneering role in the elaboration of the ideas and methods underlying developmental genetics, which were first published in German in 1955 and appeared in 1961 in its English language edition with the title *Developmental Genetics and Lethal Factors.*[6,7] Through his analysis Hadorn could show that the developmental arrest which affects *l(2)gl* larvae at the larval to pupal transition phase could be partially suppressed by implantation of a wild-type ring gland indicating that this organ is the source of the molting hormone.[8] Using *l(2)gl* larvae as a biological assay for purifying the molting hormone from silkworm Karlson identified ecdysone as the major insect molting hormone and showed that injection of ecdysone into *l(2)gl* larvae induced pupariation and formation of pseudopupae.[9] However, neither the implantation of a wild-type ring gland nor the injection of ecdysone could fully rescue the development of the *l(2)gl* larvae indicating that the hormonal imbalance was not the primary cause of the developmental arrest. Furthermore, extensive studies by Hadorn and co-workers showed that the *l(2)gl* mutation produced pleiotropic effects in numerous tissues which can already be detected at the onset of the larval life, such as the atrophy of the male germline,[10] which occurs before the appearance of any malignant growth. Although numerous comparative studies between normal and *l(2)gl* tissues and organs were conducted for more than 30 years, it was not until the late 1960s that *l(2)gl* mutations were recognized as responsible for the formation of malignancies in the brain hemispheres and the imaginal discs.[1,3,11] Subsequent to her initial studies, Elisabeth Gateff was able to isolate a series of mutations in distinct genetic loci and showed that they can produce tissue-specific tumors in either the imaginal discs, the brain hemispheres, the hematopoietic organs or the germline.[11]

Molecular studies of the *l(2)gl* gene was initiated in 1985 by Mechler and his co-workers with the cloning of the *l(2)gl* locus.[12] The molecular studies demonstrated unequivocally that the tumorous phenotype results from a lack of gene function and showed that tumorigenesis can be prevented when an intact copy of the *l(2)gl* gene was introduced into the genome of *l(2)gl*-deficient animals.[13,14] Further analyses revealed that the *l(2)gl* gene encodes a protein of 1,161 amino acids in length with a molecular mass of 127 kDa, therefore designated as p127.[14] However, the molecular determination of the *l(2)gl* gene sequence and its encoded protein revealed no direct clue to its function. No striking motif which would have suggested a plausible function could be detected in the sequence of p127 and no resemblance with any other known protein available in databases could be perceived. The only valuable information came from the absence of motifs indicating that p127 could be secreted, localized in the nucleus or act as an integral membrane protein. Neither a leader

peptide, a transmembrane domain, nor a nuclear localization signal could be detected in p127. By default the assumption was made that p127 should be a cytoplasmic protein.

Although the molecular analysis of the *l(2)gl* gene provided new and powerful ways for exploring the function of this gene, at first, it yielded perplexing information. For example, investigations on the pattern of *l(2)gl* expression revealed that the gene is intensively expressed in all embryonic cells and in numerous larval tissues, such as the gut, which display no phenotypic abnormalities in *l(2)gl* larvae. Furthermore, the *l(2)gl* gene was not expressed in the larval brain hemispheres which become massively overgrown in *l(2)gl* larvae.[15] Another difficulty originated from the genetic localization of the *l(2)gl* locus at the extreme tip on the left arm of the second chromosome. Despite numerous attempts, we were unable to induce point mutations, constituting weaker alleles which would have been more suitable for genetic analysis. All chemically induced mutations were found to be terminal deletions of the chromosome which removed part or all of the *l(2)gl* gene sequence. Under these conditions the conventional genetic tools which are routinely applied for the analysis of other *Drosophila* genes could not be used in the case of *l(2)gl*.

To circumvent these difficulties we decided first to use immunobiochemistry and histochemistry to determine the intracellular localization of p127 and then to directly determine the nature of the proteins which were recovered in association with p127. Through this approach we were able to define some partners of p127, determine their function and thus gain a better understanding on how p127 contributes to the regulation of several cellular processes controlling cell shape and proliferation.

Towards the Function of p127

p127 Participates in a Cytoskeletal Network Extending in the Cytoplasm and Covering the Innerface of the Plasma Membrane

Immunohistochemical and biochemical investigations revealed that p127 participates in a cytoskeletal network extending into the cytoplasm and in the peripheral matrix undercoating the plasma membrane.[16] In particular, p127 was found to form high molecular mass complexes made primarily of homo-oligomerized molecules[17,18] to which are associated at least 10 other proteins. Among these proteins we have so far identified three proteins: (a) nonmuscle myosin II heavy chain which binds to a relatively large domain in the center of p127[17] (G. Merdes, D. Strand, Z.W. Su, D. Kiehart, and B.M. Mechler, in preparation); (b) the Nucleosome Assembly Protein-1, or NAP-1, which appears to play a critical role in the dynamics of the cortical cytoskeleton in the cytoplasm and the spindle apparatus in the nucleus (B. Neumann, G. Merdes, D. Strand, B.M. Mechler, in preparation) and can strongly bind to cytosolic cyclin B;[19,20] and (c) a serine-kinase which specifically phosphorylates p127 at serine residues.[21] This kinase may regulate the binding of myosin to p127, as revealed by in vitro assays showing that its activation led to a specific release of nonmuscle myosin II from the p127-complexes without dissociation of the oligomerized p127 molecules. However, the molecular nature of this kinase remains unknown.

Mapping of functional domains in p127 has shown the presence of three homo-oligomerization domains which elicit intermolecular binding without the requirement of any extraneous protein,[18] a cluster of evolutionarily conserved serine residues which may constitute the target sites for phosphorylation by the p127-associated kinase,[21] two distinct amino acid substitutions, both of them conferring temperature sensitivity to the *l(2)gl* gene (de Lorenzo, C., Strand, D., and Mechler, B.M., in preparation) and two sites whose modification confers either enhancement or reduction of the in vitro binding of p127 with nonmuscle myosin II (G. Merdes, D. Strand, and B.M. Mechler, in preparation). The locations of the homo-oligomerization domains, the putative phosphorylation sites and the positions of

the modified residues in the p127 protein are indicated in Figure 5.1. In addition, previous work has shown that the terminal 140 amino acid residues located in the carboxyl terminal domain of the p127 are dispensable for the function of p127, albeit necessary for the development of the animals below 20°C.[14,22] Interestingly, the mouse and human homologous proteins to p127 are shorter proteins corresponding essentially to C-terminally truncated forms of p127, suggesting that the dispensable domain of p127 may be required for keeping the protein active at the optimal temperature for invertebrate life.

The Nonmuscle Myosin II - p127 Connection

The use of the blot overlay technique and the yeast two-hybrid system allowed us to define the interaction domains between p127 and nonmuscle myosin II. The binding domain of p127 is located in a discrete region near the carboxyl extremity of the twisting tail of myosin II between amino acid residues 1724 and 1865 whereas the binding domain of myosin II in p127 is much larger, encompassing about one-third of the total length of p127 between amino acid residues 306 and 700. The finding that p127 binds to the tail of myosin II is in contrast with all other proteins known to interact with this protein, such as actin, the essential and regulatory light chains, band 4.1 protein, telokin, or myosin light chain kinase, which are associated with to its aminomoiety. Taking into consideration that the organization of the *l(2)gl* tumorous tissues may reflect an enhanced activity of myosin II resulting in excessive foldings of the epithelial layers, we interpret that the binding of p127 to the tail of myosin II may contribute to the stabilization of the myosin II bundles.

To ascertain this hypothesis we have further analyzed the pattern of expression of both proteins during *Drosophila* embryogenesis and observed a remarkable overlapping intracellular distribution of p127 and myosin II in regions where the cytoskeleton forms an apparently stable and motionless network. In particular we found that, in quiescent cells, both proteins are essentially present in the cytoskeletal matrix and diffusely distributed in the cytoplasm. By contrast, in domains where myosin II is involved in contractile (i.e., motor) activities or in membrane assembly, we observed that p127 segregates from myosin II and is completely depleted from the myosin II structures which contribute to morphogenetic events leading to cell shape changes. These structures are: (1) the acto-myosin ring at the leading edge of the inward-growing furrow canals during cellularization of the blastoderm cells; (2) the myosin belt at the apices of cells displaying constriction during invagination; (3) the myosin belt at the leading edge of elongating epithelial cells during dorsal closure and (4) the myosin ring during cytokinesis. All together the results of the immunohistochemical analysis during *Drosophila* development show that p127 colocalizes with myosin II in sub-cellular compartments in which myosin appears to be present in a stabilized or motionless form and that p127 is dissociated from myosin II when this molecule is involved in processes leading to changes in cell shape. This finding supports the concept that p127 may contribute to the stabilization of the myosin-based cytoskeleton.

Involvement of p127 and Nonmuscle Myosin II in Dorsal Closure Processes

Proof for a role of p127 in the dynamic regulation of myosin II was obtained from genetic studies investigating the interaction between *l(2)gl* and *zipper*. The *zipper* gene encodes nonmuscle myosin II heavy chain.[23,24] Mutations in *zipper* and *l(2)gl* give rise to different phenotypes. In homozygously mutated *l(2)gl* embryos the development proceeds normally up to the beginning of larval life because the amount of maternally inherited p127 is sufficient to compensate for the absence of zygotic *l(2)gl* gene expression and permits embryogenesis to proceed normally. By contrast, in *zipper* mutants, the development is arrested at mid-embryogenesis with a characteristic irregularly shaped dorsal opening in the cuticle reflecting a failure in the completion of the migration of the ventral epidermis over

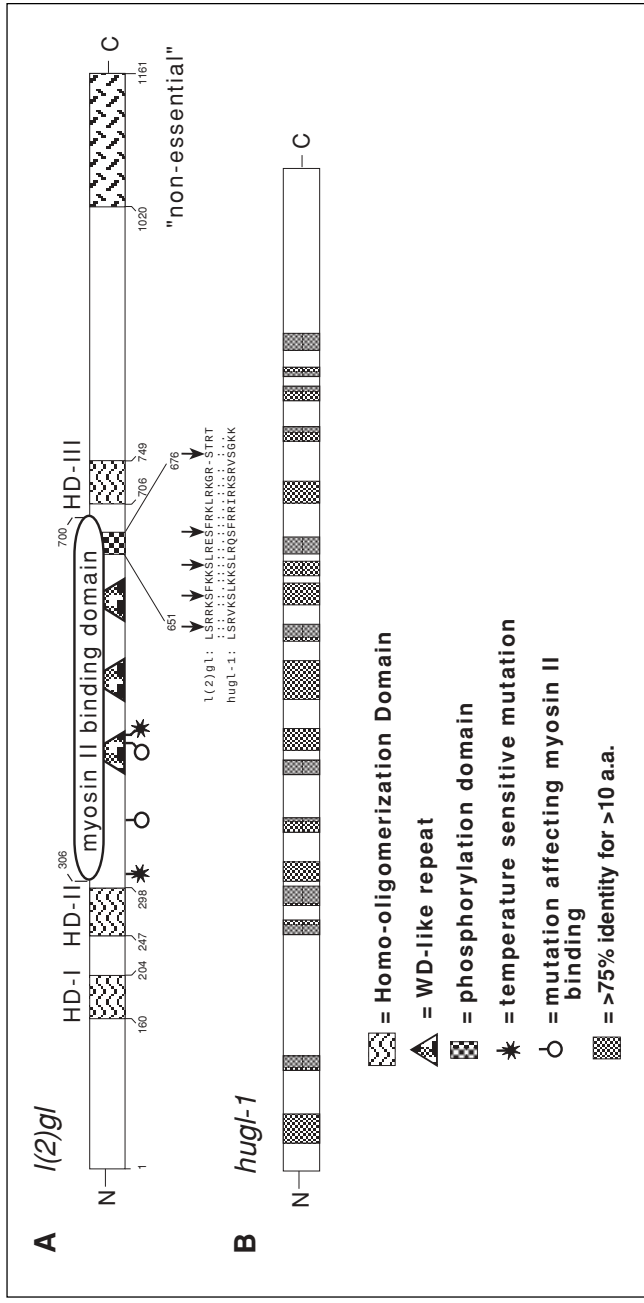


Fig. 5.1. Functional domains identified in the *l(2)gl* protein (A) compared with regions of highest sequence identity in the *hugl-1* protein (B).

the amnioserosa. In wild-type embryos, the process of dorsal closure involves the movement of the ventral epidermis made of two single-layered cell sheets which extend dorsally over a substratum of amnioserosa cells. At the rim of the ventral epidermis, the epithelial cells are elongated with a high concentration of myosin II forming a belt along the apical edge. In these cells no p127 protein could be detected along the apical edge although p127 is present in the cortical matrix underlaying the other sides of these cells. In *zipper* embryos, we observed that the belt of myosin II is absent from the apical edge and that p127 is evenly distributed all over the cell periphery. When we examined embryos mutated for both *l(2)gl* and *zipper*, we found that their development is partially rescued by comparison with embryos only mutated for *zipper*. We found that the *l(2)gl zipper* embryos can fully complete dorsal closure but are unable to complete the process of head involution which normally occurs at the same time as dorsal closure but may involve more strenuous morphogenetic movements (G. Merdes, D. Strand, and B.M. Mechler, unpublished results).

Mechanistically, these results are interpreted as follows. In the double mutant *l(2)gl zipper* embryos the low amount of maternally inherited myosin II present in the epidermis is sufficient to allow completion of dorsal closure but is insufficient to sustain the morphogenetic movements required for the involution of the head. In embryos only mutated for *zipper*, the amount of p127 resulting from the zygotic expression of *l(2)gl* may be excessive with regards to the amount of maternally inherited myosin II and the excess of p127 may sequester myosin in an inactive form. As a consequence, myosin II becomes unable to accumulate at the leading edge of the epithelial cells.

Analysis by immunostaining of the residual amounts of p127 and myosin II present in these two categories of embryos supports our model. At the time of the dorsal closure, the intracellular distribution of p127 and myosin II in the epithelial cells located at the leading edge is similar in the double mutant embryos as in wild-type embryos, albeit weaker, with a myosin belt depleted in p127. By contrast, in the *zipper* embryos, there is no belt of myosin and both proteins are evenly distributed on all faces of the epithelial cells which display an irregular shape. From this analysis, we conclude that p127 acts as a negative regulator of the activity of nonmuscle myosin II.

To strengthen this conclusion, we have introduced additional copies of the *l(2)gl* gene in embryos homozygously mutated for *zipper* and have determined whether the development of these embryos would be more drastically affected than the *zipper* embryos possessing only two normal copies of the *l(2)gl* gene. If p127 can sequester myosin II, we expect that additional copies of the *l(2)gl* gene would lead to an earlier developmental arrest which should be more pronounced as the number of copies is made higher. By using different transgenic lines containing cloned copies of the *l(2)gl* gene we were able to introduce one to four additional copies of *l(2)gl* in homozygous *zipper* embryos. The results of this analysis showed that indeed the development of the *zipper* embryos was drastically reduced and that the severity of the arrest was dependent on the number of *l(2)gl* gene copies. These data further confirmed the proposition that p127 can sequester myosin II in an inactive form and can thus regulate its activity (G. Merdes, D. Strand, and B.M. Mechler, unpublished results).

Who's Who During Dorsal Closure

Our genetic and molecular analyses indicate clearly that *l(2)gl* contributes to the regulation of nonmuscle myosin II activity during dorsal closure. However, the process of dorsal closure requires coordinate changes in cell shape and concurrent movements of the epithelial sheets. The crucial components in this process are the cytoskeleton, the cell surface and the extracellular matrix as well as regulators which modulate the activity of the cytoskeletal proteins producing the morphogenetic movements.[25,26] In eukaryotic cells, signal transduc-

tion is predominantly mediated by kinase cascades and our finding that the association between p127 and myosin II can be disrupted by the activation of a serine-kinase[21] specifically phosphorylating p127 suggests that, in order to deplete p127 from the myosin belt located at the leading edge of the epidermal cells, a similar mechanism takes place during dorsal closure. This mechanism should last during the whole process of dorsal closure, from its onset up to its completion.

The signaling pathway which precisely controls the interaction between p127 and myosin II remains unknown. However, recent genetic and molecular investigations of dorsal closure have led to the identification of two specific Jun-N-terminal kinases, JNK and JNKK, which are required in the embryo for the initiation and completion of this process.[27-31] It is therefore likely that the cascade elicited by the Jun-N-terminal kinases may ultimately result in the phosphorylation of p127 and disrupt the interaction between p127 and myosin II at the leading edge of epidermal cells.

A series of mutations are known to interrupt dorsal closure during *Drosophila* embryogenesis and have been assigned to approximately 30 different genes. Embryos with such mutations are characterized by a hole in the dorsal side of the embryos, similar to the defect observed in *zipper* embryos, or may exhibit abnormalities indicating that a particular phase in this process is affected. This is notably the case of mutations in the *puckered* gene which allow the dorsalward movement of the epidermis but give rise to abnormal alignment of the closing edges producing an irregularly folded midline (Ring and Martinez-Arias, 1993).[35] Since the duration of the dorsal closure extends over more than two hours and involves signals for initiating, maintaining and terminating the morphogenetic movements, it is plausible that numerous genes may play major and decisive roles during the different phases of dorsal closure and that more than one single signaling pathway may be involved. Although mutations in a large proportion of the genes acting during dorsal closure may never produce by themselves visible abnormalities during this process, in combination with mutations producing dorsal defects, they may either enhance or suppress the dorsal phenotype similar to what we have observed in the case of *l(2)gl* with *zipper*. Genetic analyses may therefore be extremely helpful in defining the respective roles played by the different components acting during dorsal closure and may indicate their hierarchical position with respect to the proteins encoded by the tester genes.

Among the genes involved in dorsal closure, some of them have been characterized at the molecular and genetic levels and conferred with specific roles during dorsal closure. These genes can be grouped into two classes: those involved in the regulation of dorsal closure and those coding for components implementing structural changes during dorsal closure.

The first category consists of genes essentially involved in two major pathways: (a) the Jun-N-terminal kinase (DJNK) pathway which was recently shown to govern dorsal closure in *Drosophila* and displays similarities to the mitogen-activated protein kinase (MAPK) cascade which transduces a variety of signals in eukaryotic cells in response to multiple extracellular stimuli (for a review see refs. 31 32), and (b) the transforming growth factor-β (TGF-β) or *dpp* (*decapentaplegic*) pathway.

Members of the DJNK pathway are represented by *basket* encoding the Jun-N-terminal kinase (DJNK),[33,34] *hemipterous* encoding DJNKK,[25] *puckered* which codes for a MAP-kinase phosphatase accumulating at the leading edge of the epidermal cells during dorsal closure,[33,35] as well as two small GTPases of the Rho subfamily, RacA and CDC42, whose overexpression during early embryogenesis resulted in dorsal closure phenotypes.[33,36] In mammalian cells, the RacA and CDC42 proteins modulate cytoskeletal functions and one of their known targets is the serine/threonine kinase PAK, for which a *Drosophila* homolog (D-PAK) has been identified. Although so far no mutation in this gene has been found,

immunohistochemical investigations showed a high amount of D-PAK in the epidermal cells at the rim of the dorsal opening and, more particularly, an accumulation of this protein at the apical edge suggesting that D-PAK may interact with cytoskeletal elements and may play a direct role in dorsal closure.[37]

Mutations in the Dpp/TGF-β pathway can also cause dorsal closure defects as well as affect the formation and maintenance of the amnioserosa on which the epidermal cells migrate. The apparent absence of dorsal closure could thus result from two different mechanisms, one resulting from the absence of the substrate on which the epidermis should migrate and the other from a defect in the epidermis itself. However, a direct causal link between the DJNK and Dpp pathways has recently been established. DJNK appears to regulate Dpp function by controlling the localized expression of Dpp in the dorsal most cells.[27-30] Mutations in *decapentaplegic* encoding a TGF-β homolog, as well as in the genes encoding its receptors, *thick veins*[38] and *punt,*[39] as well as in genes encoding transcription factors acting downstream of the Dpp receptors, like *schnurri*,[40,41] *anterior open/yan*[42] and *pannier*,[43] are also known to cause a dorsal closure defects lending further support to the role of Dpp/TGF-β pathway in this process. Moreover, *dpp* is expressed in the leading edge cells during dorsal closure[44] and recent experiments performed by four different groups showed that DJNK regulates the expression of *dpp* during dorsal closure.[27-30] Additional signaling pathways, such as those involving Notch, EGFR and Wnt/wingless, may also be involved in dorsal closure but their precise contribution in this process remains unclear.

The second category of genes that are needed in dorsal closure encodes structural components which are either associated with the cell membrane or the cytoskeleton. This group includes *zipper* encoding nonmuscle myosin II heavy chain,[24] *spaghetti-squash* coding for the myosin II regulatory light chain,[45] *inflated* and *myospheroid* which encode the integrin-α subunit[46] and the integrin-β subunit,[47,48] respectively, as well as *coracle* coding for a *Drosophila* homolog to the mammalian band 4.1. protein which is associated with septate junctions.[49]

Dynamics of the Interaction Between p127 and Nonmuscle Myosin II

Our current knowledge of the mechanism regulating the interaction between p127 and nonmuscle myosin II is based on the biochemical observation that the in vitro activation of a kinase tightly bound to the p127 complexes results in the release of myosin from these complexes without affecting the oligomerization of p127.[21] This release could be prevented by the presence of a synthetic 26mer peptide covering amino acid positions 651-676 of p127 and containing five serine residues surrounded by basic residues which are evolutionarily conserved from *Drosophila* to humans (see Fig. 5.1). Recent results showed that this peptide becomes phosphorylated instead of p127 indicating that the association between p127 and myosin can be disrupted by phosphorylation of one or several of the five serines located in a central domain of p127. Current investigations involving site-directed mutagenesis and reverse genetics will determine the in vivo role of these potential phosphorylation sites. Preliminary results indicate that the substitution of the five serines by aspartic acids, which mimics a constitutive phosphorylation, is dominantly lethal whereas the substitution of the serines by alanines exerts no dominant deleterious effect (F. Böhl, D. Strand, and B.M. Mechler, unpublished results). Use of a binary genetic system, which directs the expression of the modified p127 protein in defined tissues and at precise periods of *Drosophila* development, will allow us to determine more precisely the developmental abnormalities elicited by the expression of a "pseudo"-phosphorylated p127 molecule and to investigate its binding capacity with nonmuscle myosin II.

In another set of experiments we have modified a series of evolutionarily conserved sites within the myosin binding domain in p127 and were able to uncover two distinct bind-

ing sites for nonmuscle myosin II. In particular, we found that, in one case, the in vitro binding of myosin is inhibited, whereas, in the other case, it is enhanced. Interestingly the modified site displaying enhancement is located in the immediate vicinity of a substitution providing p127 with temperature sensitivity. Experiments are currently performed to determine whether this mutation affects the binding affinity between myosin and p127. However, not only post-translational modification of p127 can affect the binding between myosin and p127, but also phosphorylation of myosin. We have found that in vitro phosphorylation of the tail domain of myosin II by the catalytic subunit of PKA inhibits its binding to p127, implying that the interaction between the two molecules can be governed by different mechanisms. One mechanism may directly concern p127 whereas the other mechanism may act on myosin II. This finding suggests that the dissociation of myosin II from p127 can be directed by different signaling pathways. Molecular isolation of the kinases regulating the association between p127 and myosin II will provide ways to identify the encoding genes and to undertake studies for understanding how signals originating from distinct pathways may not only regulate the association between p127 and myosin II but may also control the interaction of p127 with other effector molecules.

As revealed by genetic and biochemical analyses, nonmuscle myosin II activity can be regulated by molecules which bind to the head domain of myosin. Recent studies have shown that the absence of the regulatory (phosphorylatable) light chain of nonmuscle myosin II encoding by the *spaghetti squash* gene leads to the aggregation of myosin II in punctate structures depleted in filamentous actin and p127[50] and affects cytokenesis,[45] whereas inactivation of the *l(2)gl* gene exhibits no effect on cytokinesis. These results demonstrate that myosin II activity can be controlled by different regulators including Ser/Thr kinases acting either on its head domain through the regulatory light chain, or on its tail domain through p127.

A New Facet of l(2)gl *Function: The Possible Involvement in the Regulation of the Cell Cycle*

The data accumulated on *l(2)gl* over more than six decades of research have shown that mutations in this gene exert numerous effects in different tissues and affect several cellular processes. We would therefore expect that the identification of other proteins interacting with p127 will provide ways to understand better the regulation exerted by p127 on important cellular functions such as the control of the cell cycle. Recent advances in the molecular and genetic analyses of *l(2)gl* have given insights into one of the partners through which p127 may affect the regulation of the cell cycle.

Studies of proteins isolated by affinity chromatography on a p127-bound matrix have shown that a 45 kDa protein corresponds to the *Drosophila* homolog of the nucleosome assembly protein-1, or NAP-1. This protein was first identified in in vitro assays as a factor assembling nucleosomes from histones and naked DNA,[51-54] and was later shown in *Xenopus* and yeast to be a component of the cyclin B-p34^{cdc2}kinase complex which plays a critical role for the entry into mitosis and progression through the cell cycle.[19,20] Our investigations showed that, during the entire interphase of the cell cycle, including the S and G2 phases, NAP-1 is present in the cytoplasm and colocalizes with p127 and nonmuscle myosin II in the cytoskeletal matrix underlaying the cell surface. However, when the components of the cytoskeletal matrix become dispersed in the cytoplasm at the onset of mitosis, we found that NAP-1 accumulates massively in the nucleus, particularly during prophase and then disappears gradually from the nucleus during metaphase becoming almost undetectable during telophase. In the nucleus NAP-1 is associated with elements of the spindle apparatus.

The high level of NAP-1 in the nucleus during prophase indicates that NAP-1 may be involved in either the condensation of the chromosomes or in the assembly/disassembly of

complexed structures required for the progression of mitosis such as the spindle apparatus that should become fully operative during metaphase. The results of two series of experiments confirmed that NAP-1 exerts a function in the organization of the spindle apparatus. On the one hand, immunohistochemical analysis showed that the treatment of embryos with taxol, an anticancer drug known to stabilize microtubules, considerably increased the association of NAP-1 with the spindle apparatus. On the other hand, micro-injection of anti-NAP-1 antibodies in preblastoderm embryos revealed that, in the region of the embryos containing high concentrations of antibodies, the nuclei stopped dividing with chromosomes remaining at the metaphase plate or failing to become properly segregated during anaphase (B. Neumann, D. Strand, and B.M. Mechler, unpublished results). These data indicate that NAP-1 may play a crucial role in the organization of the spindle apparatus.

Less is understood on the role of NAP-1 in the cytoplasm. However, analysis of the inactivation of NAP-1 in the budding yeast showed that the isomorphic growth of the bud is blocked,[20] suggesting that NAP-1 also exerts important functions in the organization of the cytoskeleton. Furthermore, our studies on *Drosophila* showed that the intracellular distribution of NAP-1 overlaps with those of p127 and nonmuscle myosin II in the peripheral cytoskeletal matrix. We also found that NAP-1 is intensively expressed in tissues in which cell division has ceased indicating further that NAP-1 exerts a cytoplasmic function, i.e., regulating the dynamics of the cytoskeleton. Insights into these processes will be gained, on the one hand, by studying mutations in NAP-1, which are not yet available and, on the other hand, by determining more precisely the molecular interactions of NAP-1 with p127 and with other cytoplasmic components.

Expansion of the *l(2)gl* Family

In recent years, the number of sequences related to the *Drosophila l(2)gl* gene has considerably increased and homologs have been found in species as diverse as human, mouse, *Caenorhabditis elegans, Dictyostelium discoideum* and yeast (Fig. 5.2). Investigations of these genes will undoubtedly shed new light on the functions of the different members of the *l(2)gl* protein family and on the variety of processes in which they participate. Moreover, by virtue of the particular advantages displayed by each organism, a larger spectrum of analytical tools is offered for decrypting unsuspected or hidden aspects of the *l(2)gl* function. For example, current analysis of mutations in the yeast *sop-1* and *sop-2* genes, which are structurally related to *l(2)gl*, shows that, as expected, mutations in both genes produce defects in the cytoskeletal architecture (R. Grabowski and L. Adler, unpublished results). However, these mutations affect also the organization of the plasma membrane, as indicated by the enhanced sensitivity of *sop* mutant cells towards NaCl. Although the mechanism by which alterations in *sop* genes lead to an enhanced sensitivity towards NaCl is not yet understood, restoration of nearly normal growth rate in NaCl was obtained by transfecting *sop* mutant cells with a normal copy of the *Drosophila l(2)gl* gene (F. Böhl, R. Grabowski, D. Strand, B.M. Mechler, and L. Adler, in preparation). This result reveals a new *l(2)gl* function which may be correlated with the recent finding that nonmuscle myosin II copurifies with chloride channel enriched membranes from bovine tracheal cells suggesting that elements of the cytoskeleton can regulate fluid secretion.[55]

In the case of cell locomotion, other organisms, such as *Dictyostelium* or mammalian cultured cells, may be more suitable than *Drosophila* for studying intracellular relocation of cytoskeletal components. In particular, studies with human cells may show whether *hugl-1*, the human homolog to *l(2)gl*, plays a role in metastasis, as suggested by the interaction that has been found between the HUGL-1 protein and nonmuscle myosin II.[4] Neither cell locomotion nor metastasis can be efficiently investigated in *Drosophila* but through the isolation of

Nomenclature	Species	Accession	Reference
l(2)gl *D-lgl*	*Drosophila melanogaster*	85047	4,14
l(2)gl D.maur	*Drosophila mauritiana*	*	
l(2)gl D.pse	*Drosophila pseudoobscura*	*	56
l(2)gl D.hyd	*Drosophila hydei*	*	
l(2)gl C.ery	*Calliphora erythrocephala*	*	
hugl-1 *Llgl* DLG4	*Homo sapiens*	X86371 D50550	4,64
hugl-2 *hgl*	*Homo sapiens*	X87342	
mgl-1 *Llglh*	*Mus musculus*	D16141	57,64
n.a.	*Caenorhabditis elegans*	U51993	
sop-1 SRO7	*Saccharomyces cerevisiae*	Z49274	65
sop-2	*Saccharomyces cerevisiae*	Z35867	
n.a.	*Dictyosteliun discoideum*	*	

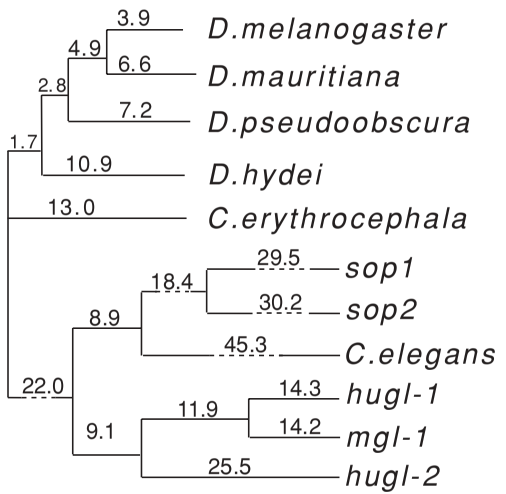


Fig. 5.2. Members of the *l(2)gl* gene family and phylogenetic tree.

l(2)gl homologs in mammals and *Dictyostelium* (H. Rubin, S. Ravid, and D. Strand, unpublished results) the involvement of *l(2)gl* in these processes can be now tested.

The identification of sequences related to *l(2)gl* was obtained either directly by cloning under reduced hybridization stringencies or serendipitously by comparing newly isolating sequences. Search of related sequences by direct hybridization with an *l(2)gl* sequence of *D. melanogaster* was only successful in the case of other insect species. This limited success resulted from the low codon bias displayed by all dipteran genes related to *l(2)gl* from the fruitfly *D. melanogaster* up to the bowfly *Calliphora erythrocephala.* In these genes, the AT content is relatively high and disfavors the possibility of recovering by hybridization procedures distantly related sequences. A low codon bias characterizes genes located in the vicinity of a telomere or a centromere and our studies showed that the *l(2)gl* gene in *D. melonagaster* as well as in other *Drosophila* species, such as *D. hydei*, is either directly located at a chromosomal end or in its near vicinity. Moreover, in *D. melanogaster* the *l(2)gl* sequence displays almost no polymorphism suggesting that it may be less prone to recombination than genes located within the chromosome. Interestingly, the frequency of mutations in the *l(2)gl* gene of *D. melanogaster* is extremely high in natural populations and molecular analysis of more than 100 different mutations shows that they consisted of terminal deletions of the chromosome with partial or complete absence of the *l(2)gl* locus.[12] The high frequency of *l(2)gl* deletions indicates that the excision-repair mechanism of mutational events in this gene is ineffective and leads to truncation of the chromosomal end. However, despite its telomere localization, the *l(2)gl* gene sequence displays normal divergence rates in its coding and noncoding sequences during insect evolution. In particular we have found that the *l(2)gl* sequence from *D. pseudoobscura*, a species which has diverged from *D. melanogaster* for more than 20 million years, contains a noncoding regulatory domain which bears no sequence relationship with that of *D. melanogaster.* However this gene is fully functional in *D. melanosgaster* as we were able to obtain full developmental rescue of *l(2)gl*-deficient *D. melanogaster* flies by gene transfer.[56] Moreover, the *D. pseudoobscura l(2)gl* gene was found to encode a protein displaying a different mobility in SDS-polyacrylamide gel electrophoresis than that of *D. melanogaster* and this characteristic allows direct experimental assessment of its biological function.

In mammals the first isolated sequence related to *l(2)gl* has been the mouse *mgl* gene which has been found fortuitously by virtue of the strong binding of its promoter to the Hox-C8 protein.[57] This finding has prompted a search for homologous sequences in humans[4] and has led to the isolation of two distinct sequences whose comparison revealed that they have recently diverged during mammalian evolution. We have found that the *hugl-1* sequence is primarily expressed in brain tissues whereas the *hugl-2* sequence is predominantly expressed in pancreas and liver tissues (D. Strand, unpublished results).

Comparison of the amino acid sequence of the different members of the *l(2)gl* protein family shows that the most conserved domains between p127and HUGL-1 are located within the central segment of these proteins where the domain of interaction with nonmuscle myosin II and NAP-1 is positioned. As shown in Figure 5.1, we have noticed that the five potential phosphorylation sites are strongly conserved as well as three WD (Trp-Asp) related sequences.[56,57a] Furthermore, two of the three homo-oligomerization domains are present in conserved regions between the two proteins and recent experiments have shown that newly synthesized HUGL-1 and p127 proteins can form mixed oligomers indicating that the oligomerization domains are functionally conserved during evolution (M. Rickert, D. Strand, and B.M. Mechler, unpublished results).

Parsimony analysis of the amino acid sequences allows a phylogenetic reconstruction of the evolution of the *l(2)gl* protein family and shows that both yeast and mammals contain two members which have recently diverged during the evolution of both phylla. In

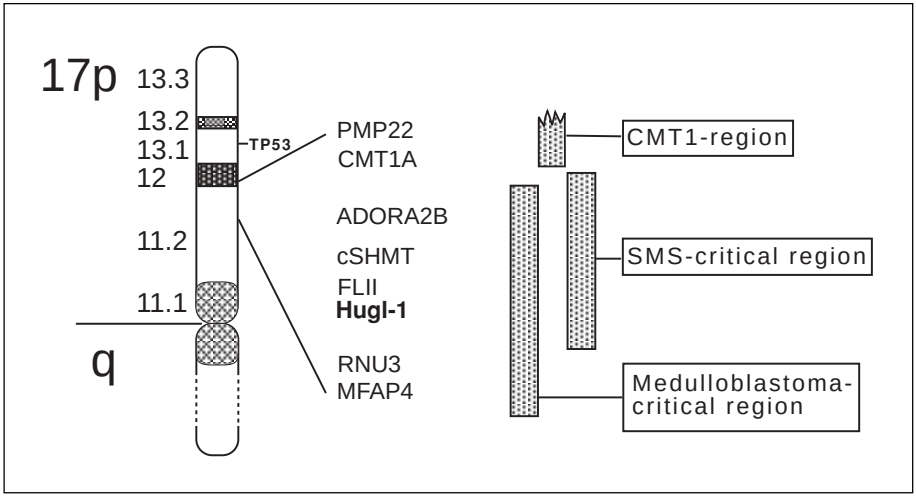


Fig. 5.3. *hugl-1* maps to chromosome 17p within a region identified as critical for medulloblastoma and the Smith-Magenis Syndrome.

particular, the two mammalian members may have diverged after the separation of mammals from the other vertebrates.

hugl-1: A New Human Tumor Suppressor?

In human, the *hugl-1* gene was found to map to a pericentromeric region on the short arm of chromosome 17, to the band 17p11.2-12, which contains a potential cancer susceptibility gene giving rise to medulloblastoma (Fig. 5.3).[4] Recent molecular characterization of this region has revealed the presence of a cluster of deletion breakpoints in cancer patients with medulloblastomas and shown that the *hugl-1* gene is frequently uncovered by a 17p interstitial deletion characterizing mentally retarded children with Smith-Magenis syndrome.[58-60]

Medulloblastoma, also described as primitive neuroectodermal tumor or PNET, is the most frequent malignant childhood brain tumor and accounts for 15-20% of brain tumors in children. Cytogenetic analysis of medulloblastoma shows that up to 60% of the cases are characterized by a hemizygous chromosomal loss of the region 17p11.2-17pter associated with the formation of a dicentric 17q isochromosome.[58,61] In at least half the cases with an i(17q) chromosome, the loss of 17p is the only detectable chromosomal aberration suggesting that this loss constitutes a critical event in the etiology of medulloblastoma.[62,63] Yet, a single defective allele presumably is not sufficient for medulloblastoma development. Full tumor conversion may require the inactivation of a tumor suppressor gene present in the remaining chromosome 17. Among the genes which have been mapped in the region 17p11.2, the *hugl-1* gene is certainly one of the most serious candidates for being the tumor suppressor gene which becomes inactivated in medulloblastoma tumors. Mutational analysis is currently performed on tumor material and normal cells from patients with medulloblastoma for determining the structure of the nondeleted *hugl-1* allele. These studies which have required the complete sequence analysis of the intron-exon structure of *hugl-1* will show whether genetic alterations may affect this gene in medulloblastoma (P. Seranski, D. Strand and A. Poustka , unpublished results).

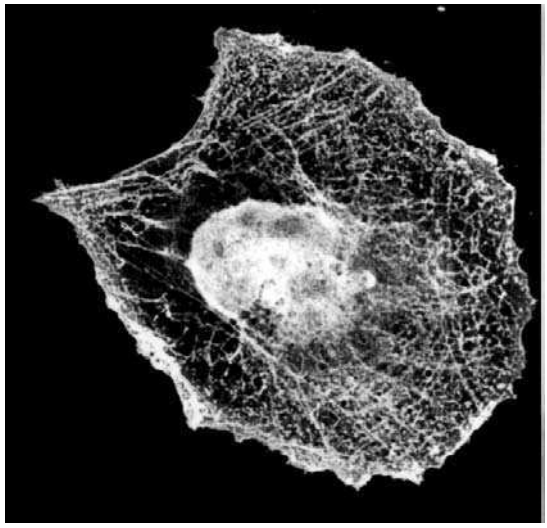


Fig. 5.4. HUGL-1 builds a cytoskeletal network.

The intense expression of *hugl-1* in brain tissues brings forward the notion that this gene may play a critical role in brain pathologies and the findings that it maps within the critical region for medulloblastoma further support this hypothesis. Our biochemical and molecular studies also show that the function of the *Drosophila* and human proteins is apparently conserved during evolution and that the *Drosophila l(2)gl* gene can even complement genetic defects in the yeast *sop* genes indicating further that the function of the *l(2)gl* gene family has been strongly conserved during evolution. Moreover, comparison of the amino acid sequences of the members of the *l(2)gl* family reveals the presence of numerous regions with high amino acid identity extending throughout the entire length of the mammalian and arthropod proteins. With the exception of the C-terminal domain of the *Drosophila* p127 protein, which exerts a dispensable role, both mammalian and insect proteins exhibit sequential arrangement of the conserved domains indicating that both proteins have retained identical functions during evolution (Fig. 5.1). Although not all the functional domains and not all the interacting partners have as yet been identified for p127, we were able to show that the known functional domains of *Drosophila* exist also in the human protein. Furthermore, the HUGL-1 protein was shown to interact with human proteins similar to those found in *Drosophila*. In particular, we have shown that HUGL-1 coimmunoprecipitates with nonmuscle myosin II heavy chain and with a specific kinase whose activation causes its phosphorylation at serine residues as in the case of p127. Interestingly, when we expressed recombinant HUGL-1 protein in *Sf9* cells, we found that the HUGL-1 protein is associated with a *Sf9* endogeneous kinase able to phosphorylate HUGL-1 at serine residues in a similar way as p127.[17,21] Furthermore, we were able to show that HUGL-1 forms high molecular mass complexes consisting of homo-oligomerized molecules and when HUGL-1 and p127 are simultaneously synthesized in *Sf9* cells we found that both molecules become intermixed. These data further indicate that the functions of both proteins have been conserved during evolution and, similar to the experiments complementing *sop* mutations in yeast, we are currently testing whether *hugl-1* can rescue the development of *l(2)gl*-deficient flies.

Further support for a functional identity between both proteins is provided by their overlapping distribution in both insect and human cells. In *Drosophila* and other dipteran species, we found that p127 is distributed in the cytoplasm and present on the plasma mem-

brane in regions of cell contacts.[16,56] We have investigated the distribution of HUGL-1 in the metastatic large cell lung carcinoma cell line LCLC-103H by transiently expressing HUGL-1 protein. Immunostaining revealed that the recombinantly expressed HUGL-1 protein forms a network in the cytoplasm which extends to the plasma membrane (Fig. 5.4). Furthermore, consistent with its role as a cytoskeletal protein interacting with nonmuscle myosin II we found that the expression of HUGL-1 dramatically reduces the motility of the LCLC-103H cells. All together these results indicate that HUGL-1 is a component of the cytoskeleton and displays a similar pattern of intracellular distribution and biochemical properties as its *Drosophila* counterpart.

Acknowledgment

The author wishes to thank B.M. Mechler for his continued support and all past and present members of the Mechler lab who have contriubted to the *1,(2)gl*-story.

References

1. Gateff E, Schneiderman HA. Developmental studies of a new mutation of *Drosophila melanogaster*: Lethal malignant brain tumor *l(2)gl*^{4}. Am Zool 1967; 7:760.
2. Gateff E, Schneiderman HA. Neoplasms in mutant and cultured wild-type tissues of *Drosophila*. Natl Cancer Inst Monogr 1969; 31:365-397.
3. Gateff E, Schneiderman HA. Developmental capacities of benign and malignant neoplasms of *Drosophila*. Wilhelm Roux Arch Entwicklungsmechn Org 1974; 176:23-65.
4. Strand D, Unger S, Corvi R et al. A human homologue of the *Drosophila* tumor suppressor gene *l(2)gl* maps to 17p11.2-12 and codes for a cytoskeletal protein that associates with nonmuscle myosin II heavy chain. Oncogene 1995; 11:291-301.
5. Bridges CB, Brehme KF. The mutation of *Drosophila melanogaster*. Carnegie Institute of Washington 1944; Publication No. 522.
6. Hadorn E. Zur Pleiotropie der Genwirkung. Arch. Jul. Klaus Stift 1955; Erg Band 20:82-85.
7. Hadorn E. Developmental Genetics and Lethal Factors. London, Great Britain: Methuen and Co. Ltd., 1961:1-355.
8. Hadorn E. An accelerating effect of normal 'ring glands' on puparium formation in lethal larvae of *Drosophila melanogaster*. PNAS USA 1937; 478-484.
9. Karlson P, Hauser G. Über die Wirkung des Puparisierungshormons bei der Wildform und der Mutante *lgl* von *Drosophila*- Z Naturforsch 1952; 7b:80-83.
10. Gloor H. Entwicklungsphysiologische Untersuchung an den Gonaden einer Letalrasse (*lgl*) von *Drosophila melanogaster*. Rev Suisse Zool 1943; 50:339-394.
11. Gateff E. Malignant neoplasms of genetic origin in the fruit fly *Drosophila melanogaster*. Science 1978; 200:1446-1459.
12. Mechler B. Molecular cloning of the *lethal(2)giant larvae*, a recessive oncogene of *Drosophila melanogaster*. EMBO 1985; 4:1551-1557.
13. Opper M, Schuler G, Mechler BM. Hereditary suppression of *lethal(2)giant larvae* malignant tumor development in *Drosophila* by gene transfer. Oncogene 1987; 1:91-96.
14. Jacob L, Opper M, Metzroth B et al. Structure of the *l(2)gl* gene of *Drosophila* and delimitation of its tumor suppressor domain. Cell 1987; 50:215-225.
15. Merz R, Schmidt M, Török I et al. Molecular action of the *l(2)gl* tumor suppressor gene of *Drosophila melanogaster*. Environ Health Persp 1990; 88:163-167.
16. Strand D, Raska I, Mechler BM. The *Drosophila lethal(2)giant larvae* tumor suppressor protein is a component of the Cytoskeleton. J Cell Biol 1994; 127(5):1345-1360.
17. Strand D, Jakobs R, Merdes G et al. The *Drosophila lethal(2)giant larvae* tumor suppressor protein forms homo-oligomers and is associated with nonmuscle Myosin II heavy chain. J Cell Biol 1994; 127(5):1361-1373.
18. Jakobs R, de Lorenzo C, Spiess E et al. Homo-oligomerization domains in the *lethal(2)giant larvae* tumor suppressor protein, p127 of *Drosophila*. J Mol Biol 1996; 264:484-496.

19. Kellog DR, Murray AW. NAP1 acts with Clb2 to perform mitotic functions and to suppress polar bud growth in budding yeast. J Cell Biol 1995; 130(3):675-685.
20. Kellogg DR, Kikuchi A, Fujii-Nakata T et al. Members of the NAP/SET family of proteins interact specifically with B-type Cyclins. J Cell Biol 1995; 130(3):661-673.
21. Kalmes A, Merdes G, Neumann B et al. A serine-kinase associated to the p127-*l(2)gl* tumor suppressor of *Drosophila* may regulate the binding of p127 to nonmuscle myosin II heavy chain and the attachment of p127 to the plasma membrane. J Cell Science 1996; 109:1359-1368.
22. Strand D, Török, I, Kalmes A et al. Molecular analysis of tumorigenesis in *Drosophila melanogaster*. In: Weber G, ed. Advances in Enzyme Regulation. Vol. 31. Oxford: Pergamon Press, 1991:339-350.
23. Ketchum AS, Stewart CT, Stewart M et al. Complete sequence of the *Drosophila* nonmuscle myosin heavy-chain transcript: conserved sequences in the myosin tail and differential splicing in the 5' untranslated sequence. PNAS NY 1990; 87:6316-6320.
24. Young PE, Richman AM, Ketchum AS et al. Morphogenesis in *Drosophila* requires nonmuscle myosin heavy chain function. Genes and Dev 1993; 7:29-41.
25. Glise B, Bourbon H, Noselli S. *hemipterous* encodes a novel *Drosophila* MAP kinase kinase, required for epithelial cell sheet movement. Cell 1995; 83:451-468.
26. Knust E. *Drosophila* morphogenesis: Follow-my-leader in epithelia. Current Biol 1996; 6(4):379-381.
27. Hou XS, Goldstein ES, Perrimon N. *Drosophila* Jun relays the Jun amino-terminal kinase signal transduction pathway to the Decapentaplegic signal transduction pathway in regulating epithelial cell sheet movement. Genes and Dev 1997; 11:1728-1737.
28. Glise B, Noselli S. Coupling of Jun amino-terminal kinase and Decapentaplegic signaling pathways in *Drosophila* morphogenesis. Genes and Dev 1997; 11:1738-1747.
29. Riesgo-Escovar JR, Hafen E. *Drosophila* Jun kinase regulates expression of *decapentaplegic* via the ETS-domain protein Aop and the AP-1 transcription factor DJun during dorsal closure. Genes and Dev 1997; 11:1717-1727.
30. Kockel L, Zeitlinger J, Staszewski LM et al. Jun in *Drosophila* development: redundant and nonredundant functions and regulation by two MAPK signal transduction pathways. Genes and Dev 1997; 11:1748-1758.
31. Marshall CJ. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr Opin Genet Dev 1994; 4:82-89.
32. Marshall CJ. Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. Cell 1995; 80:179-185.
33. Riesgo-Escovar JR, Jenni M, Fritz A et al. The *Drosophila* Jun-N-Terminal kinase is required for cell morphogenesis but not for Djun-dependent cell fate specification in the eye. Genes and Dev 1996; 10:2759-2768.
34. Sluss HK, Han Z, Barrett T et al. A JNK signal transduction pathway that mediates morphogenesis and an immune response in *Drosophila*. Genes and Dev 1996; 10:27435-2758.
35. Ring JM, Martinez-Arias A. Puckered, a gene involved in position-specific cell differentiation in the dorsal epidermis of the *Drosophila* larva. Development 1993; 119:251-259.
36. Harden N, Loh HY, Chia W et al. A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila*; Development 1995; 121:903-914.
37. Harden N, Loh HY, Ong YM et al. A *Drosophila* homolog of the Rac- and CDC42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein the colocalizes with dynamic actin strucutres. Mol Cell Biol 1996; 16:1896-1908.
38. Brummel TJ, Twombly V, Marqués G et al. Characterization and relationship of Dpp receptors encoded by the *saxphone* and *thick veins* genes in *Drosophila*. Cell 1994; 78:251-261.
39. Letsou A, Arora K, Wrana JL et al. *Drosophila* Dpp signaling is mediated by the punt gene product: a dual ligand-binding type II receptor of the TGF beta receptor family. Cell 1995; 80(6):899-908.

40. Arora K, Dai H, Kazuko SG et al. The *Drosophila schnurri* gene acts in the Dpp/TGFβ signaling pathway and encodes a transcription factor homologous to the human MBP family. Cell 1995; 81:781-790.
41. Grieder NC, Nellen D, Burke R et al. *schnurri* is required for *Drosophila* dpp signaling and encodes a zinc finger protein similar to the mammalian transcription factor PRDII-BF1. Cell 1995; 81:791-800.
42. Staehling-Hampton K, Laughon AS, Hoffmann FM. *Drosophila* protein related to the human zinc finger transcription factor PRDII/MBPI7HIV-EP1 is required for *dpp* signaling. Development 1995; 121:3393-3403.
43. Heitzler P, Haenlin M, Ramain P et al. A genetic analysis of *pannier*, a gene necessary for viability of dorsal tissues and bristle positioning in *Drosophila*. Genetics 1996; 143(3): 1271-1286.
44. St. Johnston RD, Gelbart WM. Decapentaplegic transcripts are localized along the dorsal-ventral axis of the *Drosophila* embryo. EMBO 1987; 6(9):2785-2791.
45. Karess RE, Chang XJ, Edwards KA et al. The regulatory light chain of nonmuscle myosin is encoded by *spaghetti-squash*, a gene required for cytokinesis in *Drosophila*. Cell 1991; 65(7):1177-1189.
46. Bogaert T, Brown N, Wilcox M. The *Drosophila* PS2 antigen is an invertebrate integrin that, like the fibroectin receptor, becomes localized to muscle attachments. Cell 1987; 51:929-940.
47. Wieshaus E, Nüsslein-Volhard C, Jürgens G. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*: III. Zygotic loci on the X-chromosome. Wilhelm Roux's Arch Dev Biol 1984; 193:296-307.
48. MacKrell AJ, Blumberg B, Haynes SR et al. The lethal *myospheroid* gene of *Drosophila* encodes a membrane protein homologous to vertebrate integrin β subunits. Proc. Natl. Acad. Sci. USA 1988; 85:545-557.
49. Fehon FG, Dawson IA, Artavanis-Tsakonas S. A *Drosophila* homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the *coracle* gene. Development 1994; 120:545-557.
50. Edwards KA, Kiehart D. *Drosophila* nonmuscle myosin II has multiple essential roles in imaginal disc and egg chamber morphogenesis. Development 1966; 122(5):1499-1511.
51. Ishimi Y, Sato W, Kojima M et al. Rapid purification of nucleosome assembly protein (AP-I) and production of monoclonal antibodies against it. Cell Structure and Function Function 1985; 10:373-382.
52. Ishimi Y, Kikuchi A. Identification and molecular cloning of yeast homolog of nucleosome assembly protein I which facilitates nucleosome assembly in vitro. J Biol Chem 1991; 266(11)7025-7029.
53. Simon HW, Gordon BM, Kozlowski M et al. Molecular characterization of hNRP, a cDNA encoding a human nucleosome-assembly-protein I-related gene product involved in the induction of cell proliferation. Biochem J 1994; 297:389-397.
54. Ito T, Bulger M, Kubuyashi R et al. NAP-1 is a core histone chaperone that functions in ATP-facilitated assembly of regularly spaced nucelosomal arrays. Mol Cell Biol 1996; 16:3112-3124.
55. Ecay TW, Conner TD, Decker ER. Nonmuscle myosin IIA copurifies with chloride channel-enriched membranes from epithelia. Biochem Biophys Res Commun 1997; 231:369-372.
56. Török I, Hartenstein K, Kalmes A et al. The *l(2)gl* homologue of *Drosophila pseudoobscura* suppresses tumorigenicity in transgenic *Drosophila melanogaster*. Oncogene 1993; 8:1537-1549.
57. Tomotsune D, Shoji H, Wakamatsu Y et al. A mouse homologue of the *Drosophila* tumour-suppressor gene *l(2)gl* controlled by Hox-C8 in vivo. Nature 1993; 365:69-72.
57a. Neer EJ, Schmidt CJ, Nambudriapad R et al. The ancient regulatory protein family of WD-repeat proteins. Nature 1994; 371:297-300.
58. Scheurlen W, Seranski P, Mincheva A et al. High resolution mapping of chromosome 17p in childhood primitive neuroectodermal tumors (PNET) reveals a common chromosomal

disruption within the Smith-Magenius region, an unstable region in band 17p11.2. Genes Chromosomes Cancer 1997; 18:50-58.
59. Wilgenbus KK, Seranski P, Brown A et al. Molecular characterization of a genetically unstable region containing the SMS critical area and a breakpoint cluster for human PNETs. Genomics 1997; 42:1-10.
60. Juyal RC, Figuera LE, Hauge X et al. Molecular analyses of 17p11.2 deletions in 62 Smith-Magenis syndrome patients. Am J Hum Gent 1996; 58:998-1007.
61. Cogen PH, McDonald JD. Tumor suppressor genes and medulloblastoma. J Neuro-Oncol 1996; 29:103-112.
62. Vassal G, Terrier-Lacombe M-J, Lellouch-Tubiana A et al. Tumorigenicity of cerebellar primitive neuro-ectodermal tumors in athymic mice correlates with poor prognosis in children. Int J Cancer (Pred Oncol) 1996; 69:146-151.
63. Batra SK, McLendon RE, Koo JS et al. Prognostic implications of chromosome 17p deletion in human medullablastomas. J Neuro-Oncol 1995; 24:39-45.
64. Koyama K, Fukushima Y, Inazawa J et al. The human homologue of the murine Llglh gene (LLGL) maps within the Smith-Magenis syndrome region in 17p11.2. Cytogenet Cell Genet 1996; 72:78-82.
65. Matsui Y, Toh-E A. Yeast RHO3 and RHO4 *ras* superfamily genes are necessary for bud growth, and their defect is suppressed by a high dose of bud formation genes CDC42 and BEM1. Mol and Cellular Biol 1992; 12 (12): 5690-5699.

CHAPTER 6

Focal Adhesion Kinase (FAK): Regulation of Its Tyr Phosphorylation

Enrique Rozengurt and José Luis Rodríguez-Fernández

Introduction

Multicellular organisms have developed highly efficient regulatory networks to control cell proliferation. These involve cellular interactions with stimulatory and inhibitory diffusible modulators as well as with the extracellular matrix proteins. In fully mature organisms, the cells of many tissues and organs are maintained in a nonproliferating state (the G_0/G_1 phase of the cell cycle), but they can be stimulated to resume DNA synthesis and cell division in response to external stimuli such as hormones, antigens or growth factors. In this manner, the growth of individual cells is regulated according to the requirements of the whole organism. The regulation of normal proliferation is therefore central to many physiological processes, including embryogenesis, growth and development, tissue repair and immune responses.

It has become evident that cultured cancer cells, which are characterized by unrestrained proliferation, acquire complete or partial independence of mitogenic signals in the extracellular environment through different mechanisms.[1,2] These include production of growth factors that act on the same cells that produce them (autocrine loop) or on adjacent cells (paracrine communication), and alterations in the number or structure of cellular receptors and changes in the activity of postreceptor signaling pathways that either stimulate or suppress cell growth. It has also been known for many years that most types of normal cells require attachment to a substrate to be able to proliferate, a process known as anchorage dependent growth, which is mediated by adhesive receptors of the integrin family. Many cancer cells have reduced or lost the requirement for anchorage. A fundamental problem in cancer biology is to elucidate the molecular basis of anchorage dependent and independent cell growth.

It is known that integrins, a family of heterodimeric transmembrane proteins, are involved in cell adhesion to extracellular matrix proteins on the surface of the cell.[3] There is growing evidence that shows that integrins not only mediate cell adhesion, but also transmit signals into the cell.[3-8]

An interesting hypothesis is that integrin mediated signaling converges or acts synergistically with signals elicited by growth factors.[9] In what follows, we will discuss experimental evidence indicating that integrin ligation by extracellular matrix proteins, mitogenic neuropeptides, bioactive lipids and polypeptide growth factors promote rapid and coordinate increases in the tyrosine phosphorylation of a common set of focal adhesion proteins.

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

p125^{FAK}, Paxillin and p130^{Crk}-Associated Substrate

The mechanism by which the src family of retroviral oncogenes causes malignant transformation has long proved elusive, mainly because of the difficulty in identifying substrates for the pp60$^{v\text{-}src}$ protein tyrosine kinase (PTK). The approach taken by Parsons and colleagues has been to generate monoclonal antibodies against individual proteins that are tyrosine phosphorylated in chicken embryo fibroblasts (CEF) transformed by Rous sarcoma virus or activated variants of pp60^{src}.[10] In this way, several potential substrates were identified and the antibodies subsequently used to clone the corresponding cDNAs from expression libraries.[11] This approach yielded the unexpected result that one such substrate, p125, is itself a novel type of PTK.[12] Immunostaining of CEFs with antibodies directed against p125 shows that it colocalizes with several components of cellular focal adhesions, such as tensin, vinculin and talin. Hence, it has been termed F(ocal) A(dhesion) K(inase).

Independently, Hanks et al[13] cloned the murine equivalent of p125^{FAK} using a PCR based cDNA cloning approach for the identification of novel PTKs. Like the chicken protein, the murine p125^{FAK} localizes to focal adhesions and has a similar predicted molecular mass (119.1 kDa).

p125^{FAK} is a structurally distinct nonreceptor protein tyrosine kinase characterized by a centrally located catalytic domain flanked by NH_2- and C-terminal noncatalytic domains of approximately 400 residues.[12,13] A comparison of the mouse and chicken p125^{FAK} primary structures shows a remarkable high degree of amino acid sequence similarity: 94% overall, rising to 98% within the catalytic domain. p125^{FAK} cDNAs also have been isolated from human and other sources revealing a high degree of structural conservation.[12-15] The striking species similarity of p125^{FAK} and its subcellular localization in chicken and mouse indicate a higly conserved cellular function for this tyrosine kinase.

The recently cloned PTK termed CAKβ/PYK2/RAFTK[16-18] exhibits significant homology to p125^{FAK}, suggesting that they are members of a novel family of PTKs. Nevertheless, while p125^{FAK} is expressed in many cells and tissues, CAKβ/PYK2/RAFTK is found predominantly in brain and neural cells.[16,18]

What makes the deduced amino acid sequence of p125^{FAK} interesting is the absence of noncatalytic motifs or domains found in other receptor and nonreceptor PTKs.[12,13] Strikingly, p125^{FAK} does not possess any motif determinant for either membrane association, such as acylation sites, or association with other proteins, such as Src homology 2 (SH2) and Src homology 3 (SH3) domains (see below and Fig. 6.1).

The function of the NH_2-terminal region of p125^{FAK} has not been elucidated, although "in vitro" this domain can bind peptides corresponding to the cytoplasmic region of β-integrins (Fig. 6.1). The C-terminal region contains a stretch of 159 amino acids, named focal adhesion targeting sequence (FAT), that is essential for focal adhesion localization. The C-terminal region of p125^{FAK} mediates binding to a number of signal transduction and cytoskeletal proteins, including paxillin, p130^{CAS}, talin and phosphatidylinositol 3-kinase.[19-21]

The phosphorylation of PTKs on tyrosine residues creates high affinity binding sites for proteins that contain SH2 domains. These domains are composed of a sequence of approximately 100 amino acids that contain a binding pocket for the phosphorylated tyrosine residues and a recognition site for three immediate C-terminal amino acids.[21] As SH2 mediated protein-protein interactions may alter the function of the second protein by a change in its activity or subcellular localization, such interactions are considered critical for intracellular signal transduction.[22] Expression of activated pp60^{src} with a Tyr-527-Phe mutation causes a large increase in p125^{FAK} tyrosine phosphorylation in NIH3T3 cells. By contrast, overexpression of wild type pp60^{src} results in a much more modest increase in p125^{FAK} phosphorylation, and this effect is abrogated in cells expressing nonmyristylated or kinase defective pp60^{src} mutants.[23] These findings suggest that p125^{FAK} tyrosine phosphorylation may

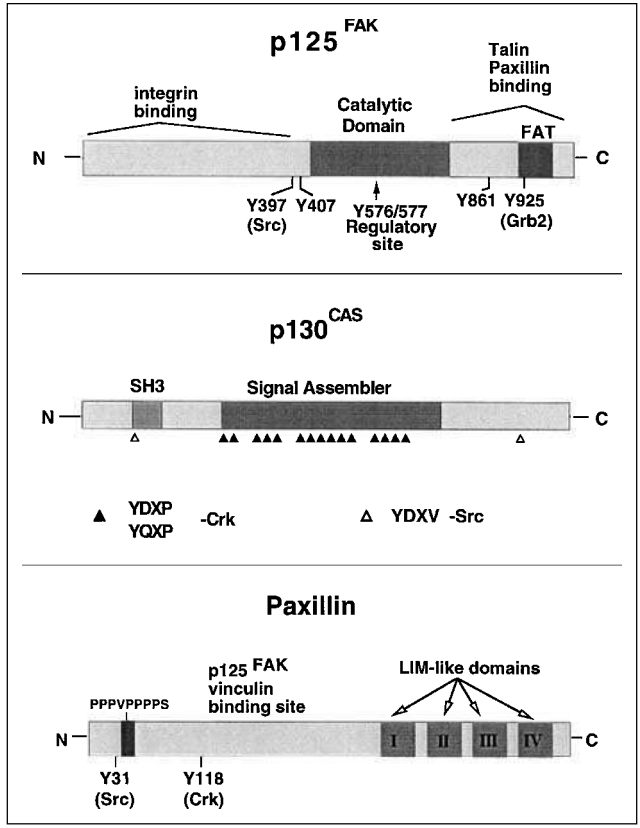


Fig. 6.1. Structures of $p125^{FAK}$, $p130^{CAS}$ and paxillin, emphasizing their organization, tyrosine phosphorylation sites and possible binding molecules. FAT sequence = focal adhesion targeting sequence; Y = tyrosine; N = amino terminal; C = carboxy terminal.

contribute to cellular transformation by $pp60^{v\text{-}src}$. Cells expressing the tyrosine kinase $pp90^{gag\text{-}yes}$ or the retroviral oncogene $p47^{gag\text{-}crk}$, which lacks intrinsic kinase activity, also exhibit increased $p125^{FAK}$ tyrosine phosphorylation.[10]

Recently it has become evident that tyrosine phosphorylated $p125^{FAK}$ associates with $pp60^{v\text{-}src}$ in v-src transformed chicken embryo cells. Complex formation requires the SH2 domain of $pp60^{v\text{-}src}$, indicating that the association is mediated by a tyrosine phosphorylated residue(s) in $p125^{FAK}$.[24] Tyr-397 has been identified as the major site of tyrosine autophosphorylation in $p125^{FAK}$.[19-21,25] Mutation of Tyr-397 to a nonphosphorylatable residue prevents $p125^{FAK}$ tyrosine phosphorylation and complex formation with $pp60^{v\text{-}src}$.

It has also been shown that "in vivo", $p125^{FAK}$ becomes phosphorylated at additional tyrosine residues (e.g., tyrosines 407, 576, 577, 861 and 925, see Fig. 6.1). It is plausible that $pp60^{src}$ phosphorylates $p125^{FAK}$ at these additional sites in intact cells.[19-21,26] In agreement with this possibility, $p125^{FAK}$ tyrosine phosphorylation is reduced in $pp60^{src}$ deficient fibroblasts[27] and Fyn-deficient brain tissues.[28] The phosphorylation of tyrosine residues 576/577 plays a role in stimulating enzyme activity,[29] whereas the phosphorylation of tyrosine 925

creates a binding site for the SH2 domain of the adaptor protein Grb-2[19-21] (see below and Fig. 6.1).

The focal adhesion proteins paxillin and p130 (Crk associated substrate (CAS) are potential downstream targets for $p125^{FAK}$ or for the complex of $p125^{FAK}$with $pp60^{src}$. Paxillin contains multiple domains that are thought to function in protein-protein interactions, including a proline-rich motif near the NH_2-terminus which could bind SH3 domains, a central region that interacts with $p125^{FAK}$ and vinculin and four regions homologous to LIM domains in the C-terminal portion of the molecule.[30-32] Phosphorylation of paxillin on tyrosine creates a binding site for Crk, Csk and $pp60^{src}$.[30,33,34] Recently, paxillin isoforms generated by alternative splicing and capable of differential association with other proteins have been identified.[35] $p130^{CAS}$ contains an SH3 domain and a cluster of 15 potential SH2 domain binding sites, nine of these are sequences (YDV/TP) that correspond to high affinity sites for SH2 domain of Crk.[36-38] $p130^{CAS}$ also contains putative binding sites for the SH2 domain of $pp60^{Src}$ and proline-rich regions that could bind SH3 domain proteins (Fig. 6.1). Thus, both paxillin and $p130^{CAS}$ could function as adaptor proteins in signal transduction at the focal adhesion plaques interacting with $p125^{FAK}$, $pp60^{src}$ and other important components of these structures.

Integrin Mediated Tyrosine Phosphorylation of Focal Adhesion Proteins

For several years, it has been known that integrins, a family of heterodimeric transmembrane proteins, are involved in cell adhesion to extracellular matrix proteins and proteins on the surface of the cell.[3] It is now evident that integrins can also transmit signals into cells.[3-8] In particular, tyrosine phosphorylation of cellular components emerged as a potential transducer of integrin generated signaling pathways. For example, tyrosine phosphorylation of proteins of 115-130 kDa occurs as a consequence of crosslinking $\alpha 3\beta 1$ integrin in KB carcinoma cells[39] and plating NIH3T3 cells on either a substrate coated with fibronectin or anti-integrin antibodies.[40] Subsequently, work from many laboratories demonstrated that integrin engagement leads to increase tyrosine phosphorylation of $p125^{FAK}$. For example, it is known that a major ~120 kDa protein phosphorylated on tyrosine after plating NIH3T3 cells on fibronectin or anti-integrin $\beta 1$ antibody is $p125^{FAK}$.[23] Tyrosine phosphorylation of $p125^{FAK}$ in response to cell attachment to fibronectin or other extracellular matrix proteins such as laminin, collagen type IV or vitronectin has also been observed in Balb/c 3T3, NIH3T3, KB carcinoma cells, endothelial cells, basophilic leukemia cells and other cell types.[13,39,41-44] Another component of the extracellular matrix, hyaluronan, has also been shown to stimulate cell motility and to cause a rapid assembly and disassembly of focal contacts.[45] Interestingly, $p125^{FAK}$ was also rapidly phosphorylated and dephosphorylated after hyaluronan stimulation, paralleling the time course of focal adhesion turnover.[45]

In blood platelets tyrosine phosphorylation occurs following ligand (fibrinogen) occupation of the major platelet specific integrin GpIIb/IIIa (reviewed in ref. 46). Brugge and co-workers reported that activation of platelets by thrombin or collagen increases tyrosine phosphorylation of $p125^{FAK}$ and that phosphorylation requires platelet aggregation mediated by the binding of fibrinogen to GpIIb/IIIa.[47] Thus, tyrosine phosphorylation of $p125^{FAK}$ failed to occur in thrombin treated platelets that were not allowed to aggregate and was abrogated either in Glanzmann's thrombasthenic platelets genetically deficient in GpIIb/IIIa or in platelets treated with an inhibitory monoclonal antibody to GpIIb/IIIa.

In both platelets and NIH3T3 cells, integrin stimulated $p125^{FAK}$ tyrosine phosphorylation correlates with an increase in the intrinsic tyrosine kinase of $p125^{FAK}$.[23,47] The implication of these findings is that the occupancy and subsequent clustering of integrins are important for tyrosine phosphorylation and activation of $p125^{FAK}$. However the possible

contribution of coprecipitating pp60^{src} in these assays requires further evaluation (see below). Cell adhesion to the extracellular matrix is also accompanied by tyrosine phosphorylation of paxillin[41,48] and p130^{CAS}.[49-51] Interestingly, tyrosine phosphorylation of p130^{CAS} induced by integrin engagement is severely reduced in cells lacking pp60^{src}.[52]

The cytoplasmic domains of the β subunits of the integrins contain sufficient information to stimulate p125^{FAK} tyrosine phosphorylation (reviewed in ref. 53). Interestingly, the conserved motif NPXY was required for β3-mediated p125^{FAK} tyrosine phosphorylation. In addition, mutation of serine 752 to proline, known to cause a variant of Glanzmann's thrombasthenia, also impairs the ability of β3 cytoplasmic domain to stimulate p125^{FAK} tyrosine phosphorylation.[54,55]

Replating of fibroblasts on fibronectin induces the formation of a stable complex between p125^{FAK} and pp60^{src}[24,25,56] which is prevented by mutation of Tyr-397 of p125^{FAK}.[25] The phosphorylation of p125^{FAK} at multiple tyrosine residues by pp60^{src} is thought to play an important role in the regulation of p125^{FAK} activity and in the ability of p125^{FAK} to associate with other SH2 containing proteins, such as the SH2/SH3 adaptor Grb-2. The possibility that a p125^{FAK}-Grb-2 complex plays a role in integrin mediated p42^{MAPK} (ERK-2) activation has been proposed.[57] It is thought that Grb-2, via the GDP/GTP exchange factor SOS, promotes Ras loading with GTP, although the role of Ras in integrin-mediated p42^{MAPK}/p44^{MAPK} activation remains controversial.[58-61] In addition, further studies indicate that integrin signals to p42^{MAPK} through multiple pathways,[52,59,60] including direct coupling of the adaptor protein Shc to β1 subunit of integrin.[62]

The precise molecular mechanism by which integrin engagement leads to p125^{FAK} tyrosine phosphorylation is not understood. Integrins could induce conformational changes and/or clustering of p125^{FAK} or stimulate an intermediate molecule that is responsible for p125^{FAK} activation and autophosphorylation. On the basis of the results discussed previously a plausible model of integrin-mediated p125^{FAK} tyrosine phosphorylation and activation is as follows. The translocation of p125^{FAK} into nascent focal adhesion plaques causes clustering and autophosphorylation of this enzyme at Tyr-397. This creates a binding site for pp60^{src} which then forms a stable complex with p125^{FAK} and phosphorylates it at tyrosines 576 and 577, which are located in the catalytic loop of p125^{FAK} and stabilizes a conformation with enhanced kinase activity. Furthermore, it has been shown that inhibitors of PKC prevented the increase in tyrosine phosphorylation of p125^{FAK} in cells plated on fibronectin.[63] In this context, interestingly, the α- isoform of protein kinase C (PKC) has also been localized to focal adhesions. These findings suggest that PKC could also play a part in mediating integrin stimulated p125^{FAK} tyrosine phosphorylation.

Neuropeptide Mediated Tyrosine Phosphorylation of Focal Adhesion Proteins

An increasing number of small regulatory peptides or neuropeptides have been discovered in the cells of the gastrointestinal tract and central nervous system. Some of these peptides are localized in neurones and act as neurotransmitters in the central or peripheral nervous system, while others are released by endocrine cells and have effects both as systemic hormones circulating through the bloodstream and by acting in a paracrine or autocrine fashion.[2] Moreover, a number of peptides are found in both neuronal and endocrine cells, and a major effect of some regulatory peptides in vivo, for example, bombesin/gastrin-releasing peptide (GRP), is to stimulate the release of other biologically active peptides. The classic view was that these peptides act as fast-acting neuroendocrine signalers eliciting contractile or secretory response in their target cells. Subsequently, it has become evident that small regulatory peptides can act as potent growth factors both in cultured cells and in vivo. Moreover, neuropeptides, particularly those of the bombesin/GRP family, are

implicated in the autocrine growth of small cell lung carcinoma (reviewed in ref. 2). Consequently, it is important to understand in detail the receptors and signal transduction pathways that mediate the mitogenic action of neuropeptides because they may provide potential targets for therapeutic intervention.

Many studies to identify the molecular pathways by which neuropeptide mitogens elicit cellular growth have exploited cultured murine Swiss 3T3 cells as a model system.[64] The list of neuropeptides that can act as a mitogens in these cells has now grown considerably and includes bombesin, bradykinin, endothelin, and vasopressin.[2]

Binding of neuropeptides such as bombesin to their receptors initiates a cascade of intracellular signals culminating in DNA synthesis 10 to 15 h later (reviewed in ref. 65). The bombesin receptor like many other neuropeptide receptors belongs to the superfamily of G-protein coupled receptors. These are characterized by seven transmembrane domains which are thought to cluster to form a ligand-binding pocket. The binding of bombesin to its specific receptor activates the heterotrimeric G protein Gαq, which in turn stimulates phospholipase Cβ (PLCβ). This catalyses the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP_2) in the plasma membrane to produce inositol 1,4,5-trisphosphate (IP-3) and 1,2-diacylglycerol (DAG). IP-3 binds to a specific intracellular receptor which releases Ca^{2+} from internal stores. Depletion of Ca^{2+} from these stores, as induced by bombesin, other mitogenic neuropeptides and growth factors could play a role as one of the synergistic signals that contribute to stimulating the transition from G_0 to DNA synthesis.[66]

DAG, the other product of bombesin induced PLC mediated hydrolysis of PIP_2, directly activates protein kinase C (PKC). In accord with this, bombesin rapidly increases the phosphorylation of a major PKC substrate known as 80K or myristoylated alanine rich C-kinase substrate (80K/MARCKS). PKC activation induced by bombesin causes translocation of 80K/MARCKS from the membrane to the cytosolic fraction and a dramatic downregulation of 80K/MARCKS mRNA and protein in Swiss 3T3 cells.[67-69] PKC also plays a pivotal role in transducing neuropeptide signals into activation of protein kinase cascades in Swiss 3T3 cells, including $p42^{MAPK}$/$p44^{MAPK}$,[70] $p70^{S6k}$[71] and the recently cloned protein kinase D.[72]

In addition to these serine/threonine protein kinases, bombesin and other neuropeptides (e.g., vasopressin, endothelin and bradykinin) also stimulate a rapid increase in tyrosine phosphorylation of multiple proteins, including an heterogeneous band of 110-130 kDa in Swiss 3T3 cells.[9,73-77] Neuropeptide stimulation of these cells also increases tyrosine phosphorylation in cell free preparations.[76] These results suggested that neuropeptide stimulation of tyrosine phosphorylation reflected activation of a tyrosine kinase.

Subsequent studies demonstrated that bombesin, vasopressin and endothelin stimulate tyrosine phosphorylation of $p125^{FAK}$ in Swiss 3T3 cells.[75] Neuropeptide induced $p125^{FAK}$ phosphorylation occurs at concentrations of the peptide that closely parallel those necessary for mitogenic stimulation. The rapidity of neuropeptide stimulated phosphorylation (detectable within seconds) is consistent with a pathway leading from neuropeptide receptors to $p125^{FAK}$.[75] Thus $p125^{FAK}$ may function as a downstream element in a neuropeptide stimulated tyrosine pathway, as depicted in Figure 6.2.

In addition to $p125^{FAK}$, neuropeptides strikingly increase the tyrosine phosphorylation of paxillin and $p130^{CAS}$ and promote the formation of a complex between $p130^{CAS}$ and the protooncogene c-Crk.[37,74] These studies provided evidence indicating that ligation of G-protein coupled receptors not only elicits the synthesis of second messengers (such as Ca^{2+}, DAG or cAMP), but also induces tyrosine phosphorylation of focal adhesion proteins in their target cells.

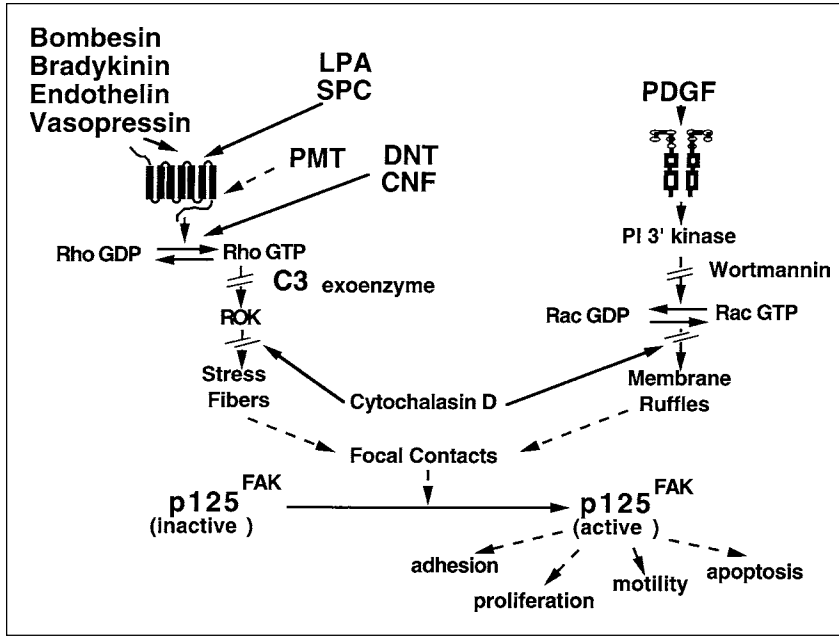


Fig. 6.2. The nonreceptor tyrosine kinase p125^{FAK} is a point of convergence in the action of mitogenic neuropeptides, lysophosphatidic acid (LPA), sphingosylphosphorylcholine (SPC), cytotoxic necrotizing factor 1 (CNF), dermonecrotic toxin (DNT) and platelet-derived growth factor (PDGF). Bombesin, bradykinin, endothelin, vasopressin, LPA and SPC act via distinct seven transmembrane domain receptors (although note that a single receptor is shown in the scheme). PMT might increase the coupling of heptahelical receptors to heterotrimeric G proteins, but its precise mechanism of action has not been elucidated. DNT and CNF act directly on Rho. See the text for details. Neuropeptides, bioactive lipids and PDGF are known to induce many other early events such as phosphoinositide breakdown, PKC activation, Ca^{2+} mobilization from internal stores, arachidonic acid release and serine/threonine phosphorylation of many cellular proteins. These events and others induced by PDGF are not represented in this scheme but can be found in complementary reviews.[83,132]

Signal Transductional Pathways in Neuropeptide Stimulated p125^{FAK} Tyrosine Phosphorylation

The molecular mechanisms by which neuropeptide receptor activation leads to rapid increase in p125^{FAK} tyrosine phosphorylation are only beginning to emerge. As described previously, bombesin and other neuropeptides are known to generate the intracellular second messenger diacylglycerol and inositol 1,4,5-trisphosphate, which activate PKC and mobilize Ca^{2+}, respectively. Direct activation of PKC, either by biologically active phorbol esters or by membrane permeant diacylglycerols, stimulates tyrosine phosphorylation of p125^{FAK}.[73] These results indicate that activation of PKC in intact cells is a potential signaling pathway leading to enhanced tyrosine phosphorylation of p125^{FAK}. However, several lines of evidence show that neuropeptides, unlike integrins[63] stimulate tyrosine phosphorylation of p125^{FAK} through a signal transduction pathway that is largely independent of PKC.[73] For example, downregulation of PKC by chronic treatment with phorbol esters or addition of a

selective inhibitor of PKC blocked the increase in $p125^{FAK}$ tyrosine phosphorylation induced by phorbol 12,13-dibutyrate (PDB) but did not impair the response to bombesin.

In view of the rapid kinetics of the neuropeptide stimulated Ca^{2+} mobilization and $p125^{FAK}$ tyrosine phosphorylation, a role for Ca^{2+} in neuropeptide stimulated tyrosine phosphorylation was also examined. Elevation of intracellular Ca^{2+} using a Ca^{2+} ionophore did not increase $p125^{FAK}$ tyrosine phosphorylation in Swiss 3T3 cells.[73] Furthermore, depletion of the intracellular Ca^{2+} pool by treating cells with the tumor promoter thapsigargin, a selective inhibitor of Ca^{2+} ATPase of the endoplasmic reticulum that causes depletion of Ca^{2+} from internal stores and thereby blocks agonist-mediated mobilization of Ca^{2+}, had no effect on bombesin stimulated $p125^{FAK}$ tyrosine phosphorylation. In addition, bombesin stimulated $p125^{FAK}$ tyrosine phosphorylation with a half maximal concentration that was 6-fold lower than that required for mobilization of Ca^{2+}. Thus, several lines of evidence indicate that increases in the intracellular concentration of Ca^{2+} do not mediate neuropeptide stimulated $p125^{FAK}$ tyrosine phosphorylation.[73] Consequently, the two major early signals generated by activation of phospholipase C are not responsible for neuropeptide stimulation of $p125^{FAK}$ tyrosine phosphorylation in Swiss 3T3 cells.

A salient feature of $p125^{FAK}$ is its subcellular localization to focal adhesions that form at the termini of actin stress fibers. Several recent reports have implicated the Rho family of G proteins,[78] in the assembly of focal adhesion plaques and in the regulation of the actin cytoskeleton in Swiss 3T3 cells.[79,80] Microinjection of Rho into these cells increases the formation of actin fibers and the assembly of focal adhesion plaques. Conversely, microinjection of botulinum C3 exoenzyme, an ADP ribosyltransferase that impairs Rho function, causes the disruption of the actin filament network. Bombesin has been shown to promote a rapid increase in stress fibers and focal adhesions, an effect apparently mediated by Rho.[80] Interestingly, bombesin induced $p125^{FAK}$ tyrosine phosphorylation is completely blocked by treatment with cytochalasin D, which caps the plus end of the actin filament, induces actin depolymerization and consequently prevents focal adhesion assembly.[73] By contrast, disassembly of microtubules did not exert any inhibitory effect on $p125^{FAK}$ tyrosine phosphorylation. These findings suggest that $p125^{FAK}$ tyrosine phosphorylation induced by bombesin depends on the focal adhesion assembly and on the integrity of the actin filaments.

Subsequent studies indicated that treatment of Swiss 3T3 cells with C3 exoenzyme attenuates $p125^{FAK}$ tyrosine phosphorylation induced by bombesin.[81] Furthermore, addition of nonhydrolyzable GTP analogs to permeabilized Swiss 3T3 cells induces tyrosine phosphorylation of $p125^{FAK}$ in a Rho- dependent manner.[82] Further experiments with bioactive lipids and bacterial toxins (see below) also indicate a Rho-dependent tyrosine phosphorylation of $p125^{FAK}$. These findings suggest the existence of a pathway activated by seven transmembrane domain receptors in which Rho is upstream of cytoskeletal responses and tyrosine phosphorylation of focal adhesion proteins as illustrated in Figure 6.2.

Paxillin and $p130^{CAS}$ have also been identified as targets for neuropeptide stimulated tyrosine phosphorylation. Similar to the findings with $p125^{FAK}$, bombesin induced tyrosine phosphorylation of paxillin and $p130^{CAS}$ also occurs through a PKC and Ca^{2+} independent pathway, which is critically dependent on the integrity of the actin cytoskeleton and focal adhesions plaques.[37,74] We conclude that tyrosine phosphorylation of paxillin, $p130^{CAS}$ and $p125^{FAK}$ is coordinately regulated. It is possible that paxillin is a cellular substrate for $p125^{FAK}$ in a neuropeptide stimulated tyrosine kinase pathway. The fact that paxillin can be phosphorylated in vitro by $p125^{FAK}$[33] is consistent with this hypothesis.

As mentioned previously, bombesin induces a rapid and striking stimulation of $p42^{MAPK}$/$p44^{MAPK}$ in Swiss 3T3 cells.[70] Given that $p125^{FAK}$ has been implicated in the pathway leading to MAPK activation in integrin stimulated cells (see above), it is of interest to elucidate whether $p125^{FAK}$ tyrosine phosphorylation is also involved in bombesin-mediated $p42^{MAPK}$/

$p44^{MAPK}$ activation. Disruption of the actin cytoskeleton by cytochalasin D at concentrations that completely prevented tyrosine phosphorylation of $p125^{FAK}$ in response to bombesin did not interfere with $p42^{MAPK}/p44^{MAPK}$ activation by these growth factors.[83] These results imply that cytochalasin D dissociates MAPK activation from 125^{FAK} tyrosine phosphorylation in bombesin stimulated Swiss 3T3 cells.[83]

Lysophosphatidic Acid and Sphingosylphosphorylcholine Stimulate Tyrosine Phosphorylation of $p125^{FAK}$, Paxillin and $p130^{CAS}$

Lysophosphatidic acid (LPA) elicits a wide range of biological responses including changes in cell shape and mitogenesis via specific cell surface receptors linked to G protein signal transduction pathways.[84] LPA also induces rapid assembly of focal adhesions and the formation of actin stress fibers in Swiss 3T3 cells through a pathway that requires the function of Rho.[80] As could be predicted from the links illustrated in the diagram shown in Figure 6.2, LPA also induces tyrosine phosphorylation of $p125^{FAK}$, paxillin and $p130^{CAS}$ in Swiss 3T3 cells. The kinetics of LPA stimulated tyrosine phosphorylation, detectable within seconds, suggests that this effect could be functionally important in the action of LPA.

LPA is known to induce the rapid formation of second messenger diacylglycerol and inositol 1,4,5-trisphosphate which activates PKC and mobilize Ca^{2+} from intracellular stores.[85] However, neither inhibition of PKC nor depletion of Ca^{2+} from intracellular stores prevented LPA induced tyrosine phosphorylation of $p125^{FAK}$.[86] Thus LPA, like bombesin, induces tyrosine phosphorylation through a pathway largely independent of PKC activation and Ca^{2+} mobilization.[37,86] The concentration dependence of LPA induced tyrosine phosphorylation closely paralleled the concentration dependence for LPA induced accumulation of stress fibers and assembly of focal adhesions. Pretreatment of quiescent Swiss 3T3 cells with cytochalasin D completely blocks LPA induced tyrosine phosphorylation of $p125^{FAK}$, $p130^{CAS}$ and paxillin. Thus, the integrity of the actin cytoskeleton is essential for the effects of LPA on tyrosine phosphorylation. Furthermore, treatment of Swiss 3T3 cells with C3 exoenzyme to inactivate Rho attenuates the ability of LPA to induce tyrosine phosphorylation of $p125^{FAK}$.[87] These findings suggest the existence of a signal transduction link that involves $p125^{FAK}$, $p130^{CAS}$ and paxillin tyrosine phosphorylation, Rho function, focal adhesion assembly and stress fiber formation, as predicted by the scheme presented in Figure 6.2.

Lysosphingolipids such as sphingosylphosphorylcholine (SPC) are potential derivatives of sphingolipids. SPC accumulates in patients with Niemann-Pick disease, a lipid storage disorder. SPC is a potent inducer of multiple signal transduction pathways and DNA synthesis in Swiss 3T3 cells, presumably via a G protein coupled receptor.[88] In particular, SPC elicits tyrosine phosphorylation of $p125^{FAK}$, $p130^{CAS}$ and paxillin and induces concomitant actin reorganization and focal adhesion assembly in these cells[37,89] Treatment with cytochalasin D or microinjection of C3 exoenzyme prevented the change in actin organization, the assembly of focal adhesions and tyrosine phosphorylation of focal adhesion proteins in response to SPC. These studies with LPA and SPC also support the existence of a signal transduction pathway in which Rho, via actin cytoskeleton, leads to the tyrosine phosphorylation of $p125^{FAK}$, $p130^{CAS}$ and paxillin.

Cytotoxic Necrotizing Factor 1 from *Escherichia coli* and Dermonecrotic Toxin from *Bordetella bronchiseptica* Induce Rho-Dependent Tyrosine Phosphorylation of $p125^{FAK}$ and Paxillin in Swiss 3T3 Cells

The mechanism of action of bacterial toxins has provided novel insights into the control of cellular regulatory processes, including signal transduction and cell proliferation.

For example, the *Clostridium botulinum* C3 exoenzyme and the enterotoxins A and B from *Clostridium difficile,* which selectively inactivate members of the Rho subfamily, have provided useful tools to evaluate the role of these small G proteins in signal transduction and cytoskeletal organization.[90-92] The potent mitogenic toxin from *Pasteurella multocida* (PMT) induces Rho-dependent actin stress fiber formation, focal adhesion assembly and tyrosine phosphorylation of p125^{FAK} and paxillin but its molecular mechanism of action has not been elucidated yet.[93-96] In contrast to these toxins, cytotoxic necrotizing factors (CNF) produced by some pathogenic strains of *E. coli*[97] and dermonecrotic toxin (DNT) from *Bordetella bronchiseptica*[98] directly target and activate Rho.[99,100]

Recently, two independent laboratories demonstrated that CNF selectively activates Rho by deaminating a glutamine residue at position 63 (Gln 63), thereby locking Rho in the active (i.e., GTP-bound) state.[101,102] In accord with this mechanism of action, CNF and DNT induce actin reorganization in several cell types.[100,103-105] These toxins provide a powerful tool to explore further the connection between Rho activation and tyrosine phosphorylation of p125^{FAK} and paxillin.

Recently CNF1 and DNT have been shown to stimulate tyrosine phosphorylation of p125^{FAK} and paxillin and induce a concomitant increase in the formation of actin stress fibers and in the assembly of focal adhesion plaques in Swiss 3T3 cells. Microinjection of C3 exoenzyme prevented both the cytoskeletal responses and the increase in tyrosine phosphorylation of focal adhesion proteins. In contrast to most other stimuli that promote tyrosine phosphorylation of p125^{FAK}, p130^{CAS} and paxillin,[73-75,86,89,96,106-110] CNF1 and DNT do not activate phospholipase C-mediated events including inositol phosphate production, Ca^{2+} mobilization and PKC activation. In addition, CNF1 and DNT stimulated reinitiation of DNA synthesis but neither induce activation of p42^{MAPK} (ERK2). These results lead to the important conclusion that activation of the endogenous Rho (i.e., without overexpression) can promote cytoskeletal responses and tyrosine phosphorylation of focal adhesion proteins in the absence of p42^{MAPK}/p44^{MAPK} activation. Reciprocally, these findings also indicate that tyrosine phosphorylation of p125^{FAK} does not necessarily leads to MAPK activation.

The results with CNF1 and DNT provide novel evidence for the existence of a signal transduction pathway that links Rho activation to tyrosine phosphorylation of focal adhesion proteins.

Neuropeptides and Phorbol Esters Activate Src-Family Tyrosine Kinases in Swiss 3T3 Cells Independently from Tyrosine Phosphorylation of p125^{FAK}

As mentioned in the preceding sections, p125^{FAK} activation and tyrosine autophosphorylation creates a high affinity binding site for the SH2 domain of members of the Src-kinase family which could, therefore, play a role as signal transducers of tyrosine phosphorylated p125^{FAK}. The kinase activity of pp60^{src}-kinase family members (such as Src, Yes and Fyn) is repressed when a key tyrosine residue in the carboxy-terminal region (corresponding to Tyr-527 of the chicken protein) is phosphorylated by Csk.[111,112] Phosphorylation at Tyr-527 creates a binding site for Src SH2 domain and allows an intramolecular interaction that locks pp60^{src} in an inactive conformation.[22,113,114] Two mechanisms that may "unlock" and activate Src family members are currently considered. In one case, dephosphorylation of Tyr-527 by a tyrosine phosphatase may destabilize the complex, releasing the SH2 domain thereby activating the kinase activity.[115-117] An alternative mechanism, involving competition for the SH2 domain of pp60^{src} by a high affinity allosteric ligand, would also lead to enzymatic activation of this kinase.[22,118] In this context, autophosphorylation of p125^{FAK} at Tyr-397 creates a putative competing binding site for the SH2 domain of Src, and thus would lead to the formation of a signaling complex in which Src kinases are active.[22,25,56,119]

Thus, it was of interest to examine the effect of bombesin and other neuropeptides on the activity of Src-family kinases.[120]

We have shown recently that treatment of quiescent Swiss 3T3 cells with bombesin induces a rapid and transient increase in the kinase activity of Src-family of tyrosine kinases.[120] Src-family kinase activity was also increased by treatment of intact cells with phorbol 12,13-dibutyrate, a direct activator of PKC. However, Src-family kinase activation by bombesin was not dependent either on PKC or Ca^{2+}.

As previously pointed out, bombesin-induced $p125^{FAK}$ tyrosine phosphorylation is completely blocked by treatment with cytochalasin D, a drug that disrupts the actin filament network and the assembly of focal adhesion plaques.[73,106] Cytochalasin D, at concentrations that profoundly inhibited $p125^{FAK}$ tyrosine phosphorylation, does not impair the striking increase in Src family kinase activity induced by bombesin. Furthermore, bombesin also induces Src kinase family activation in Swiss 3T3 cells placed in suspension, a condition that also prevents the tyrosine phosphorylation of $p125^{FAK}$ induced by bombesin. These findings indicate that Src-family kinase activation can be dissociated from $p125^{FAK}$ tyrosine phosphorylation in bombesin-treated Swiss 3T3 cells and demonstrate that two distinct signal transduction pathways lead to protein tyrosine phosphorylation in bombesin-stimulated Swiss 3T3 cells.[120]

As indicated previously, engagement of integrins leads to a stable complex between $p125^{FAK}$ and $pp60^{src}$. In contrast, we did not detect the formation of an immunoprecipitable complex between $p125^{FAK}$ and $pp60^{src}$ in Swiss 3T3 cells stimulated with neuropeptides.[120] These results can not exclude the possibility that once activated, $pp60^{src}$ can transiently associate with $p125^{FAK}$ in bombesin treated cells. The basis of this difference between integrin and neuropeptide stimulated $p125^{FAK}$ /$pp60^{src}$ association is not known.

A Model of Rho-Dependent Tyrosine Phosphorylation of Focal Adhesion Proteins

The signal transduction steps that link the heptahelical receptors for neuropeptides and bioactive lipids to Rho-dependent tyrosine phosphorylation of focal adhesion proteins remains undefined. It is clear that a single receptor subtype mediates coupling to PLCβ as well as tyrosine phosphorylation of $p125^{FAK}$.[121,122] A plausible model is that one domain of the seven transmembrane receptors couples to Gq and thereby to PLCβ, whereas a separate domain could lead to activation of Rho and consequently to cytoskeletal responses and tyrosine phosphorylation of focal adhesion proteins via interaction with a different heterotrimeric G protein. In this context, the demonstration that constitutively active mutants of $G_{\alpha 12}$ and $G_{\alpha 13}$ but not $G_{\alpha q}$ stimulate Rho-dependent actin stress fibers formation and focal adhesion assembly is highly relevant.[123] This suggests that seven transmembrane domain receptors could couple to Rho via different heterotrimeric G-proteins (i.e., $G_{\alpha 12}/G_{\alpha 13}$) than those that couple to PLCβ. The activated forms of α_{12}/α_{13} could activate or recruit Rho exchange factors that would promote the GTP-bound form of Rho in vivo. Further experimental work will be required to demonstrate that active forms of $G_{\alpha 12}$ and $G_{\alpha 13}$ can induce tyrosine phosphorylation of focal adhesion proteins via a Rho-dependent signal transduction pathway.

An important step in the understanding of the mechanism(s) by which Rho promotes cytoskeletal responses has been the identification of the protein kinases PKN and ROK (that bind GTP-Rho) as downstream targets of Rho.[124-128] Microinjection of a constitutively active form of ROK has been shown to induce the formation of actin stress fibers and focal adhesion plaques.[129] Thus, it is likely that ROK plays a role in transducing Rho activation into cytoskeletal responses. The demonstration that ROK leads to myosin II light chain (MLC) phosphorylation, either by inhibition of the 130kDa myosin-binding subunit of

myosin phosphatase (mPP)[130] and/or by phosphorylation and activation of MLC kinase,[131] has suggested a molecular mechanism by which Rho-mediated ROK activation triggers cytoskeletal reorganization. MLC phosphorylation leads to myosin filament formation and stimulates the actin-activation of myosin ATPase.[131] It has been suggested that the tension generated by actomyosin plays a key role in the formation of stress fibers and in the clustering of integrins to which they are attached, giving rise to observable focal adhesions.[132,133] In agreement with this hypothesis, MLC inhibitors also prevent LPA-mediated formation of stress fibers and focal adhesions. As suggested previously, the translocation of $p125^{FAK}$ into nascent focal adhesions is thought to induce its activation and autophosphorylation, as a result of the clustering and/or conformational change. The inhibition by cytochalasin D of $p125^{FAK}$, $p130^{CAS}$ and paxillin tyrosine phosphorylation induced by bombesin, LPA, SPC and bacterial toxins can be readily accounted for in the framework of this model.

Platelet-Derived Growth Factor Modulation of $p125^{FAK}$, $p130^{CAS}$ and Paxillin Tyrosine Phosphorylation

Platelet-derived growth factor (PDGF), a potent mitogen for mesenchymal cells, has been reported to play a role in development, wound healing, inflammation and oncogenesis.[1,134] The biological effects of PDGF are mediated through receptors that possess an intrinsic tyrosine kinase activity. The binding of PDGF, a disulfide linked dimer of two homologous polypeptides, A and B, to individual receptor chains (α and β) results in their dimerization and the subsequent transphosphorylation of specific tyrosine residues in the receptor chain.[134] These phosphorylated tyrosine residues serve as attachment sites for cytoplasmic effector proteins. The interaction between receptor and effector proteins is mediated by SH2 domains in the effector proteins. Several of these effector proteins have been identified, including phospholipase C-γ, Ras GAP of 120 kDa, the p85 subunit of PI-3 kinase, Grb-2, and the Src family of tyrosine kinases.[135] Once bound to the receptor, many of these proteins are phosphorylated on tyrosine by intrinsic kinase activity of the receptor. In addition, to its mitogenic affect, PDGF has been reported to induce changes in cell morphology and stimulate chemotaxis.[1] The substrates of PDGF induced tyrosine phosphorylation identified so far may not account for all the cellular effects of this growth factor.

Tyrosine phosphorylation of $p125^{FAK}$ is markedly stimulated in response to low concentrations of PDGF in Swiss 3T3 cells.[106,136] Surprisingly, at higher concentration of PDGF, $p125^{FAK}$ tyrosine phosphorylation is dramatically decreased in these cells. Paxillin and $p130^{CAS}$ are also phosphorylated in response to low but not high concentrations of PDGF.[37,106] This unexpected bell shape dose-response relationship appears to be unique to PDGF. Tyrosine phosphorylation of $p125^{FAK}$, $p130^{CAS}$ and paxillin induced by neuropeptides, LPA or SPC exhibits a sigmoidal dose-response curve, which does not decrease at high agonist concentrations.[37,75,86,89] The precise molecular mechanism by which PDGF stimulates a biphasic $p125^{FAK}$ tyrosine phosphorylation is intriguing. This cytosolic tyrosine kinase does not possess an SH2 domain (Fig. 6.1), and therefore it is unlikely that $p125^{FAK}$ associates with and is directly phosphorylated by the PDGF receptor.

Examination of the actin cytoskeleton showed that a low concentration of PDGF (5 ng/ml) causes accumulation of actin in membrane ruffles, whereas a high concentration of PDGF (30 ng/ml) induces actin disorganization.[106] By contrast, bombesin or LPA increased the formation of stress fibers with no evidence of disruption of the actin cytoskeleton network at high concentrations. Thus, PDGF modulation of the actin cytoskeleton, as with tyrosine phosphorylation of proteins, is critically dependent on PDGF concentrations. As dissolution of actin stress fibers by cytochalasin D also prevented PDGF-mediated tyrosine phosphorylation of $p125^{FAK}$, paxillin and $p130^{CAS}$, the inhibitory limb in the dose-response curve of PDGF may be related to the fact that at high concentrations, PDGF dis-

rupts the actin cytoskeleton.[106] Interestingly, addition of PDGF at high concentrations rapidly and completely blocked the accumulation of stress fibers and the stimulation of $p125^{FAK}$ and paxillin tyrosine phosphorylation induced either by bombesin, LPA or SPC.

A model to explain the intriguing effects of PDGF on tyrosine phosphorylation of focal adhesion proteins and actin cytoskeleton organization is suggested by the striking difference in the concentration of PDGF that is required to induce tyrosine phosphorylation of PI-3-kinase and GAP. PI-3-kinase phosphorylates the head group of phosphatidyl inositol 4,5-bisphosphate to yield phosphatidyl inositol (3,4,5) trisphosphate and this lipid has been postulated to act as a second messenger.[137-141] PI-3-kinase is stimulated by PDGF at low concentrations, similar to those required to elicit $p125^{FAK}$ and paxillin tyrosine phosphorylation and membrane ruffles.[106]

It has been shown that PI-3-kinase activation is required for the formation of membrane ruffles and the stimulation of chemotaxis induced by growth factors.[142-144] Recently it has been shown that the small G protein Rac lies downstream of PI-3-kinase, and there is evidence that D3 phosphatidyl inositides may promote Rac-GTP formation.[145] Furthermore, it has been demonstrated that there is a GTP-dependent and PDGF-stimulated association of Rac with PI-3-kinase in Swiss 3T3 cells.[146] Activated Rac has been demonstrated to direct the formation of membrane ruffles and the assembly of small focal contacts.[80] As mentioned above, PDGF-stimulated tyrosine phosphorylation of $p125^{FAK}$ and paxillin is dependent on the integrity of the actin cytoskeleton.[106] Furthermore, PI-3-kinase is required in the pathway leading to the formation of membrane ruffles and focal contacts and tyrosine phosphorylation of $p125^{FAK}$, $p130^{CAS}$ and paxillin induced by a low concentration of PDGF.[107] These results establish another link between the reorganization of the actin cytoskeleton and the tyrosine phosphorylation of these focal adhesion proteins. Taken together, all these findings suggest that there is a linear signal transduction pathway whereby ligation of the PDGF receptor activates PI-3-kinase and thereby stimulates Rac-GTP formation. Activated Rac induces the formation of focal contacts, reorganization of the actin cytoskeleton and thereby the tyrosine phosphorylation of $p125^{FAK}$ and paxillin (Fig. 6.2).

In contrast to PI-3-kinase, GAP tyrosine phosphorylation is induced by high concentrations of PDGF. Overexpression of a truncated GAP lacking the Ras GAP domain causes disruption of the actin stress fibers and a reduction in cell adhesion, presumably reflecting the disassembly of focal contacts.[147]

Biological Significance of the $p125^{FAK}$ Pathway

The findings discussed above have important implications for signal transduction and cell regulation. Most obviously, they suggest that tyrosine phosphorylation of the tyrosine kinase $p125^{FAK}$ is a point of convergence in the action of integrins, oncogenic forms of $pp60^{src}$, mitogenic neuropeptides, bioactive lipids, bacterial toxins and growth factors. One inference is that the signal transduction pathways initiated by these diverse groups of molecules have, at least in part, similar consequences for cell function.

Several lines of evidence indicate that $p125^{FAK}$ and its downstream targets play a central role in a number of fundamental cellular processes. Cells isolated from FAK (-/-) "knockout" embryos lacking $p125^{FAK}$ exhibit impaired cell locomotion.[27] These cells contain a large number of focal adhesions, suggesting that $p125^{FAK}$ plays a role in focal adhesion turnover rather than in focal adhesion assembly, as originally proposed.[27,148] The alteration in cell locomotion could be responsible for the embryonic lethality of FAK "knock-outs".[27,149,150] In addition, overexpression of FAK in CHO cells enhanced cell migration on fibronectin.[151] Interestingly, $p125^{FAK}$ expression and activity is increased in invasive and metastatic colon and breast cancers and in melanoma cell lines.[152-156] Tyrosine phosphorylation of $p125^{FAK}$ also increases in migrating human vascular endothelial cells,[157] motile keratinocytes during

the process of wound healing[150] and vascular smooth muscle cells stimulated to migrated with PDFG.[136]

There is evidence indicating that $p125^{FAK}$ is also involved in the control of cell proliferation. Thus, a dominant negative C-terminal fragment of $p125^{FAK}$, which displaces endogenous $p125^{FAK}$ from focal adhesions and prevents its activation, attenuates serum-induced stimulation of cellular DNA synthesis.[158] In addition, treatment of cells with cytochalasin D, which prevents cytoskeletal signaling and tyrosine phosphorylation of $p125^{FAK}$, $p130^{CAS}$ and paxillin inhibits the passage of the cells through the restriction point of the cell cycle.[83] Furthermore, a constitutively activated form of $p125^{FAK}$ which has been directed to the cell membrane prevents apoptosis resulting from loss of contact with extracellular matrix proteins and induces neoplastic transformation in MDCK epithelial cells.[159] Reciprocally, antisense mediated downregulation of expression[160] or inhibition of activation of $p125^{FAK}$ by microinjection of antibodies or peptides that prevents the binding of $p125^{FAK}$ to endogenous integrins induced apoptosis in tumor cells.[161] Similarly, $p130^{CAS}$ has been implicated in cellular transformation and signal transduction.[37,38,162]

All these findings indicate that the pathway discussed in this review plays an important role in transducing integrin, neuropeptide and growth factor signals into locomotive, proliferative and apoptotic responses (Fig. 6.2).

References

1. Westermark B, Heldin C-H. Platelet-derived growth factor in autocrine transformation. Cancer Res 1991; 51:5087-5092.
2. Rozengurt E. Polypeptide and neuropeptide growth factors: Signalling pathways and role in cancer. Oxford: Oxford University Press, 1995:12-20.
3. Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 1992; 69:11-25.
4. Juliano RL, Harskill S. Signal transduction from the extracellular matrix. J Cell Biol 1993; 120:577-585.
5. Lafrenie RM, Yamada KM. Integrin-dependent signal transduction. J Cell Biochem 1996; 61:543-553.
6. Clark EA, Brugge JS. Integrins and signal transduction: the road taken. Science 1995; 268:233-239.
7. Schwartz MA, Scaller MD, Ginsberg MA. Integrins: Emerging paradigms of signal transduction. Annu Rev Cell Dev Biol 1995; 11:549-599.
8. Yamada KM, Miyamoto S. Integrin transmembrane signaling and cytoskeletal control. Current Biol 1995; 7:681-689.
9. Rozengurt E. Convergent signalling in the action of Integrins, Neuropeptides, Growth Factors and Oncogenes. Cancer Surveys 1995; 24:81-96.
10. Kanner SB, Reynolds AB, Vines RR, Parsons JT. Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. Proc Natl Acad Sci USA 1990; 87:3328-3332.
11. Schaller MD, Bouton AH, Flynn DC, Parsons JT. Identification and characterization of novel substrates for protein tyrosine kinases. Prog Nucleic Acid Res Mol Biol 1993; 44:205-226.
12. Schaller MD, Borgman CA, Cobb BS et al. $pp125^{FAK}$, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc Natl Acad Sci USA 1992; 89:5192-5196.
13. Hanks SK, Calalb MB, Harper MC, Patel SK. Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. Proc Natl Acad Sci USA 1992; 89:8487-8491.
14. Andre E, Becker-Andre M. Expression of a N-terminally truncated form of human focal adhesion kinase in brain. Bichim Biophys Res Commun 1993; 190:140-147.
15. Zhang X, Wright CVE, Hanks SK. Cloning ofa Xenopus laevis cDNA encoding focal adhesion kinase (FAK) and expression during early development. Gene 1995; 160:219-222.

16. Lev S, Moreno H, Martinez et al. Protein tyrosine kinase PYK2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. Nature 1995; 376:737-745.
17. Sasaki H, Nagura K, Ishino M et al. Cloning and characterization of cell adhesion kinase b, a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. J Biol Chem 1995; 270:21206-21219.
18. Abraham S, Londoa R, Fu Y et al. Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. J Biol Chem 1995; 270:27742-27751.
19. Hanks SK, Polte TR. Signalling through focal adhesion kinase. Bioessays 1997; 19:137-145.
20. Ilic D, Damsky CH, Yamamoto T. Focal adhesion kinase: At the crossroads of signal transduction. J Cell Sci 1997; 110:401-407.
21. Richardson A, Parsons JT. Signal transduction through integrins: A central role for focal adhesion kinase? BioEssays 1995; 17:229-236.
22. Pawson T. Protein modules and signalling networks. Nature 1995; 373:573-580.
23. Guan JL, Shalloway D. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature 1992; 358:690-692.
24. Xing Z, Chen H-C, Nowlen JK et al. Direct Interaction of v-src with the Focal Adhesion Kinase Mediated by the Src SH2 Domain. Mol Biol Cell 1994; 5:413-421.
25. Schaller MD, Hildebrand JD, Shannon JD et al. Autophosphorylation of the focal adhesion kinase, $pp125^{FAK}$, directs SH2-dependent binding of $pp60^{src}$. Mol Cell Biol 1994; 14:1680-1688.
26. Calalb MB, Zhang X, Polte TR, Hanks SK. Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. Biochem Biophys Res Comm 1996; 228:662-668.
27. Ilic D, Furuta Y, Kanazawa S et al. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature 1995; 377:539-544.
28. Grant SGN, Karl KA, Kiebler MA, Kandel ER. Focal adhesion kinase in the brain: novel subcellular localization and specific regulation by Fyn tyrosine kinase in mutant mice. Genes & Dev 1995; 9:1909-1921.
29. Calalb MB, Polte TR, Hanks SK. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain refgulates kinase activity: A role for Src family kinases. Mol Cell Biol 1995; 15:954-963.
30. Turner CE. Paxillin: A cytoskeletal target for tyrosine kinases. Bioessays 1994; 16:47-52.
31. Brown MC, Perrotta JA, Turner CE. Identification of LIM3 as the principal determinat of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. J Cell Biol 1996; 135:1109-1123.
32. Salgia R, Li J-L, Lo SH et al. Molecular cloning of human paxillin, a focal adhesion protein phosphorylated by $p210^{BCR/ABL}$. J Biol Chem 1995; 270:5039-5047.
33. Bellis SL, Miller JT, Turner CE. Characterisation of tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. J Biol Chem 1995; 270:17437-17441.
34. Schaller MD, Parsons JT. $pp125^{FAK}$ -dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. Mol Cell Biol 1995; 15:2635-2645.
35. Mazaki Y, Hashimoto S, Sabe H. Monocyte cells and cancer cells express novel paxillin isoforms with different binding specificities. J Biol Chem 1997; 272:7437-7444.
36. Harte MT, Hildebrand JD, Burnham MR et al. p130cas, a substrate associated with v-src and v-crk, localizes to focal adhesions and binds to focal adhesion kinase. J Biol Chem 1996; 271:13649-13655.
37. Casamassima A, Rozengurt E. Tyrosine phosphorylation of $p130^{cas}$ by bombesin, lysophosphatidic acid, phorbol esters, and platelet-derived growth factor. J Biol Chem 1997; 272:9363-9370.
38. Sakai R, Iwamatsu A, Hirano N et al. A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation dependent manner. EMBO J 1994; 13:3748-3756.
39. Kornberg LJ, Earp HS, Turner CE et al. Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of β_1 integrins. Proc Natl Acad Sci USA 1991; 88:8392-8396.

40. Guan J-L, Trevithick JE, Hynes RO. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. Cell Regul 1991; 2:951-964.
41. Burridge K, Turner CE, Romer LH. Tyrosine phosphorylation of paxillin and pp125^{FAK} accompanies cell adhesion to extracellular matrix: A role in cytoskeletal assembly. J Cell Biol 1992; 119:893-903.
42. Kornberg L, Juliano RL. Signal transduction from the extracellular matrix- the integrin tyrosine kinase connection. Trends Pharm Sci 1992; 13:93-95.
43. Takahashi M, Berk BC. Mitogen activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells. Essential role for herbamycin-sensitive kinase. J Clin Invest 1996; 98:2623-2631.
44. Hamawy MM, Minoguchi K, Swaim WD et al. A 77-kDa protein associates with pp125FAK in mast cells and becomes tyrosine phosphorylated by high affinity IgE receptor aggregation. J Biol Chem 1995; 270:12305-12309.
45. Hall CL, Wang C, Lange LA, Turley EA. Hyaluronan and the hgyaluronan receptor receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity. J Cell Biol 1994; 126:575-588.
46. Clark E, Shattil S, Brugge J. Regulation of protein tyrosine kinases in platelets. Trends Biochem Sci 1994; 19:464.
47. Lipfert L, Haimovich B, Schaller MD et al. Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125^{FAK} in platelets. J Cell Biol 1992; 119:905-912.
48. Turner CE. Paxillin is a major phosphotyrosine-containing protein during embryonic development. J Cell Biol 1991; 115:201-207.
49. Petch LA, Bockholt SM, Bouton A et al. Adhesion-induced tyrosine phosphorilation of the p130 SRC substrate. J Cell Biol 1995; 108:1371-1379.
50. Vuori K, Ruoslahti E. Tyrosine phosphorylation of p130^{cas} and cortactin accompanies integrin-mediated cell adhesion to extracellular matrix. J Biol Chem 1995; 270:22259-22262.
51. Nojima Y, Morino N, Mimura T et al. Integrin-mediated cell adhesion promotes tyrosine phosphorylation of p130cas, a Src homology 3-containing molecule having multiple Src homology 2-binding motifs. J Biol Chem 1995; 270:15398-15402.
52. Schlaepfer DD, Broome MA, Hunter T. Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130^{cas}, and Nck adaptor proteins. Mol Cell Biol 1997; 17:1702-1713.
53. Shattil SJ, Ginsberg MH. Integrin signaling in vascular biology. J Clin Invest 1997; 100:1-5.
54. Tahiliani PD, Singh L, Auer KL, LaFlamme SE. The role of conserved amino acid motifs within the integrin β3 cytoplasmic domain in triggering focal adhesion kinase phosphorylation. J Biol Chem 1997; 272:7892-7898.
55. Leong L, Hughes PE, Schwartz MA et al. Integrin signaling: roles for the cytoplasmic tails of αIIβ3 in the tyrosine phosphorylation of pp125^{FAK}. J Cell Sci 1995; 108:3817-3825.
56. Cobb BS, Schaller MD, Leu T-H, Parsons JT. Stable association of pp60^{src} and pp59^{fyn} with the focal adhesion-associated protein tyrosine kinase, pp125^{FAK}. Mol Cell Biol 1994; 14:147-155.
57. Schlaepfer DD, Hanks S, Hunter T, Geer PVD. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. Nature 1994; 372:786-791.
58. Clark EA, Hynes RO. Ras activation is necessary for integrin-mediated activation of extracellular signal-regulated kinase 2 and cytosolic phospholipase A2 but for cytoskeletal organization. J Biol Chem 1996; 271:14814-14818.
59. Lin TH, Aplin AE, Shen YS et al. Integrin-mediated Activation of MAP kinase is independent of FAK: Evidence for Dual Integrin Signalling Pathways in fibroblasts. J Cell Biol 1997; 136:1385-1395.
60. Schlaepfer DD, Hunter T. Focal Adhesion kinase Overexpression Enhances Ras-dependent Integrin Signalling to ERK2/Mitogen-activated Protein Kinase through Interactions with and Activation of c-Src. J Biol Chem 1997; 272:13189-13195.
61. Chen Q, Lin TH, Der CJ, Juliano RL. Integrin-mediated activation of mitogen-activated protein (MAP) or extracellular signal-related kinase kinase (MEK) and kinase is independent of Ras. J Biol Chem 1996; 271:18122-18127.

62. Wary KK, Mainiero F, Isakoff SJ et al. The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. Cell 1996; 87:733-743.
63. Vuori K, Ruoslahti E. Activation of protein kinase C precedes $\alpha_5\beta_1$integrin-mediated cell spreading on fibronectin. J Biol Chem 1993; 268:21459-21462.
64. Rozengurt E. Early signals in the mitogenic response. Science 1986; 234:161-166.
65. Rozengurt E. The mitogenic response of cultures 3T3 cells: Integration of early signals and synergistic effects in a unified framework. In: Molecular Mechanisms of Transmembrane Signalling. 1985; 429-452.
66. Charlesworth A, Rozengurt E. Thapsigargin and di-*tert*-butylhydroqunione induce synergistic stimulation of DNA synthesis with phorbol ester and bombesin in Swiss 3T3 cells. J Biol Chem 1994; 269:32528-32535.
67. Brooks SF, Hergert T, Broad S, Rozengurt E. The expression of 80K/MARCKS, a major substrate of PKC, is down-regulated through both PKC-dependent and -independent pathways: effects of bombesin, PDGF and cAMP. J Biol Chem 1992; 267:14212-14218.
68. Erusalimsky JD, Friedberg I, Rozengurt E. Bombesin, diacylglycerols, and phorbol esters rapidly stimulate the phosphorylation of an Mr = 80,000 protein kinase C substrate in permeabilized 3T3 cells. Effect of guanine nucleotides. J Biol Chem 1988; 263:19188-19194.
69. Herget T, Broad S, Rozengurt E. Overexpression of the myristoylated alanine-rich C kinase substrate in Rat 1 cells increases sensitiviy to calmodulin antagonists. Eur J Biochem 1994; 225:549-566.
70. Seufferlein T, Withers DJ, Rozengurt E. Reduced requirement of mitogen-activated protein kinase (MAPK) activity for entry into the S phase of the cell cycle in Swiss 3T3 fibroblasts stimulated by bombesin and insulin. J Biol Chem 1996; 271:21471-21477.
71. Withers DJ, Seufferlein T, Mann D et al. Rapamycin dissociates $p70^{S6K}$ activation from DNA synthesis stimulated by bombesin and insulin in Swiss 3T3 cells. J Biol Chem 1997; 272:2509-2514.
72. Zugaza JL, Sinnett-Smith J, Lint JV, Rozengurt E. Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. EMBO J 1996; 15:6220-6230.
73. Sinnett-Smith J, Zachary I, Valverde AM, Rozengurt E. Bombesin stimulation of p125 focal adhesion kinase tyrosine phosphorylation: role of protein kinase C, Ca^{2+} mobilization and the actin cytoskeleton. J Biol Chem 1993; 268:14261-14268.
74. Zachary I, Sinnett-Smith J, Turner CE, Rozengurt E. Bombesin, vasopressin and endothelin rapidly stimulate tyrosine phosphorylation of the focal adhesion associated protein paxillin in Swiss 3T3 cells. J Biol Chem 1993; 268:22060-22065.
75. Zachary I, Sinnett-Smith J, Rozengurt E. Bombesin, vasopressin and endothelin stimulation of tyrosine phosphorylation in Swiss 3T3 cells: Identification of a novel tyrosine kinase as a major substrate. J Biol Chem 1992; 267:19031-19034.
76. Zachary I, Sinnett-Smith J, Rozengurt E. Stimulation of tyrosine kinase activity in antiphosphotyrosine immune complexes of Swiss 3T3 cell lysates occurs rapidly after addition of bombesin, vasopressin, and endothelin to intact cells. J Biol Chem 1991; 266:24126-24133.
77. Zachary I, Gil J, Lehmann W et al. Bombesin, vasopressin and endothelin rapidly stimulate tyrosine phosphorylation in intact Swiss 3T3 cells. Proc Natl Acad Sci U S A 1991; 88:4577-4581.
78. Nobes C, Hall A. Regulation and function of the Rho subfamily of small GTPases. Curr Opin Gen Dev 1994; 4:77-81.
79. Miura Y, Kikuchi A, Masha T. Regulation of morphology by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI) in Swiss 3T3 cells. J Biol Chem 1993; 268:510-515.
80. Ridley, A. J. Membrane ruffling and signal transduction. BioEssays 1994; 16:312-327.
81. Rankin S, Morii N, Narumiya S, Rozengurt E. Botulinum C3 exoenzyme blocks the tyrosine phosphorylation of $p125^{FAK}$ and paxillin induced by bombesin and endothelin. FEBS Lett 1994; 354:315-319.
82. Seckl MJ, Morii N, Narumiya S, Rozengurt E. Guanosine 5'-3-0-(thio)triphosphate stimulates tyrosine phosphorylation of $p125^{FAK}$ and paxillin in permeabilized Swiss 3T3 cells. J Biol Chem 1995; 270:6984-6990.

83. Seufferlein T, Withers DJ, Mann D, Rozengurt E. Dissociation of Mitogen-activated protein kinase activation from p125 Focal Adhesion Kinase tyrosine phosphorylation in Swiss 3T3 Cells stimulated by bombesin, lysophosphatidic acid, and platelet-derived growth factor. Mol Biol Cell 1996; 7:1865-1875.
84. Moolenaar WH. LPA: A novel lipid mediator with diverse biological actions. Trends Cell Biol 1994; 4:213-219.
85. Moolenaar WH, Jalink K, Van Corven EJ. Lysophosphatidic acid: A bioactive phospholipid with growth factor-like properties. Rev Physiol Biochem Pharmacol 1992; 119:48-65.
86. Seufferlein T, Rozengurt E. Lysophosphatidic acid stimulates tyrosine phosphorylation of focal adhesion kinase, paxillin and p130. J Biol Chem 1994; 269:9345-9351.
87. Kumagai N, Morii N, Fujisawa K et al. ADP-ribosylation of rho p21 inhibits lysophosphatidic acid-induced protein tyrosine phosphorylation and phosphatidylinositol 3-kinase activation in cultured Swiss 3T3 cells. J Biol Chem 1993; 238:24535-24538.
88. Seufferlein T, Rozengurt E. Sphingosylphosphorylcholine activation of Mitogen-activated protein kinase in Swiss 3T3 cells requires protein kinase C and pertussis toxin-sensitive G protein. J Biol Chem 1995; 270:24334-24342.
89. Seufferlein T, Rozengurt E. Sphingosylphosphorylcholine rapidly induces tyrosine phosphorylation of $p125^{FAK}$ and paxillin, causing rearrangement of the actin cytoskeleton and focal contact assembly. J Biol Chem 1995; 270:24343-24351.
90. Aktories K, Braun U, Habermann B, Rosener S. Botulinum ADP-ribosyltransferase C3. In: Moss J, Vaughn M, eds. ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction. Washington, DC: American Society for Microbiology, 1990:97-115.
91. Just I, Wilm M, Selzer J et al. The enterotoxin from Clostridium difficile (ToxA) monoglucosylates the Rho proteins. J Biol Chem 1995; 270:13932-6.
92. Just I, Selzer J, Wilm M, von-Eichel-Streiber C, Mann M, Aktories K. Glucosylation of Rho proteins by Clostridium difficile toxin B. Nature 1995; 375:500-3.
93. Staddon JM, Barker CJ, Murphy AC et al. Pasteurella multocida toxin, a potent mitogen, increases inositol 1,4,5-trisphosphate and mobilizes Ca^{2+} in Swiss 3T3 cells. J Biol Chem 1991; 266:4840-7.
94. Rozengurt E, Higgins T, Chanter N et al. Pasteurella multocida toxin: Potent mitogen for cultured fibroblasts. Proc Natl Acad Sci USA 1990; 87:123-127.
95. Murphy AC, Rozengurt E. *Pasteurella multocida* toxin selectively facilitates phosphatidylinositol 4,5-bisphosphate hydrolysis by bombesin, vasopressin and endothelin. J Biol Chem 1992; 267:25296-25303.
96. Lacerda HM, Lax AJ, Rozengurt E. Pasteurella multocida toxin, a potent Intracellular acting mitogen, induces $p125^{FAK}$ and paxillin tyrosine phosphorylation, actin stress fiber formation, and focal contact assembly in Swiss 3T3 cells. J Biol Chem 1996; 271:439-445.
97. Falbo V, Pace T, Picci L et al. Isolation and nucleotide sequence of the gene encoding cytotoxic necrotizing factor 1 of *Escherichia coli*. Infection and Immunity 1993; 61:4909-4914.
98. Horiguchi Y, Nakai T, Kume K. Purification and characterization of *Bordetella bronchiseptica* dermonecrotic toxin. Microbial Pathogen 1989; 6:361-368.
99. Oswald E, Sugai M, Labigne A et al. Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. Proc Natl Acad Sci USA 1994; 91:3814-3818.
100. Horiguchi Y, Senda T, Sugimoto et al. *Bordetella bronchiseptica* dermonecrotizing toxin stimulates assembly of actin stress fibers and focal adhesions by modifying the small GTP-binding protein rho. J Cell Sci 1995; 108:3243-3251.
101. Schmidt G, Sehr P, Wilm M et al. Gln 63 of Rho is deamidated by *Escherichia coli* cytotoxic necrotizing factor-1. Nature 1997; 387:725-729.
102. Flatau G, Lemichez E, Gauthier M et al. Toxin-induced activation of G protein p 21 Rho by deamination of glutamine. Nature 1997; 387:729-733.
103. Caprioli A, Falbo V, Roda LG et al. Partial purification and characterization of an *Escherichia coli* toxic factor that induces morphological cell alterations. Infect Immun 1983; 39:1300-1306.

104. Caprioli A, Donelli G, Falbo V et al. A cell division-active protein from *E. coli*. Biochem Biophys Res Comm 1984; 118:587-593.
105. Horiguchi Y, Sugimoto N, Matsuda M. Stimulation of DNA synthesis in osteoblast-like MC3T3-E1 cells by *Bordetella bronchiseptica* dermonecrotic toxin. Infect Immun 1993; 61:3611-3615.
106. Rankin S, Rozengurt E. Platelet-derived growth factor modulation of focal adhesion kinase ($p125^{FAK}$) and paxillin tyrosine phosphorylation in Swiss 3T3 cells. Bell-shaped dose response and cross-talk with bombesin. J Biol Chem 1994; 269:704-710.
107. Rankin S, Hooshmand-Rad R, Claesson-Welsh L, Rozengurt E. Requirement for phosphatidylinositol 3'-kinase activity in platelet-derived growth factor-stimulated tyrosine phosphorylation of p125 focal adhesion kinase and paxillin. J Biol Chem 1996; 271:7829-7834.
108. Kumagai N, Morii N, Fujisawa K et al. Lysophosphatidic acid induces tyrosine phosphorylation and activation of MAP-kinase and focal adhesion kinase in cultured Swiss 3T3 cells. FEBS Lett 1993; 329:273-276.
109. Hordijk PL, Verlaan I, van Corven EJ, Moolenaar WH. Protein tyrosine phosphorylation induced by lysophosphatidic acid in Rat-1 fibroblasts. Evidence that phosphorylation of map kinase is mediated by the G_i-$p21^{ras}$ pathway. J Biol Chem 1994; 269:645-651.
110. Seufferlein T, Rozengurt E. Sphingosine induces $p125^{FAK}$ and paxillin tyrosine phosphorylation, actin stress fiber formation and focal contact assembly in Swiss 3T3 cells. J Biol Chem 1994; 269:27610-27617.
111. Imamoto A, Soriano P. Disruption of csk gene, encoding a negative regulator of Src family tyrosine kinases, leads to neural tube defects and embryonic lethality in mice. Cell 1993; 73:1117-1124.
112. Nada S, Yagi T, Takeda H et al. Constitutive activation of Src family kinases in embryos that lack Csk. Cell 1993; 73:1125-1135.
113. Roussel RR, Brodeur SR, Shalloway D, Laudano AP. Selective binding of activated $pp60^{c\text{-}src}$ by an immobilized synthetic phosphopeptide modeled on the carboxyl terminus of $pp60^{c\text{-}src}$. Proc Natl Acad Sci USA 1991; 88:10696-10700.
114. Liu X, Brodeur SR, Gish G et al. Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. Oncogene 1993; 8:1119-1126.
115. Kaplan KB, Swedlow JR, Morgan DO, Varmus HE. c-Src enhances the spreading of src-/- fibroblasts on fibronectin by a kinase-independent mechanism. Genes and Develop 1995; 9:1505-1517.
116. Brady-Kalnay SM, Tonks N. Protein tyrosine phosphatases: from structure to function. Trends Cell Biol 1994; 4:73-76.
117. Courtneidge SA. Activation of $pp60^{c\text{-}src}$ kinase by middle-T antigen binding or by dephosphorylation. EMBO J 1985; 4:1471-1477.
118. Parsons JT, Parsons SJ. Src family protein tyrosine kinases: cooperating with growth factor and adhesion signaling pathways. Curr Opin Cell Biol 1997; 9:187-192.
119. Schaller MD, Parsons JT. Focal adhesion kinase and associated proteins. Curr Opin Cell Biol 1994; 6:705-710.
120. Rodríguez-Fernández JL, Rozengurt E. Bombesin, bradykinin, vasopressin, and phorbol esters rapidly and transiently activate Src family tyrosine kinases in Swiss 3T3 cells. J Biol Chem 1996; 271:27895-27901.
121. Lach EB, Broad S, Rozengurt E. Mitogenic signaling by transfected neuromedin B receptors in Rat-1 cells. Cell Growth & Differ 1995; 6:1427-1435.
122. Charlesworth A, Broad S, Rozengurt E. The bombesin/GRP receptor transfected into Rat-1 fibroblasts couples to phospholipase C activation, tyrosine phosphorylation of $p125^{FAK}$ and paxillin and cell proliferation. Oncogene 1996; 12:1337-1345.
123. Buhl AM, Johnson NL, Dhanasekaran N, Johnson GL. $G\alpha_{12}$ and $G\alpha_{13}$ stimulate Rho-dependent stress fibre formation and focal adhesion assembly. J Biol Chem 1995; 270:24631-24634.
124. Amano M, Mukai H, Ono Y et al. Identification of a putative target for Rho as the serine-threonine protein kinase N. Science 1996; 271:648-650.

125. Matsui T, Amano M, Yamamoto T et al. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. EMBO J 1996; 15:2208-2216.
126. Fujisawa K, Fujita A, Ishizaki T et al. Identification of Rho-binding domain of p160ROCK, a Rho-associated coiled-coil containing protein kinase. J Biol Chem 1996; 271:23022-23028.
127. Watanabe G, Saito Y, Madaule P et al. Protein kinase N (PKN) and PKN-related protein Rhophilin as targets of small GTPase Rho. Science 1996; 271:645-650.
128. Tapon N, Hall A. Rho, Rac and CDC42 CDC42 GTPases regulate the organization of the actin cytosketon. Curr Opin Cell Bio 1997; 9:86-92.
129. Amano M, Chihara K, Kimura K et al. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 1997; 275:1308-1311.
130. Kimura K, Ito M, Amano M et al. Regulation of myosin phosphatase by Rho and Rho-asssociated kinase (Rho-kinase). Science 1996; 273:245-248.
131. Kureishi Y, Kobayashi S, Amano M et al. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. J Biol Chem 1997; 272:12257-12260.
132. Chrzanowska-Wodnicka M, Burridge K. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J Cell Biol 1996; 133:1403-1415.
133. Burridge K, Chrzanowska-Wodnicka M, Zhong C. Focal adhesions assembly. Trends Cell Biol 1997; 7:342-347.
134. Heldin C-H. Structural and functional studies on platelet-derived growth factor. EMBO J 1992; 11:4251-4259.
135. Claesson-Welsh L. Platelet-derived growth factor receptor signals. J Biol Chem 1994; 269:32023-32026.
136. Abedi H, Dawes KE, Zachary I. Differential effects of platelet-derived growth factor BB on p125FAK focal adhesion kinase and paxillin tyrosine phosphorylation and on cell migration in rabbit aortic vascular smooth muscle cells and Swiss 3T3 fibroblasts. J Biol Chem 1995; 270:11367-11376.
137. Downes CP, Carter AN. Phosphoinositide 3-kinase: a new effector in signal transduction? Cell Signal 1991; 3:501-503.
138. Stephens LR, Jackson TR, Hawkins PT. Agonist-stimulated synthesis of phosphoinositol (3,4,5)-trisphosphate: a new intracellular signalling system? Biochem Biophys Acta 1993; 1179:27-75.
139. Auger KR, Seruian LA, Soltoff SP et al. PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell 1989; 57:167-175.
140. Stephens LR, Hughes KT, Irvine RF. Pathway of phosphatidyl inositol (3,4,5)-trisphosphate synthesis in activated neutrophils. Nature 1991; 351:33-39.
141. Hawkins PT, Jackson TR, Stephens LR. Platelet-derived growth factor stimulates synthesis of PtdIns (3,4,5)P_3 by activating PtdIns (4,5)P_2 3-OH kinase. Nature 1992; 358:157-159.
142. Wennström S, Siegbahn A, Yokote K et al. Membrane ruffling and chemotaxis transduced by the PDGF b-receptor require the binding site for phosphatidylinositol 3' kinase. Oncogene 1994; 9:651-660.
143. Nobes CD, Hawkins P, Stephens L, Hall A. Activation of the small GTP-binding proteins rho and rac by growth factor receptors. J Cell Sci 1995; 108:225-233.
144. Wennström S, Hawkins P, Cooke F et al. Activation of phosphoinositide 3-kinase is required for PDGF-stimulated membrane ruffling. Curr Biol 1994; 4:385-393.
145. Hawkins PT, Eguinoa A, Qui R-G et al. PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. Curr Biol 1995; 5:393-403.
146. Tolias KF, Cantley LC, Carpenter CL. Rho family GTPases bind to phosphoinositide kinases. J Biol Chem 1995; 270:17656-17659.
147. McGlade J, Brunkhorst B, Anderson D et al. The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. EMBO J 1993; 12:3073-3081.
148. Fincham VJ, Wyke JA, Frame MC. v-Src induced degradation of focal adhesion kinase during morphological transformation of chicken embryo fibroblasts. Oncogene 1995; 10:2247-2252.

149. Ilic D, Kanazawa S, Furuta Y et al. Impaiment of mobility in endodermal cells by FAK deficiency. Exp Cell Res 1996; 222:298-303.
150. Gates RE, King LEJ, Hanks SK, Nanney LB. Potential role for focal adhesion kinase in migrating and proliferating keratinocytes near epidermal wounds and in culture. Cell Growth Differ 1994; 5:891-899.
151. Cary LA, Chang JF, Guan J-L. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. J Cell Science 1996; 109:1787-1794.
152. Malik RK, Parsons JT. Integrin-mediated signaling in normal and malignant cells: a role of protein tyrosine kinases. Biochim Biophys Acta 1996; 1287:73-76.
153. Withers BE, Hanks SE, Fry DW. Correlation between the expression, phosphotyrosine content and enzymatic activity of focal adhesion kinase, pp125^{FAK}, in tumor and nontransformed cells. Cancer Biochem Biophys 1996; 15:127-139.
154. Owens LV, Xu L-H, Craven RJ et al. Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. Cancer Res 1995; 55:2752-2755.
155. Weiner TM, Liu ET, Craven RJ, Cance WG. Expression of focal adhesion kinase and invasive cancer. Lancet 1993; 342:1024-1025.
156. Akasaka T, van-Leeuwen RL, Yoshinaga G Jr, MCM, Byers HR. Focal adhesion kinase (p125^{FAK}) expression correlates with motility of human melanoma cell lines. J Inv Dermatol 1995; 105:104-108.
157. Romer LH, McLean N, Turner CE, Burridge K. Tyrosine kinase activity, cytoskeletal organization, and motility in human vascular endothelial cells. Mol Biol Cell 1994; 5:349-361.
158. Gilmore AP, Romer LH. Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. Mol Biol Cell 1996; 7:1209-1224.
159. Frisch SM, Vuori K, Ruoslahti E, Chan-Hui P-Y. Control of adhesion-dependent cell survival by focal adhesion kinase. J Cell Biol 1996; 134:793-799.
160. Xu L-H, Owens LW, Sturge GC et al. Attenuation of the expression of the focal adhesion kinase induces apoptosis in tumor cells. Cell Growth & Differ 1996; 7:413-418.
161. Hungerford JE, Compton MC, Matter ML, Hoffstrom BG, Otey CA. Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. J Cell Biol 1996; 135:1383-1390.
162. Auvinen M, Paasinen-Sohns A, Hirai H, Andersson LC, Holtta E. Ornithine decarboxylase and ras-induced cell transformations: reversal by protein tyrosine kinase inhibitors and role of pp130^{CAS}. Mol Cell Biol 1995; 15:6513-6525.

CHAPTER 7

Cytoskeletal Plaque Proteins: Their Role in the Regulation of Tumorigenesis

Avri Ben-Ze'ev

Introduction

Cell adhesion to neighboring cells and to the extracellular matrix (ECM) plays important roles in the regulation of cell motility, growth, differentiation and survival.[1-4] The molecular interactions at cell adhesion sites include transmembrane integrin-type receptors that link cells to the ECM, and cadherin receptors at cell-cell contact sites that link cells to each other by homophilic interactions (Figs. 7.1 and 7.2). These receptors are associated with submembranal plaque proteins that bridge between the cytoskeleton and the transmembrane adhesion receptors.[3] Major junctional plaque proteins of adherens-type junctions are vinculin, α-actinin and the catenins (α-, β- and γ-catenin, or plakoglobin). In addition to these structural components, recent studies have demonstrated the localization in the submembranal plaque area of a large number of regulatory molecules, known to be involved in signal transduction (Figs. 7.1 and 7.2; for reviews see refs. 2-5). The submembranal plaque area is now viewed not only as a structural link that mediates cell adhesion, but also as an important component in the control of signal transduction that regulates cell behavior.

Tumor cells are often characterized by altered adhesion, disorganized cytoskeletal architecture and impaired adhesion-mediated signaling.[2,6] Many cancer cells are "anchorage independent" and less susceptible to cell density-dependent inhibition of growth. Our previous studies have demonstrated that both the organization and expression of cytoskeletal plaque proteins is regulated during growth activation, differentiation and cell transformation.[1] Here, I summarize our studies on the targeted changes in the expression of the adhesion plaque proteins vinculin and α-actinin, and the cell-cell junctional plaque proteins plakoglobin and β-catenin, to define their role in cell physiology, with special emphasis on their effect on the tumorigenic ability of cells.

Regulation of Vinculin and α-Actinin Expression after Growth Activation

Vinculin and α-actinin are major focal adhesion plaque proteins that link actin stress fibers to areas of cell adhesion to the ECM (Fig. 7.1). While these proteins are among the most abundant constitutive cellular proteins, their expression is extensively modulated in response to growth stimulation of quiescent 3T3 cells by serum factors. When such cells are

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

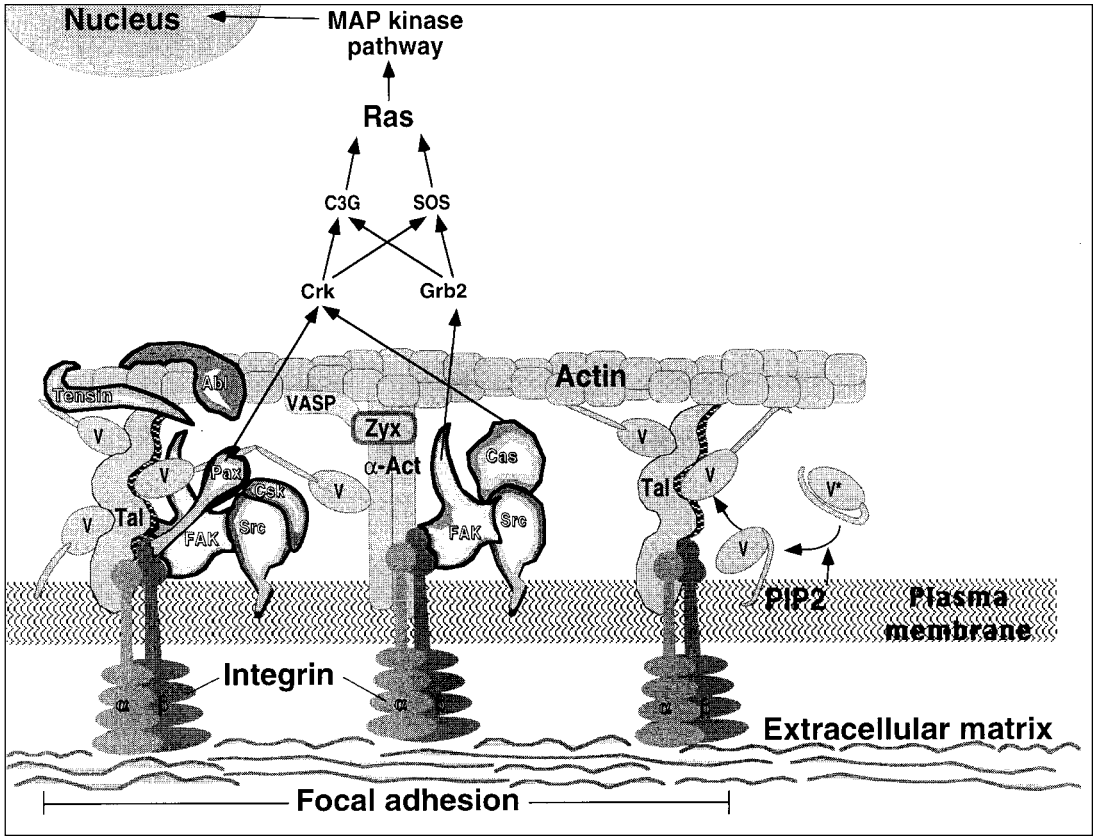


Fig. 7.1. A scheme depicting the organization of structural and signaling molecules at focal adhesions. Actin filaments are linked to the extracellular matrix via transmembrane integrin receptors by talin (Tal) and/or α-actinin (α-Act). Vinculin (V) has talin- and α-actinin-binding sites. Vinculin can fold into a conformation where its binding sites are masked (V*). Zyxin (Zyx) is an α-actinin-binding protein that can bind to VASP which binds to actin filaments. The assembly of focal adhesions involves PIP_2 that stimulates the transition of vinculin from the nonactive conformation (V*) to one that binds talin- and actin. Protein tyrosine kinases localized at focal adhesions are: focal adhesion kinase (FAK) that binds the cytoplasmic domain of β-integrin, Src and Src-family kinases, Csk (a negative regulator of Src kinases), and Abl (binds actin). Other components of focal adhesions, including paxillin (Pax), tensin, and $p130^{Cas}$ (p130), are common substrates for tyrosine phosphorylation. The adhesion of cells to the extracellular matrix induces tyrosine phosphorylation of FAK, increases its tyrosine kinase activity and induces tyrosine phosphorylation of paxillin, tensin and $p130^{Cas}$. Cell adhesion can activate the Ras-MAP kinase signaling pathway: Tyrosine phosphorylated FAK binds to the SH2/SH3 adaptor protein Grb2, tyrosine phosphorylated paxillin, that can bind Crk—another SH2/SH3 adapter protein. $p130^{Cas}$ can bind Crk and Grb2. The SH3 domains of both Crk and Grb2 bind to the guanine nucleotide exchange factors SOS and C3G that activates Ras and stimulates the MAP kinase pathway leading to nuclear transmission of the adhesive signal. Modified from Ben-Ze'ev and Bershadsky.[5]

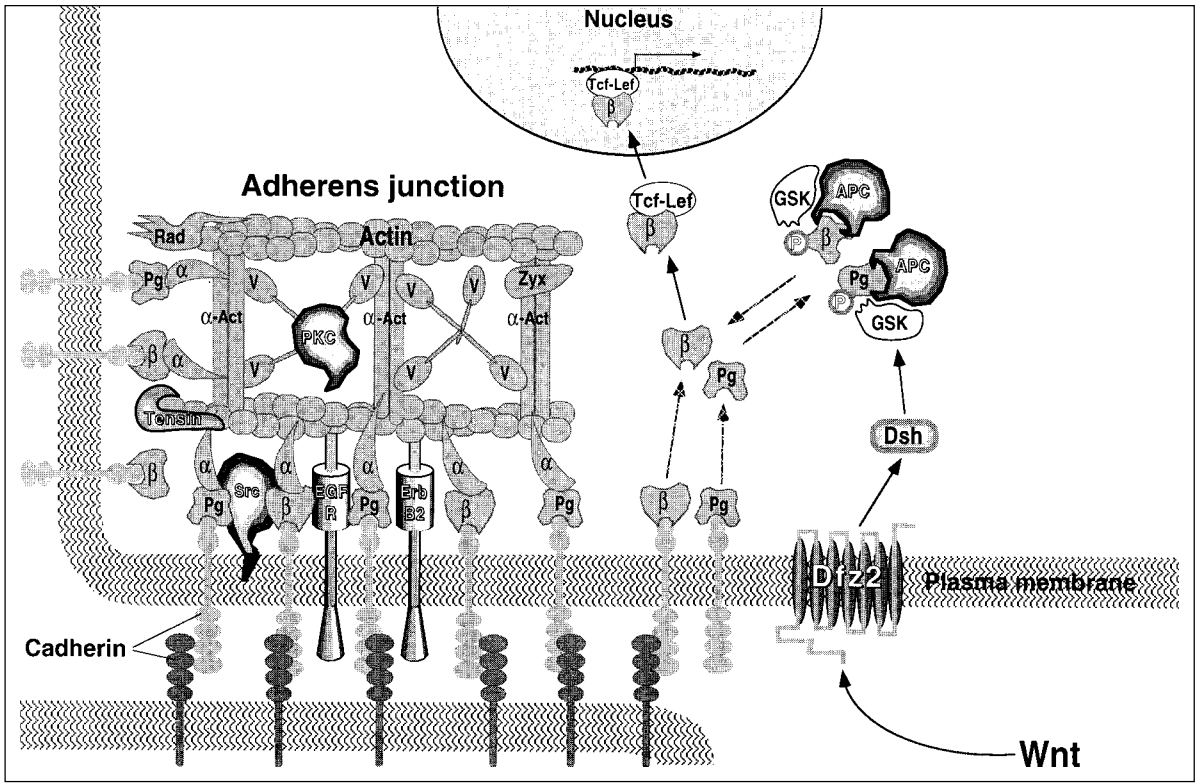


Fig. 7.2. A model for the structure and signaling by cell-cell adherens junctions. Signals generated by cell-cell adhesion via cadherins and their assembly with catenins can modulate the level of free β-catenin (β), or plakoglobin (Pg) that is available for interaction with the APC tumor suppressor molecule, or with transcription factors of the LEF-1 family (Tcf-Lef). The complex between LEF-1 and β-catenin, or plakoglobin, can translocate into the nucleus and directly bind to the 5' end of target genes to regulate their expression. Signaling that involves β-catenin and plakoglobin can also be elicited by the Wnt pathway, that includes the Wnt receptor (Dfz2), Dsh, and glycogen synthase kinase (GSK). The association between APC and β-catenin and GSK, regulates the stability of β-catenin (by phosphorylation, (P)), and may control its translocation into the nucleus. An association between receptor tyrosine kinases such as the EGF receptor (EGFR) and Erb-B2-receptor with β-catenin and plakoglobin may also affect signaling. Note the presence of protein kinases such as Src and PKC in the junctional plaque. α, α-catenin; Rad, radixin; Zyx, zyxin; α-Act, α-actinin; V, vinculin; PKC, protein kinase C. Modified from Ben-Ze'ev and Bershadsky.[5]

treated with serum, a major transient increase in the level of vinculin synthesis is observed.[7,8] The synthesis of α-actinin is also induced by serum, but follows a different kinetics of induction.[9] These changes in vinculin and α-actinin synthesis are accompanied by parallel changes in the levels of the corresponding mRNAs. A rapid, yet transient, induction of vinculin gene transcription precedes the increase in vinculin mRNA and protein synthesis in serum stimulated cells,[7,8] and involves the serum responsive elements at the 5'-end of the vinculin gene.[10] Post-transcriptional regulation of vinculin expression is also implicated in the induction of vinculin expression. This is inferred from studies showing that in 3T3 cells stably expressing a transfected vinculin under a constitutive viral promoter, there is a similar yet more moderate elevation in the synthesis of the transfected vinculin after serum stimulation.[11]

The possible physiological relevance of these changes in vinculin and α-actinin expression in response to growth stimulation was examined in regenerating liver, a system widely used as an in vivo model for studying gene expression at early stages of induced cell proliferation. Northern blots of RNA isolated at different times after two-thirds hepatectomy show an early, transient elevation in vinculin RNA level and latter, in the abundance of α-actinin RNA which persists into the S-phase.[9] These regulated changes in the expression of vinculin and α-actinin expression may be required for the cellular response to growth stimulation.

The Effects of Vinculin and α-Actinin Overexpression

To investigate the role of these changes in vinculin and α-actinin expression on cell behavior, 3T3 clones stably expressing transfected vinculin or α-actinin were isolated. In stably transfected 3T3 cells the transgenes are expressed at levels that are usually less than 50% of the endogenous protein level. This is expected since 3T3 cells cultured on plastic substrate express very high levels of these adhesion plaque proteins, unlike fibroblasts in vivo, or fibroblasts cultured on an ECM. Nevertheless, overexpression of vinculin by only 20% is sufficient to confer phenotypic and functional changes on 3T3 cells. Such cells assemble more abundant stress fibers, terminating in larger vinculin-containing plaques, than control 3T3 cells.[11] Moreover, cells overexpressing either vinculin or α-actinin display a decrease in cell motility, resulting in the formation of shorter phagokinetic tracks on colloidal gold-coated substrates, and a reduced ability to close an artificial wound created in a confluent monolayer of cells.[12,13] These results suggest that moderate elevations in vinculin or α-actinin level may have a major impact on cell morphology, cytoskeletal assembly, and the adhesive and motile abilities of the cells.

The Effects of Suppressing Vinculin and α-Actinin Levels and Targeted Inactivation of the Vinculin Gene

To study the effect of forced reduction in vinculin and α-actinin levels, 3T3 cells were transfected with antisense cDNA constructs of these genes and clones stably expressing reduced levels of either vinculin or α-actinin were isolated. Antisense vinculin cDNA transfection generated clones displaying vinculin levels equal to only 10%-30% of the control levels.[14] Such cells show a marked change in morphology, with reduced capacity to spread on the substrate, and small vinculin-containing plaques at the cell periphery. Moreover, the suppression in vinculin expression results in enhanced cell motility.[14] These changes in the morphological and motile properties of cells correlate with the degree of suppression in vinculin expression. Only cells displaying over 70% reduction in vinculin levels show the increase in motility and reduced spreading on the substrate. Cells displaying a more moderate decrease in vinculin, and revertants of clones originally showing effective vinculin suppression, revert to the control motile characteristics.[14]

Vinculin expression was completely eliminated in the F9 embryonal carcinoma and embryonal stem (ES) cells by targeted disruption of the vinculin gene after homologous recombination.[15] Vinculin deficient F9 cells show unchanged levels of α-actinin, paxillin and talin, but an enhanced assembly of these molecules at focal adhesions.[16] Vinculin-null cells also display a significantly reduced ability to spread on the substrate, and a slower rate of initial adhesion on ECM components,[16] reminiscent of the phenotype of antisense vinculin transfected 3T3 cells.[14] Moreover, vinculin-deficient F9 cells have increased locomotory capacity, in agreement with the results obtained with antisense vinculin transfection. This increase in the motility of vinculin-null F9 cells is also manifested when these cells are induced to differentiate into the more motile parietal endodermal (PE) cells.[15] The vinculin null F9 cells can be induced to express the differentiation markers in the absence of vinculin,[15] and their ability to form stress fibers and assemble focal adhesions containing α-actinin, paxillin, talin and phosphotyrosinated components is not impaired.[16] Taken together, these findings suggest that there are multiple molecular mechanisms for focal adhesion formation in the absence of vinculin, including one that is based on α-actinin bridging between integrin receptors and actin (Fig. 7.1).

Vinculin and α-Actinin Expression in Transformed Cells

Suppression of vinculin expression decreases the rate of cell adhesion and spreading, and also confers anchorage independent growth on 3T3 cells.[14] Moreover, in antisense α-actinin transfected 3T3 clones, when α-actinin levels are reduced by 40%-75%, the cells are tumorigenic when injected into nude mice.[13] The appearance of tumors correlates with the level of α-actinin suppression, and cells expressing lower levels of the protein cause faster developing tumors. Furthermore, revertants of such clones that regain control levels of α-actinin become nontumorigenic. These results support the notion that vinculin and α-actinin may have a tumor suppressive activity. In agreement with this view are studies showing that SV40-transformed 3T3 cells (SVT2) display diminished levels of vinculin and α-actinin,[17,18] and vinculin is absent in a highly metastatic adenocarcinoma cell line (ASML) that expresses α-actinin and talin.[19] Reduced expression of various junctional plaque proteins is thus characteristic of certain tumor cells and may play a role in the tumorigenic ability of these cells.

Transfection with Vinculin and α-Actinin Can Suppress Tumorigenicity

To examine the effect of restoring vinculin and α-actinin levels in transformed cells on their tumorigenic phenotype, 3T3 fibroblasts transformed by SV40 (SVT2) and the highly metastatic ASML epithelial cells were transfected with either full length vinculin or α-actinin. Clones stably expressing different levels of these transgenes were isolated. High levels of vinculin expression in SVT2 results in cells with more abundant stress fibers and larger vinculin-positive focal adhesions.[18] The tumorigenicity of cells overexpressing vinculin is dramatically affected, and cells expressing the transfected vinculin at levels similar to that in nontransformed 3T3 cells, completely lose their tumorigenic ability in syngeneic and nude mice.[18] Similarly, the overexpression of α-actinin in SVT2 cells, results in suppression of their tumorigenic capacity, that correlates with the level of the transfected α-actinin in the different clones.[17] The expression of the viral (SV40) T-antigen in SVT2 cells however, and that of mutant p53 molecules accumulating in these cells, is not altered in the vinculin- and α-actinin-transfected clones. This implies that these junctional molecules use alternative route(s) to influence the tumorigenic ability of cells.

The effect of vinculin overexpression on the metastatic spread of tumor cells was examined in a highly metastatic adenocarcinoma (ASML) cell line that does not express

vinculin. Expression of high levels of the vinculin transgene suppresses the malignant metastatic ability of these cells, while low levels of vinculin only partially alter the number of lung metastases that form with these ASML clones.[18] Vinculin and α-actinin may thus act as potential suppressors of the tumorigenic ability of cancer cells.

Plakoglobin and β-Catenin: Junctional Molecules Involved in Signaling and Regulation of Tumorigenesis

In addition to their role in cell-ECM adhesion, junctional plaque proteins are also found in cell-cell adhesion sites (Fig. 7.2).[3] The most direct effect of cell-cell adhesion is on morphogenesis, i.e., the assembly of individual cells into highly ordered tissues through cell-cell adhesion junctions.[4] These interactions among cells involve transmembrane cell adhesion receptors of the cadherin family that link cells to each other.[20] Constitutive expression and function of cadherin receptors are essential for the development and maintenance of epithelial cell interaction. Targeted genetic inactivation of E-cadherin in mice is embryonic lethal with the embryonal cells dissociating an failing to form the trophectoderm.[21] Effective cell-cell adhesion requires, in addition, an association of the transmembrane cadherin receptors with the cytoskeleton that is mediated by junctional plaque proteins including plakoglobin (γ-catenin), β-catenin and α-catenin (Fig. 7.2, and refs. 2-4). α-Catenin, that has structural similarity to vinculin, has actin binding properties, suggesting that it links the catenin complex to the actin-cytoskeleton.[22] Restoration of α-catenin levels in lung carcinoma cells restored cell-cell adhesion and the assembly of various intercellular junctions.[23] In prostate cancer cells and in a human ovarian carcinoma cell line, wt α-catenin expression results in the induction of E-cadherin function, cell-cell adhesion and suppression of tumorigenesis in nude mice.[24,25]

In addition to their function in cell adhesion, β-catenin and plakoglobin are highly homologous to Drosophila armadillo that is also found in adherens junctions of flies.[26] Armadillo in Drosophila and β-catenin/plakoglobin in Xenopus have been shown to play a role in the transduction of transmembrane signals initiated by the extracellular glycoprotein wg/Wnt that regulates cell growth, differentiation and fate.[4,27-29] Activation of this pathway results in the elevation of β-catenin levels and its nuclear localization in a complex with the TCF/LEF family of transcription factors[30-32] (Fig. 7.2), suggesting that β-catenin may have a role in regulating gene expression by transactivating target genes.[33,34] In the absence of wg/Wnt signaling, excess β-catenin is degraded in mammalian cells by a process involving the adenomatous polyposis coli (APC) tumor suppressor protein[35] and the ubiquitin-proteasome degrading pathway.[36] Mutations in the APC gene that constitute the major genetic defect in inherited colon cancer and certain melanoma, or mutations in the amino terminus of β-catenin, both result in the accumulation of β-catenin,[37-39] and most probably cause inappropriate activation of target genes by the β-catenin-LEF/TCF complex.[40-42]

In contrast, the involvement of plakoglobin in suppressing tumorigenesis is inferred from studies showing loss of heterozygosity of the plakoglobin gene in certain types of tumors,[43] its reduction in several tumor cell types,[44-46] and by demonstrating that plakoglobin overexpression can suppress the tumorigenicity of mouse and human cells while localized in the nuclei of such cells.[46] The regulation of β-catenin and plakoglobin level may therefore be a key element in their nuclear localization and signal transduction.

Interestingly, overexpression of plakoglobin leads to a decrease in β-catenin level, and plakoglobin competes with β-catenin for N-cadherin binding, thus directing the displaced β-catenin molecules for degradation by the ubiquitin-proteasome system.[47] Inhibition of the proteasome degradation system in these cells leads to the accumulation of both catenins in the nucleus.[47]

Wnt-induced signaling during development includes the accumulation of β-catenin in the cell, but artificially elevated cadherin expression in Xenopus can antagonize the propagation of the Wnt signal by sequestering "free pools" of β-catenin into a complex with cadherin and thus probably limiting its function in extra-junctional signaling.[39,48,49] Plakoglobin therefore, may serve as an additional regulator of β-catenin level acting upstream of the APC step by competing on the cadherin binding site and thus releasing β-catenin and exposing it to the degradation fate.

The accumulation of β-catenin and its nuclear translocation in complex with transcription factors, its aberrant effect on the transcription of genes during development of colon cancer and melanoma,[40-42] as well as the ability of plakoglobin to influence the tumorigenicity of cells when overexpressed and localized in the nucleus[46] highlight the importance of mechanisms that regulate the level of β-catenin in the cell. Interestingly, in tumor cells where plakoglobin overexpression resulted in suppression of the tumorigenic ability,[46] the level of β-catenin was reduced.[47] This may indicate that plakoglobin confers a tumor suppressive phenotype in these cells by decreasing the level of β-catenin whose abnormally increased level can be oncogenic.[40-42,50]

The challenge for future studies is to determine whether elevated β-catenin can confer tumorigenicity on nontransformed cells, the physiological conditions that are associated with the regulated expression and translocation of β-catenin and plakoglobin into the nuclei of mammalian cells, and the target genes whose expression is modulated by transactivation involving complexes that contain these junctional plaque proteins.

Conclusions

Our earlier studies have shown modulations in both the organization and expression of cytoskeletal and junctional plaque proteins during growth activation, differentiation and cell transformation. The studies summarized here strongly imply that such changes in expression may have important long-term effects on cell behavior. This is demonstrated by the studies showing that moderate overexpression of vinculin or α-actinin dramatically influence cell motility, and restoration to control levels of expression of these molecules in transformed cells effectively suppresses their tumorigenicity. Recent studies on the dual role of plakoglobin and β-catenin in both adhesion and signaling by affecting the transcription of target genes, provide another exciting direction for future studies on deciphering the molecular mechanisms by which adhesion mediated signaling is regulating tumorigenesis.

Acknowledgment

I would like to express my gratitude to all my colleagues and collaborators who have participated in the studies summarized in this manuscript. To JL Rodríguez Fernández, U Glück, I Simcha, D Salomon, T Volberg, B Geiger, M Zöller, R Pankov, JL Coll, E Adamson, S Guha Roy, P. Sacco, KR Johnson and MJ Wheelock. These studies were supported by grants from the USA-Israel Binational Fund, by a grant from the Leo and Julia Forchheimer Center for Molecular Genetics, by the German Israeli Foundation for Scientific Research and Development, and the MOSA-BMBF, Joint German Israeli Research Program. A. B-Z holds the Lunenfeld-Kunin Professorial Chair in Genetics and Cell Biology.

References

1. Ben-Ze'ev A. Animal cell shape changes and gene expression. BioEssays 1991; 13:207-212.
2. Ben-Ze'ev A. Cytoskeletal and adhesion proteins as tumor suppressors. Curr Opin Cell Biol 1997; 9:99-108.
3. Geiger B, Yehuda-Levenberg S, Bershadsky AD. Molecular interactions in the submembrane plaque of cell-cell and cell-matrix adhesions. Acta Anat 1995; 154:46-62.

4. Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 1996; 84:345-357.
5. Ben-Ze'ev A, Bershadsky AD. The role of the cytoskeleton in adhesion-mediated signaling and gene expression. Adv Mol Cell Biol 1997; 24:125-163.
6. Ben-Ze'ev A. The cytoskeleton in cancer cells. Biochim Biophys Acta 1985; 780:197-212.
7. Ben-Ze'ev A, Reiss R, Bendori R et al. Transient induction of vinculin gene expression in 3T3 fibroblasts stimulated by serum growth factors. Cell Regul 1990; 1:621-636.
8. Bellas RE, Bendori R, Farmer SR. Epidermal growth factor activation of vinculin and β-integrin gene transcription in quiescent Swiss 3T3 cells. J Biol Chem 1991; 266: 12008-12014.
9. Glück U, Rodríguez Fernández JL, Pankov R et al. Regulation of adherens junction protein expression in growth-activated 3T3 cells and in regenerating liver. Exp Cell Res 1992; 202:477-486.
10. Moiseyeva EP, Weller PA, Zhidkova NI et al. Organization of the human gene encoding the cytoskeletal protein vinculin and the sequence of the vinculin promoter. J Biol Chem 1993; 268:4318-4325.
11. Geiger B, Ayalon O, Ginsberg D et al. Cytoplasmic control of cell adhesion. Cold Spring Harb Symp Quant Biol 1992; 57:631-642.
12. Rodríguez Fernández JL, Geiger B, Salomon D et al. Overexpression of vinculin suppresses cell motility in Balb/c 3T3 cells. Cell Motil Cytoskel 1992; 22:127-134.
13. Glück U, Ben-Ze'ev A. Modulation of α-actinin levels affects cell motility and confers tumorigenicity on 3T3 cells. J Cell Sci 1994; 107:1773-1782.
14. Rodríguez Fernández JL, Geiger B, Salomon D et al. Suppression of vinculin expression by antisense transfection confers changes in cell morphology, motility and anchorage-dependent growth of 3T3 cells. J Cell Biol 1993; 122:1285-1294.
15. Coll J-L, Ben-Ze'ev A, Ezzell RM et al. Targeted disruption of vinculin genes in F9 and ES cells changes cell morphology, adhesion and locomotion. Proc Natl Acad Sci USA 1995; 92:9161-9165.
16. Volberg T, Geiger B, Kam Z et al. Focal adhesion formation by F9 embryonal carcinoma cells after vinculin gene disruption. J Cell Sci 1995; 108:2253-2260.
17. Glück U, Kwiatkowski DJ, Ben-Ze'ev A. Suppression of tumorigenicity in Simian virus 40-transformed cells transfected with α-actinin cDNA. Proc Natl Acad Sci USA 1993; 90:383-387.
18. Rodríguez Fernández JL, Geiger B, Salomon D et al. Suppression of tumorigenicity in transformed cells after transfection with vinculin cDNA. J Cell Biol 1992; 119:427-438.
19. Raz A, Zöller M, Ben-Ze'ev A. Cell configuration and adhesive properties of metastasizing and non-metastasizing Bsp73 rat adenocarcinoma cells. Exp Cell Res 1986; 162:127-141.
20. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science 1991; 251:1451-1455.
21. Larue L, Oshugi M, Hirchenhain J et al. E-cadherin null mutant embryos fail to form a trophectoderm epithelium. Proc Natl Acad Sci USA 1994; 91:8263-8267.
22. Rimm DL, Koslov ER, Kebriaei P et al. α_1(E)-catenin is a novel actin binding and bundling protein mediating the attachment of F-actin to the membrane adhesive complex. Proc Natl Acad Sci USA 1995; 92:8813-8817.
23. Watabe M, Nagafuchi A, Tsukita S et al. Induction of polarized cell-cell association and retardation of growth by activation of the E-cadherin-catenin adhesion system in a disperced carcinoma cell line. J Cell Biol 1994; 127:247-256.
24. Bullions LC, Notterman DA, Chung LS et al. Expression of wild type α-catenin protein in cells with mutant α-catenin gene restores both growth regulation and tumor suppressor activities. Mol Cell Biol 1997; 17:4501-4508.
25. Ewing CM, Ru N, Morton RA et al. Chromosome 5 suppresses tumorigenicity of PC3 prostate cancer cells: correlation with re-expression of α-catenin and restoration of E-cadherin. Cancer Res 1995; 55:4813-4817.
26. Peifer M, Wieschaus E. The segment polarity gene armadillo encodes a functionally modular protein that is the Drosophila homolog of human plakoglobin. Cell 1990; 63:1167-1176.

27. Huber O, Bierkamp C, Kemler R. Cadherins and catenins in development. Curr Opin Cell Biol 1996; 8:685-691.
28. Miller JR, Moon RT. Signal transduction through β-catenin and specification of cell fate during embryogenesis. Genes Dev 1996; 10:2527-2539.
29. Rubenstein A, Merriam J, Klymkowsky M. Localizing the adhesive and signaling functions of plakoglobin. Dev Genetics 1997; 20:91-102.
30. Behrens J, von Kries JP, Kuhl M et al. Functional interaction of β-catenin with the transcription factor LEF-1. Nature 1996; 382:38-642.
31. Huber O, Korn R, McLaughlin J et al. Nuclear localization of β-catenin by interaction with the transcription factor LEF-1. Mech Dev 1996; 59:3-10.
32. Molenaar M, van de Wetering M, Oosterwegel M et al. XTcf-3 transcription factor mediates β-catenin-induced axis formation in Xenopus embryos. Cell 1996; 86:391-399.
33. van de Wetering M, Cavallo R, Dooijes D et al. Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell 1997; 88:789-799.
34. Riese J, Yu X, Munnerlyn A et al. LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. Cell 1997; 88:777-787.
35. Polakis P. Mutations in the APC gene and their implications for protein structure and function. Curr Opin Gen Dev 1995; 5:66-71.
36. Aberle H, Bauer A, Stappert J et al. β-Catenin is a target for the ubiquitin-proteasome pathway. EMBO J 1997; 16:3797-3804.
37. Munemitsu S, Albert I, Souza B et al. Regulation of intracellular β-catenin levels by the adenomatous polyposis coli (APC) tumor suppressor protein. Proc Natl Acad Sci USA 1995; 92:3046-3050.
38. Munemitsu S, Albert I, Rubinfeld B et al. Deletion of an amino-terminal sequence stabilizes β-catenin in vivo and promotes hyperphosphorylation of the adenomatous polyposis coli tumor suppressor protein. Mol Cell Biol 1996; 16:4088-4094.
39. Yost C, Torres M, Miller JR et al. The axis inducing activity, stability, and subcellular distribution of β-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3. Genes Dev 1996; 10:1443-1454.
40. Korinek V, Backer N, Morin PJ et al. Constitutive transcriptional activation by a β-catenin-Tcf complex in $APC^{-/-}$ colon carcinoma. Science 1997; 275:1784-1787.
41. Morin PJ, Sparks AB, Korinek V et al. Activation of β-catenin Tcf-signaling in colon cancer by mutations in β-catenin or APC. Science 1997; 275:1787-1790.
42. Rubinfeld B, Robbins P, El-Gamil M et al. Stabilization of β-catenin by genetic defects in melanoma cell lines. Science 1997; 275:1790-1792.
43. Aberle H, Bierkamp C, Torchard D et al. The human plakoglobin gene localizes on chromosome 17q21 and is subject to loss of heterozygosity in breast and ovarian cancer. Proc Natl Acad Sci USA 1995; 92:6384-6388.
44. Sommers CL, Gelmann EP, Kemler R et al. Alterations in β-catenin phosphorylation and plakoglobin expression in human breast cancer cells. Cancer Res 1994; 54:3544-3552.
45. Navarro P, Lozano E, Cano A. Expression of E- or P-cadherin is not sufficient to modify the morphology and the tumorigenic behavior of murine spindle carcinoma cells: possible involvement of plakoglobin. J Cell Sci 1993; 105:923-934.
46. Simcha I, Geiger B, Yehuda-Levenberg S et al. Suppression of tumorigenicity by plakoglobin: an augmenting effect of N-cadherin. J Cell Biol 1996; 133:199-209.
47. Salomon D, Sacco PA, Guha Roy S et al. Regulation of β-catenin levels and localization by overexpression of plakoglobin and inhibition of the ubiquitin-proteasome system. J Cell Biol 1997; 139:1325-1335.
48. Heasman J, Crawford A, Goldstone K et al. Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in Xenopus embryos. Cell 1994; 79:791-803.
49. Fagotto F, Funayama N, Glück U et al. Binding of cadherins antagonizes the signaling activity of β-catenin during axis formation in Xenopus. J. Cell Biol 1996; 132:1105-1114.
50. Peifer M. β-Catenin as oncogene: the smoking gun. Science 1997; 275:1752-1753.

CHAPTER 8

Regulation of α-Actinin and Vinculin Functions by PIP_2

Kiyoko Fukami and Tadaomi Takenawa

Introduction

The ligand-receptor interactions results in the establishment of a physical connection between the extracellular ligand and the actin cytoskeleton. Dynamic changes in the actin cytoskeleton of cells in response to extracellular stimuli are fundamental functions, which induce the formation of stress fibers at the center, and the formation of membrane ruffles, microspikes, and lamellipodia at the edge of motile cells. Many actin-binding proteins are involved in the regulation of the actin filament. They are mainly categorized as the severing proteins (gelsolin and severin), G-actin-binding protein (profilin), capping protein (gCap 39), crosslinking proteins (α-actinin and filamin), and the ezrin-radixin-moesin (ERM) family, which cooperates with each other to organize the actin cytoskeleton.

α-Actinin was originally discovered in skeletal muscle as a protein factor promoting the superprecipitation of actomyosin and inducing the formation of actin fibers. α-Actinin is densely located in Z-bands of the sarcomere of the muscle and at sites where actin is attached to the plasma-membrane-associated structures in nonmuscle cells. On the other hand, vinculin is a highly conserved cytoskeletal protein found at both cell-cell and cell-extracellular matrix type junctions. The fact that α-actinin and vinculin are found at focal contacts where actin is anchored to a variety of intercellular structures in nonmuscle cells suggests that α-actinin and vinculin may play important roles in the linkage between the plasma membrane and actin. Efforts to elucidate these linkages have implicated α-actinin and vinculin as one of the connecting molecules in a chain that involves binding of the β1 subunit of integrin to talin, talin to vinculin, vinculin to α-actinin, and α-actinin to F-actin. Therefore, it is important to analyze what regulates these protein-protein interactions when cells are stimulated with ligand in order to understand the mechanism of cell migration or invasion.

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a metabolically active phospholipid. PIP_2 generates two second messengers, inositol 1,4,5-trisphosphate (IP-3) and diacylglycerol (DAG) upon phospholipase C (PLC) activation by a variety of physiological stimuli. IP-3 and DAG are known to mobilize Ca^{2+} from the endoplasmic reticulum[1] and to activate protein kinase C (PKC),[2] respectively. To date, many actin binding proteins, such as gelsolin,[3] gCap 39,[4] profilin,[5] cofilin,[6] vinculin and α-actinin[7] have been found to bind specifically to PIP_2, which regulates polymerization and depolymerization of actin (Table 8.1). Although PIP_2 has been reported to regulate the function of these actin-binding proteins in vitro, so far only α-actinin and vinculin have been shown to bind PIP_2 in vivo. Thus, the physiological

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

Table 8.1. Actin regulating proteins and their interactions with lipids

Actin Regulating Proteins	MW (kDa)	Effect of PIP_2 on Activity of the Protein	Lipids Bound to the Protein
G-actin binding protein			
profilin	12-15	↓	PIP_2, palmitic acid, PS, PI
Depolymerization protein			
cofilin	21	↓	PIP_2, PIP, PI
destrin	19	↓	PIP_2, PIP, PI
F-actin severing protein			
gelsolin	84	↓	PIP_2, PIP
severin	40	↓	PIP_2, PIP
villin	90	↓	PIP_2, PIP
F-actin crosslinking protein			
α-actin	100	↑	PIP_2, palmitic acid, DG
vinculin	120	↑	PIP_2, PI, PS, PA
spectrin	240	–	PS, PE, PC
dystrophin	400	↓	PIP_2
filamin	270	↑	PIP_2
Capping protein			
gCap39	39	↓	PIP_2

role of PIP_2 in the regulation of other proteins is still obscure. Here we focused on the effect of PIP_2 on the function of α-actinin and vinculin, and protein-protein interactions found at focal adhesions.

Effect of PIP_2 on the Function of α-Actinin In Vitro

Previous studies demonstrated the physiological significance of the interaction between PIP_2 and cytoskeletal proteins. Although PIP_2 is a component of phospholipid membrane, it is also located densely in Z-bands of the sarcomere of the muscle.[8] These results suggested the possibility that endogenous PIP_2 binding to cytoskeletal proteins exists in Z-bands of the myofibrils. Westernblot analysis with anti-PIP_2 antibody showed that α-actinin was a major component of the striated muscle which contained endogenous PIP_2. In addition, purified α-actinin from striated muscle stained strongly with anti-PIP_2 antibody, but that from smooth muscle stained only slightly. Furthermore smooth muscle α-actinin binds markedly to exogenously added PIP_2. To clarify the physiological relevance of PIP_2-binding to striated α-actinin, the effects of inositol phospholipids on the gelating activity of smooth muscle and striated muscle α-actinin were compared by falling ball viscometry (Fig. 8.1). Striated muscle α-actinin formed a gel in the absence of inositol phospholipids, but smooth muscle α-actinin showed only a weak gelating activity. It is worth noting that PIP_2 greatly stimulates the gelating activity of smooth muscle α-actinin, while phosphatidylinositol (PI) has no effect and phosphatidylinositol 4-monophosphate (PIP) has only a slightly activating effect. None of the inositol phospholipids produced changes in the viscosity of F-actin. These results indicate that α-actinin is an F-actin cross-linker and that striated α-actinin shows a much higher F-actin-gelation activity than smooth muscle α-actinin simply because of the endogenous PIP_2 bound to striated α-actinin. In turn, the interaction of spe-

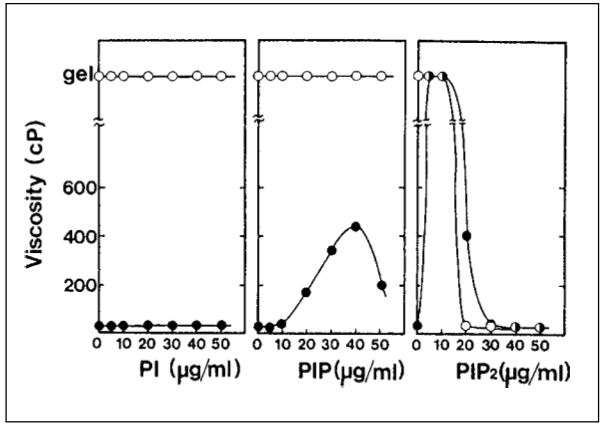


Fig. 8.1. Effects of exogenous inositol phospholipids on the gelating activity of α-actinin. The effects of PI, PIP, and PIP_2 on the interaction of striated muscle α-actinin (open circles) and smooth muscle α-actinin (closed circles) with F-actin were measured by the falling-ball methods. The gel represents the viscosity at which the ball does not fall. Final PI, PIP, and PIP_2 concentration in the reaction medium are indicated. Reprinted with permission from Nature 1992; 359:150-152.

cific lipids with α-actinin may regulate α-actinin function at focal adhesions. The associations of PIP_2 with gelsolin, cofilin, or profilin have been reported to promote actin stress fiber formation by inhibiting the function of these proteins,[3,5,6] while association of PIP_2 with α-actinin promotes stress fiber formation by stimulating the function of α-actinin, suggesting that the level of PIP_2 bound to these proteins is critical for their function.

α-Actinin and Vinculin are PIP_2-Binding Proteins Involved in Signal Transduction by Tyrosine Kinases

α-actinin and vinculin have been detected as PIP_2 abundant proteins[8] by W estern blot analysis using an antibody specific to PIP_2.[7] In Balb/c 3T3 cells, α-actinin in the cytoskeleton contains PIP_2, while α-actinin in cytosol does not. The levels of PIP_2 bound to α-actinin decrease in response to PDGF. Similarly, PIP_2 bound to vinculin is decreased upon PDGF stimulation. By immunofluorescent staining, PIP_2 was found to be present densely in the central areas around the nuclei, microfilament bundles, and focal contacts, where α-actinin and vinculin are distributed. PDGF stimulation decreases the intensity of PIP_2 staining in these areas. Double immunofluorescent staining of quiescent cells with anti-α-actinin and anti-PIP_2 antibodies shows that PIP_2 colocalizes with α-actinin on microfilament bundles.

Tyrosine phosphorylation of proteins in response to growth factors such as EGF and PDGF is thought to play a crucial role in signal transduction.[9,10] The tyrosine phosphorylation promotes the binding of key effector molecules containing the SH2 domain such as PLCγ,[9] Ras GTPase activating protein, phosphatidylinositol 3-kinase[11] and Ash/Grb2[12] to the activated receptor tyrosine kinases. This clustering mechanism of signal transduction leads to multiple cellular responses, including the mitogenic response and reorganization of the actin cytoskeleton. Recently, profilin has been found to play an important role in the hydrolysis of PIP_2 by PLCγ. Profilin binds to PIP_2 and inhibits its hydrolysis by unphosphorylated PLCγ.[13] However, the phosphorylation of PLCγ by tyrosine kinase overcomes the inhibitory effect of profilin, resulting in the effective activation of PLCγ. In this case, the V_{max} of PLCγ is not affected by growth factor-induced tyrosine phosphorylation, instead, the K_m of PLCγ for PIP_2 is lowered.[14] Therefore, it is possible to demonstrate that the tyrosine kinase linked regulation of PIP_2 levels cause a reorganization of the cytoskeleton. Since PIP_2 is a very potent stimulator of actin bundle formation by α-actinin, the decrease in the amount of PIP_2 bound to α-actinin may induce actin depolymerization. All these data suggest that the loss of PIP_2 bound to actin binding proteins in response to PDGF may cause the disruption of focal adhesion and the disassembly of stress fibers. Therefore,

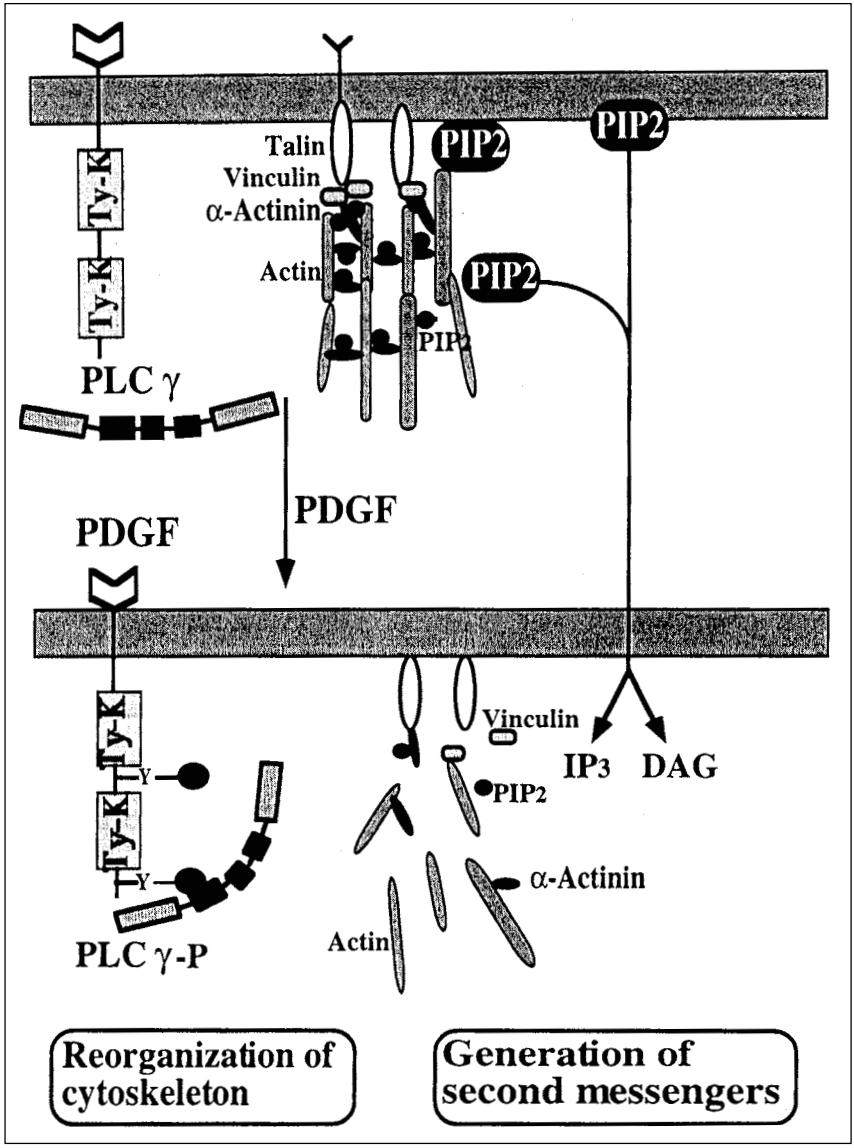


Fig. 8.2. α-actinin and vinculin are PIP_2-binding proteins involved in signal transduction by tyrosine kinases. The tyrosine kinase activation by PDGF promote binding of PLCγ, and the tyrosine phosphorylation of PLCγ might overcome the inhibitory effect of α-actinin and vinculin. PIP_2 hydrolysis by PLCγ not only generates the second messengers IP-3 and DAG, but also causes a decrease in the PIP_2 content of some actin binding proteins, thus inducing the reorganization of the cytoskeleton.

PIP_2 hydrolysis by PLCγ not only generates the second messengers IP-3 and DAG, but also causes a decrease in the PIP_2 content of some actin binding proteins, thus inducing reorganization of the actin cytoskeleton (Fig. 8.2). Furthermore, the IP-3-mediated Ca^{2+} increase activates actin-severing and barbed end nucleating proteins such as gelsolin.[3] All of these events favor actin depolymerization. Thus, changes in cell shape following stimulation by growth factors may be a result of the increased hydrolysis of PIP_2 by cytoskeletal associated PLCγ.

PIP_2-Binding Site in α-Actinin and Vinculin

PIP_2 has been shown to modulate the functions of various proteins such as PKC, μ-calpain, ADP-ribosylation factor (ARF) 1,[15] and phospholipase D,[16] in addition to actin-regulating proteins. To understand the role of PIP_2 in protein function or in protein-protein interactions, it is important to identify the PIP_2-binding site on these proteins.

α-Actinin belongs to the spectrin/dystrophin family, which is characterized by a N-terminal actin-binding domain and a central rod domain consisting of four spectrin-like repeats, and forms heterodimers in solution (Fig. 3.8). The residues 120-134 contain an actin-binding site,[17] although the N-terminal 247 amino acids have also been reported to be necessary for actin-binding. This N-terminal domain appears to contain a binding site for another cytoskeletal protein, zyxin.[18] The central region of α-actinin is thought to contain binding sites for the cytoplasmic domains of β1- and β3 subunit of integrin,[19] providing a possible route linking integrin to F-actin. Recently α-actinin has also been reported to bind a to the cytoplasmic domain of the intercellular adhesion molecule-2 (ICAM-2).[20] On the other hand, the C-terminal domain of α-actinin contain two EF-hand motif which account for the ability of calcium to inhibit the binding of nonmuscle α-actinin isoforms to F-actin[21] and the vinculin-binding domain (residues 713-749).[22] We have provided evidence[23] that amino acids 168-184 (TAPYRNVNIQNFHLSWK) in chicken skeletal muscle α-actinin comprise of a PIP_2-binding site and that the two basic amino acids, arginine 172 and lysine 184, are important for this interaction. Mutants in which either arginine 172 or lysine 184 is replaced by isoleucine partially lose their direct binding with PIP_2. However, this PIP_2-binding site shares no homology sequence with either gelsolin or cofilin.

Recently, the pleckstrin homology (PH) domain was found in a variety of proteins,[24] including protein kinases, substrates for kinases, regulators of small G proteins, PLC isozymes, and cytoskeletal proteins. This domain has been reported to bind to PIP_2,[25] although it also associates with the βγ subunit of trimeric G proteins[26,27] and PKC.[28] So the PH domain is thought to be involved in protein-protein or lipid-protein interactions, and in that case, PIP_2 may act as a target for PH domain-containing proteins in the membrane. Interestingly, regions homologous to the PIP_2-binding site in α-actinin also exist in the β1- and β2-sheets of the PH domains of PLCδ1 and Grb7. Another report showed that IP-3 binds to PLCδ1 and that this interaction is inhibited by PIP_2.[29] The IP-3 binding site on PLCδ1 is thought to comprise of amino acids 30 to 43, which overlaps with the site (amino acids 23-37) which is aligned to α-actinin.

Vinculin is comprised of a globular head and an extended tail (Fig. 8.3). The head region (residues 1-398) contains a talin-binding site, and three 112-residue repeats of unknown function. Evidence for an α–actinin-binding site between residues 1-107 has recently been presented.[31] A proline rich region, which spans residues 837-878 and contains two sites for the V8-protease, is thought to separate the globular head from the extended tail.[32] The C-terminal tail region has been shown to contain a binding site (residue 893-1016) for F-actin[33] and for paxillin[34] (residue 978-1000), another protein localized to focal adhesions. It was also shown that residues 935-978 and 1020-1040 in vinculin interact with acidic

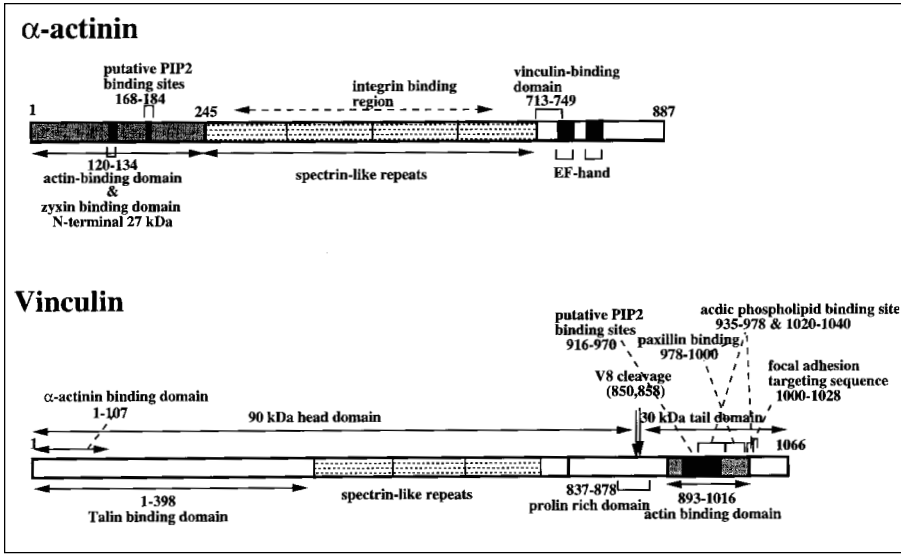


Fig. 8.3. The structure of chicken smooth muscle α-actinin and vinculin. Structure and interaction sites of α-actinin with actin, zyxin, vinculin, and PIP_2 are shown, respectively. Structure and interaction sites of vinculin with actin, talin, paxillin, focal adhesion targeting sequence and PIP_2 are also presented.

phospholipids such as phosphatidyl serine.[35] Our experiment provides evidence that residues 916-970 in mouse fibroblast vinculin comprised of a PIP_2-binding site.[35a]

Binding of Vinculin to Talin and Actin Are Regulated by PIP_2 In Vivo

Vinculin has been reported to interact with the other cytoskeletal proteins, talin and actin. Recently, Gilmore et al[36] examined the effect of PIP_2 on the association of the 90 kDa N-terminal head domain of vinculin and the 30 kDa C-terminal tail domain and on the binding of vinculin to talin and actin. An intramolecular interaction between the head and tail domain masks the binding sites for both talin and actin.[37] The exposure of the masked binding sites seems to be important for promoting the formation of focal contacts and adherent junctions. Based on the fact that the small GTP-binding protein Rho activates PIP(5) kinase which produces PIP_2,[38] and PIP_2 binds to many actin-regulating proteins including vinculin, they examined the role of PIP_2 on the formation of focal contacts. They showed that 10 μg/ml PIP_2 dissociated vinculin's head-tail interaction, exposing its binding sites for talin and actin. PIP_2 increased talin binding to vinculin, with a K_d of approximately 30 nM. In the absence of PIP_2, there was little talin binding to intact vinculin, indicating that PIP_2 induces a conformational change in vinculin. In the same way, PIP_2 produced an increase in actin binding, although intact vinculin bound actin poorly. Furthermore they showed that cells microinjected with antibodies to PIP_2 did not respond to serum stimulation and did not form stress fibers and focal adhesions, indicating that the Rho-induced assembly of focal adhesions requires PIP_2. Further reports show that Rho regulates the association of vinculin with the plasma membrane at focal adhesions in MDCK cells[39] and that elevated levels of cytoskeletally associated PIP_2 in thrombin-stimulated aggregation of human platelet are mediated by translocation of PIP(5) kinase.[40] These results suggest a model

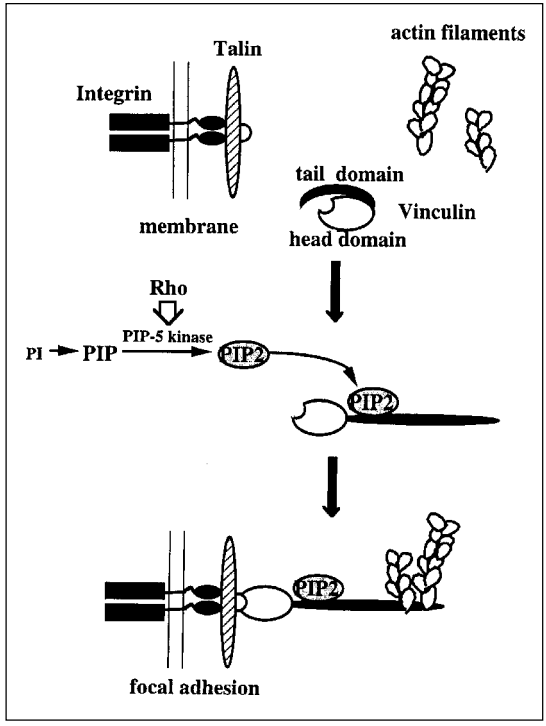


Fig. 8.4. Binding of vinculin to talin and actin are regulated by PIP_2 in vivo. In a resting cell, vinvulin is in the inactive conformation with the C-terminal tail bound to the head. Activation of PIP(5) kinase by Rho elevates PIP_2, which induces a conformational change in vinculin, exposing binding sites for talin and actin. These promote the assembly of focal adhesions.[36] Modified from Gilmore AP. Nature 1996;l 381: 531-535.

in which cells activation of PIP(5) kinase by Rho elevates PIP_2, inducing a conformational change in vinculin, and promoting its association with other components of focal adhesions and the attachment of the microfilament to the membrane (Fig. 8.4).

Conclusions

Here we focused on the functions of inositol phospholipids in the actin cytoskeleton. Phospholipids involved in the control of actin cytoskeleton are metabolically active and abundant in cells and therefore play a very important role linking signal transduction molecules to the reorganization of the actin cytoskeleton. In addition to the actin-regulating function, other functions PIP_2 have been proposed.

Interestingly, it has become clear that PIP_2 synthesis by PIP(5) kinase is also involved in exocytosis.[41] Additional evidence for a role of PIP_2 in vesicular trafficking was provided by Cantley et al.[42] They reported that PIP_2 stimulates the in vitro activity of partially purified membrane phospholipase D(PLD) in which PIP_2 functions as a PLD cofactor.[16]

On the other hand, some functional proteins have recently been reported to associate with α-actinin. α-Actinin was identified as a brain postsynaptic density protein that colocalizes in dendritic spines of the N-metyl-D-aspartate (NMDA) receptor, a neurotransmitter receptor.[43] α-Actinin binding to the NMDA receptor was antagonized by Ca^{2+}/calmodulin in a Ca^{2+}-dependent fashion, indicating that Ca^{2+}/calmodulin displaces α-actinin from the NMDA receptor in response to a postsynaptic Ca^{2+} influx and therefore, leads to Ca^{2+}-dependent detachment of the NMDA receptor from the actin cytoskeleton on cell activation. In addition, rabphilin-3A, a downstream target of Rab3, has been shown to directly interact with α-actinin and stimulate its actin filament bundling activity.[44] Both Rab3 and rabphilin-3A are associated with synaptic vesicles. As rabphilin-3A has two C2-like domains that interact

with Ca^{2+} and phospholipid as well as synaptotagmin, the rabphilin-3A/α-actinin complex may be involved in the Ca^{2+}-and phospholipid-dependent neurotransmitter release. Other evidence for the interaction of α-actinin with protein kinase N (PKN), one of the target molecules of Rho kinase, has also been reported.[45] This interaction occurs in a PIP_2-dependent manner, while PIP_2 has been reported to directly affect the kinase activity of PKN in vitro. Although these data are very complicated, they all suggest that a Rho kinase (Rab3/rabphilin-3A)/PIP_2/Ca^{2+}/α-actinin system may be essential not only for the rearrangement of actin cytoskeleton on cell activation, but also for the exocytosis of neurotransmitters. Alternatively this regulation system seems to be a more common phenomenon in Ca^{2+}-sensitive proteins. Therefore, one possible speculative role of inositolphospholipid is to bring these cellular modulators to the membrane or to their respective ligands as cluster core molecules, and to regulate these interactions by Ca^{2+} or lowering the Ca^{2+}-dependency of Ca^{2+}-sensitive proteins.

References

1. Berridge MJ, Irvine RF. Inositol phosphates and cell signaling. Nature 1989; 341:197-205.
2. Nishizuka Y. The role of protein kinase C in cell surface transduction and tumour promotion. Nature 1984; 308:693-698.
3. Janmey PA, Stossel TP. Modulation of gelsolin function by phosphatidylinositol-4-5-bisphosphate. Nature 1987; 325:362-364.
3. Yu FX, Johnston PA, Sudhof TC et al. gCap39, a calcium ion-and polyphosphoinositide-regulated actin capping protein. Science 1990; 250:1413-1415.
5. Lassing I, Lindberg U. Specific interaction between and profilactin. Nature 1985; 314:472-474.
6. Yonezawa N, Homma Y, Yahara I et al. A short sequence responsible for both phosphoinositide binding and actinbinding activities of cofilin. J Biol Chem 991; 266:17218-17221.
7. Fukami K, Endo T, Imamura M et al. α-Actinin and vinculin are PIP_2-binding proteins involved in signaling by tyrosine kinase. J Biol Chem 1994; 269:1518-1522.
8. Fukami K, Furuhashi K, Inagaki M et al. Requirement of phosphatidylinositol-4-5-bisphosphate for α-actinin function. Nature 1992; 359:150-152.
9. Cantley LC, Auger KR, Carpenter C et al. Oncogenes and signal transduction. Cell 1991; 64:281-302.
10. Hunter T, Cooper JA. Proetin-tyrosine kinases. Ann Rev Biochem 1985; 54:897-930.
11. Aaronson SA. Growth factors and Cancer. Science 1991; 254:1146-1153.
12. Matsuoka K, Shibata M, Yamakawa A et al. Cloning of ASH, a ubiquitous protein composed of one SH 2 and two SH3 domains, from human and rat cDNA libraries. Proc Natl Acad Sci USA 1992; 89:9015-9019.
13. Goldschmidt-Clermont PJ, Kim JW, Machesky LM et al. Regulation of phospholipase C-γ1 by profilin and tyrosine phospholilation. Science 1991; 251:1231-1233.
14. Wahl MI, Jones GA, Nishibe S et al. Growth factor stimulation of phospholipase C-gammmal activity. Comparative propaties of control and activated enzymes. J Biol Chem 1992; 267:10447-10456.
15. Randazzo PA. Functional interaction of ADP-rybosylation factor 1 with phosphatidylinositol 4,5-bisphosphate. J Biol Chem 1997; 272:7688-7692.
16. Liscovitch M, Chalifa V, Pertile P et al. Novel function of phosphatidylinositol-4-5-bisphosphate as a cofactor for brain membrane phospholipase D. J Biol Chem 1994; 269:21403-21406.
17. Hemmings L, Kuhlman PA, Critchley DR. Analysis of the actin-binding domain of α-actinin by mutagenesis and demonstration that dystrophin contains a functionally homologous domain. J Cell Biol 1992; 116:1369-1380.
18. Crawford AW, Michelsen JW, Beckerle MC. An interaction between zyxin and α-actinin. J Cell Biol 1992; 116:1381-1393.

19. Otey CA, Pavalko FM, Burridge K. An interaction between α-actinin and the β1 integrin subunit in vitro. J Cell Biol 1990; 111:721-729.
20. Heika L, Kantor C, Parr T et al. Binding of the cytoplasmic domain of intercellular adhesion molecule-2(ICAM) to alpha-actinin. J Biol Chem 1996; 271:26214-26219.
21. Arimura C, Suzuki T, Yanagisawa M et al. Primary structure of chicken skeletal muscle and fibroblast alpha-actinins deduced from cDNA sequences. Eur J Biochem 1988; 177:649-655.
22. McGregor A, Blancherd A, Rowe AV. Identification of the vinculin-binding site in the cytoskeletal protein α-actinin. Biochem J 1994; 301:225-233.
23. Fukami K, Sawada N, Endo T et al. Identification of a phosphatidylinositol-4-5-bisphosphate-binding site in chicken skeletal muscle α-actinin. J Biol Chem 1996; 271:2646-2650.
24. Mayer BJ, Ren R, Clark K et al. A putative modular domain present in diverse signaling proetins. Cell 1993; 73:629-630.
25. Harlan JE, Hajduk PJ, Yoon, HS et al. Pleckstrin homology domains bind to phosphatidylinositol-4-5-bisphosphate. Nature 1994; 371:168-170.
26. Touhara K, Inglese J, Pitcher JA et al. Binding of G protein βγ-subunits to pleckstrin homology domains. J Biol Chem 1994; 269:10217-10220.
27. Tsukada S, Simon MI, Witte ON et al. Binding of βγ-subunits of heterotrimeric G proetins to the PH domain of Bruton tyrosine kinase. Proc Natl Acad Sci USA 1994; 91:11256-11260.
28. Yao L, Kawakami Y, Kawakami T. The pleckstrin homology domain of Bruton tyrosine kinase interact with protein kinase C. Proc Natl Acad Sci USA 1994; 91:9175-9179.
29. Yagisawa H, Hirata M, Kanematsu T et al. Expression and characterization of an inositol1,4,5-trisphoaphate binding domain of phosphoinositol-specific phospholipase C-δ1. J Biol Chem 1994; 269:20179-20188.
30. Gilmore AP, Jackson P, Waites GT et al. Further characterisation of the talin-binding site in the cytoskeletal protein vinculin. J Cell Sci 1992; 103:719-731.
31. Kroemker, MP, Rudiger AH, Jockusch BM et al. Intramolecular interactions in vinculin control α-actinin binding to the vinculin head. FEBS Lett 1994; 355:259-262.
32. Price GJ, Jones P, Davison MD et al. Primary sequence and domain structure of chicken vinculin. Biochem J 1989; 259:453-461.
33. Menkel AR, Kroemker MP, Bubeck M et al. Characterization of an F-actin-binding domain in the cytoskeletal protein vinculin. J Cell Biol 1994; 126:1231-1240.
34. Wood CK, Turner CE, Critchley DR. Characterisation of the paxillin-binding site and C-terminal focal adhesion targeting sequence in vinculin. J Cell Sci 1994; 107:709-717.
35. Tempel M, Goldmann WH, Isenberg G et al. Interaction of the 47-kDa fragment and the 32-kDa vinculin fragment with acidic phospholipids: a computer analysis. Biophysic J 1995; 69:228-241.
35a. Miyamoto R, Fukami K, Takenawa T. Polyphosphoinositide-binding site in vinculin. (In preparation).
36. Gilmore AP, Burridge K. Regulation of vinculin binding to talin and actin by phosphatidylinositol-4-5-bisphosphate. Nature 1996; 381:531-535.
37. Johnson RP, Craig SW. F-actin binding site masked by the intramolecular association of vinculin head and tail domains. Nature 1995; 373:261-264.
38. Chong LD, Traynor-Kaplan A, Bokoch GM et al. Th esmall GTP-binding protein rho regulates a phosphatidylinositol-4-phosphate 5-kinase in mammalian cells. Cell 1994; 79:507-513.
39. Kotani H, Takaishi K, Sasaki T et al Rho regulates association of both the ERM family and vinculin with the plasma memblane in MDCK cells. Oncogene 1997; 14:1705-1713.
40. Hinchlitte KA, Irvine RF, Divecha N. Aggregation-dependent, integrin-mediated increases in cytoskeletally associated PtdInsP2(4,5) levels in platelets are controled by translocation of PtdIns 4-P 5-kinase C to the cytoskeleton. EMBO J 1996; 15:6516-6524.
41. Hay JC, Fisette PL, Jenkins GH et al. ATP-dependent inositide phosphorylation required for Ca^{2+}-activated secretion. Nature 1995; 374:173-177.
42. Liscovitch M, Cantley LC. Signal transduction and membrane taraffic: the PITP/phosphoinositide connection. Cell 1995; 81:659-662.

43. Wyszynski M, Lin J, Rao A et al. Competitive binding of α-actinin and calmodulin to NMDA receptor. Nature 1997; 385:439-442.
44. Kato M, Sasaki T, Ohya T et al Physical and functional interaction of rabphilin-3A with α-actinin. J Biol Chem 1997; 271:31775-31778.
45. Murai H, Toshimori M, Shibata H et al. Interaction of PKN with α-actinin. J Biol Chem 1997; 272:4740-4746.

CHAPTER 9

Tumor Suppressive Function of Gelsolin

Noboru Kuzumaki, Hisakazu Fujita, Motoyoshi Tanaka, Norio Sakai and Makoto Ohtsu

Introduction

Actin regulatoryproteins play important roles in the morphology and motility of cells. An F-actin severing/capping protein, gelsolin, which was first isolated from rabbit lung macrophages as a modulator of the cytoplasmic actin gel-sol transformation by Yin and Stossel in 1979,[1] can be found in almost all mammalian organs.[2] The molecular weight of gelsolin is about 90 kDa, and there are two isoforms of gelsolin, i.e., the cytoplasm and plasma gelsolins.[3] Gelsolin has three functions for regulating the size of the actin filament: (1) severing the actin filaments (2) nucleating to induce actin polymerization and (3) blocking growth of actin filaments at the barbed ends.[4] These functions are regulated by calcium ions or protons positively, and polyphosphoinositides, especially phosphatidylinositol 4,5-bisphosphate (PIP_2) negatively.[5] Recently, several reports have suggested that PIP_2/actin-binding proteins such as profilin, gelsolin, CapG (gCap39), and cofilin are involved in cellular signal transduction.[6-9]

Mutant Gelsolin and Tumor Suppression

Malignant transformation is now regarded widely as a multistep process involving alterations at several gene loci. Activation of oncogenes and/or functional loss of tumor-suppressor genes are thought to play key roles widely. As an approach to elucidate mechanisms underlying the suppression of malignant transformation, we characterized two flat revertant cell lines, R1 and R2. Both of the flat revertants were isolated from human activated c-Ha-ras-1 (hu-ac-Ha-ras) gene-transformed NIH 3T3 cells (EJ-NIH 3T3) treated with a mutagen, ethyl methanesulfonate. R1 contained unchanged transfected hu-ac-Ha-ras DNA and expressed high levels of hu-ac-Ha-ras mRNA and protein.[10] Transfection experiments revealed that NIH 3T3 cells could be transformed by high molecular weight genomic DNA from R1 cells, but R1 cells could not be retransformed by Kirsten sarcoma virus, DNA from EJ-NIH 3T3 cells, hu-ac-Ha-ras, v-src, v-mos, simian virus 40 large T antigen or polyomavirus middle T antigen. Somatic cell hybridization studies showed that R1 was not retransformed by fusion with NIH 3T3 cells, and suppressed anchorage independence of EJ-NIH 3T3 and hu-ac-Ha-ras gene-transformed rat W31 cells in soft agar. These results suggest that the phenotypic reversion of R1 and its resistance to several oncogenes is due not to the loss of positively acting transforming factors but rather to enhanced production of proteins that suppress oncogenic transformation.[10] R2 cells had reduced colony forming ability and

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

tumorigenicity in syngeneic mice as well, but the degree of the reduction was lower than that in R1 cells.

Many cancer cells display quantitative and/or qualitative alterations of cytoskeletal components.[11-13] The downregulation of smooth-muscle α-actin expression is a transformation-sensitive marker in rodent fibroblasts.[14] Considering gelsolin's role as an actin regulatory protein, we have investigated the steady-state levels of α-actin and gelsolin mRNA in R1 cells in comparison with those of EJ-NIH/3T3, NIH/3T3 and R2 cell lines.[15] The expression of α-actin mRNA was restored in R1 cells to the level seen in NIH/3T3 cells. In R2 and EJ-NIH/3T3 cell lines, no α-actin transcript was detected. Southern blot analysis gave neither signs of gross rearrangements nor amplification of the gelsolin gene. Gelsolin mRNA expression was highest in R1 cells, intermediate in R2 and NIH/3T3 cells, and low in EJ-NIH/3T3 cells. Restoration of α-actin expression and high levels of gelsolin expression may be associated with the acquisition of flat morphology and an ordered cell growth pattern, which imply loss of tumorigenicity of R1 cells.

In order to further investigate the changes in protein expression accompanying the reversion of transformed cells, we have analyzed polypeptide patterns in the mouse embryo fibroblast NIH/3T3 cell line, EJ-NIH/3T3 cells, and the two flat revertant cell lines, R1 and R2, by two-dimensional gel electrophoresis.[16] Common alterations of polypeptide patterns were observed in the two revertants compared with NIH/3T3 and EJ-NIH/3T3 cells. One polypeptide, p92-5.7, appeared as a newly detected spot in the two revertants. Moreover, the expression level of p92-5.7, judged by visual assessment, seems to be correlated with the morphology and reduced tumorigenicity of R1 and R2 cells. This stems from the fact that p92-5.7 was relatively highly expressed in R1 cells which completely lost their malignant phenotypes, such as colony-forming ability in soft agar and tumorigenicity in syngeneic newborn or adult NIH Swiss mice, despite the expression of activated c-Ha-ras (EJ-ras).[10] On the other hand, p92-5.7 had a relatively low level of expression in R2 cells that had partially lost malignant phenotypes. Briefly, R2 cells have about 50% of the colony-forming ability in soft agar and tumorigenicity in syngeneic newborn NIH Swiss mice seen in EJ-NIH/3T3 cells. The amounts of p92-5.7 were confirmed by densitometric analysis. Furthermore, we analyzed the total proteins extracted from BALB/3T3 cells or NIH Swiss mouse primary embryo fibroblasts, normal rat cell lines, NRK and L6, by two-dimensional gel electrophoresis. The p92-5.7 spot could not be detected in these cell extracts. Fey et al reported the catalog of polypeptides from secondary mouse kidney fibroblasts.[16] Although we compared the two-dimensional electrophoretograms of R1 cells and secondary mouse kidney fibroblasts, we could not identify p92-5.7 in the two-dimensional electrophoretogram of the latter. Therefore, it is conceivable that the expression of p92-5.7, which is specifically detected in the revertants, is associated, at least in part, with the cause of the reversion of R1 and R2 cells rather than as a secondary consequence of the reversion.

Western blot analysis demonstrated that p92-5.7, in addition to the wild-type gelsolins (more acidic compared with p92-5.7), were stained with an anti-gelsolin antibody.[16] The p92-5.7 spot, hence, may be a variant form of gelsolin or a gelsolin-like protein with shared antigenic epitopes. There are several possibilities to explain why p92-5.7 has a different pI value compared to wild-type gelsolins: (1) mutation of the wild-type gelsolin gene; (2) the expression of an unknown gelsolin isoform coding for p92-5.7 or a gelsolin-like protein; (3) a post-translational modification of wild-type gelsolin. To clone the cDNA encoding a variant form of gelsolin (p92-5.7), clones from an unamplified cDNA library of R1 cells were screened with a human gelsolin cDNA probe. We then determined and compared the nucleotide sequences of the clones which showed inserts of full-length murine gelsolin cDNA. Within the coding region, we detected only one difference between nucleotides.[17] A cytosine in the second position of codon 321 in a wild-type clone was replaced by an adenine

in the clones, thus causing an amino acid change from proline to histidine. We called this variant gene His321 (Fig. 9.1). These sequencing results agree with the in vitro transcription-translation data. Furthermore, the change from the neutral amino acid proline to the basic amino acid histidine could explain the altered mobility and the more basic isoelectric point (PI) of His321 gelsolin, as compared with the wild-type gelsolin protein in two-dimensional gels. In order to understand the role of His321 gelsolin in reversion of ras-transformed cells, cDNAs encoding His321 gelsolin or human wild-type gelsolin as a control were transfected into EJ-NIH3T3 cells. All the transfectants that produced His321 gelsolin and one of three transfectants that produced human wild-type gelsolin either lost or reduced tumorigenicity in syngeneic mice. These results demonstrate that His321 mutated gelsolin can suppress a ras-induced murine tumor and suggest that wild-type gelsolin, if expressed at increased levels, may have a similar suppressive potential. From our data, we propose an important role for gelsolin in the cell signal transduction pathways that involve the mammalian ras protooncogene.

Wild-Type Gelsolin and Tumor Suppression

Variations in gelsolin expression have been associated with major cytoskeletal changes that occur during carcinogenesis and differentiation. For example, gelsolin was undetectable, or barely detectable, in murine fibroblasts transformed by the H-ras oncogene and in undifferentiated embryonal carcinoma cell lines.[15,18] Gelsolin is one of most prominently downregulated polypeptides in several simian virus 40 (SV40)-transformed cell lines[19] and diminished gelsolin expression has been documented in invasive human mammary tumor cells compared with normal breast ductal epithelium.[20] On the other hand, when murine L929 cells were exposed to dexamethasone, synthesis of gelsolin was induced and the cells acquired a flat morphology.[21] Levels of gelsolin mRNA and protein also increase during the differentiation of murine embryonal carcinoma cells.[18] An increase in gelsolin mRNA and protein levels has been found to accompany the tetradecanoyl-phorbolacetate(TPA)-induced differentiation of human myelogenic leukemia cell lines into macrophage-like cells.[22] These observations suggest that an increase in gelsolin is strongly correlated to the differentiation of embryonal and myeloid cells, and may contribute to the differentiation process.

We have examined the expression of gelsolin in human gastric and colon cancer cell lines and tissues compared with normal tissues, using Western blot analysis with a monoclonal anti-gelsolin antibody. The production of gelsolin was notably reduced in 7 out of 8 gastric carcinoma cell lines[23] in 4 of 7 colon cell lines and in 9 of the 15 colon cancer tissues.[24] Furthermore, upon the introduction of the exogenous human wild-type gelsolin cDNA into a human colon cancer cell line, the gelsolin transfectants greatly reduced in vivo both the cancer's colony-forming ability and its tumorigenicity. These results confirm that gelsolin plays a key role as a tumor suppressor of carcinogenesis in human gastric and colon tumors.

A loss of chromosome 9, especially allelic losses of chromosome 9q, on which the gelsolin gene is located[25] is one of the most frequent genetic alterations in low-grade, low-stage bladder cancers.[26-28] The inactivation of putative tumor suppressor genes on chromosome 9q may lead to abnormal uroepithelial proliferation and differentiation in the early stages of bladder carcinogenesis, but the presence of tumor suppressor genes on chromosome 9q has not yet been determined. We examined the expression of gelsolin in a number of human bladder cancer cell lines and tissues. In all 6 cell lines and in 14 of the 18 tumor tissues (77.8%), gelsolin expression was undetectable or extremely low in comparison with its expression in normal bladder epithelial cells.[29] Furthermore, upon the introduction of the exogenous human or mouse wild-type gelsolin cDNA into a human bladder cancer cell line, gelsolin transfectants greatly reduced the colony-forming ability and the tumorigenicity in

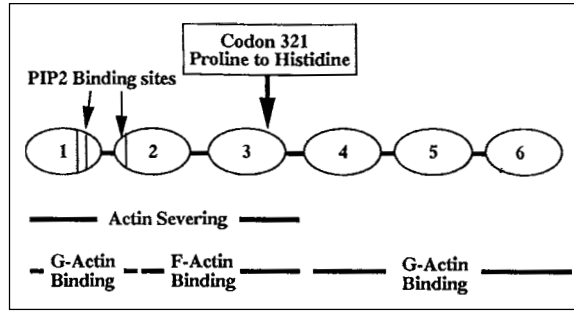


Fig. 9.1. Domain structure of mutant gelsolin His321.

vivo. These results also suggest the idea that gelsolin plays a key role as a tumor suppressor in human urinary bladder carcinogenesis.

To investigate the in vivo efficacy of gene therapy with the gelsolin gene, we tested the effects of adding retroviral vector constructs containing gelsolin cDNA on human urinary bladder cancer cells (UMUC-2 or DAB-1) that were previously inoculated into nude mice.[30] We used retroviral packaging cells that produce the same retroviral DNA constructs at high levels. This gelsolin-treatment resulted in marked and reproducible tumor growth inhibition and prolonged survival time in the majority of animals tested. Sections taken from injection sites 10 days after grafting of producer cells showed many gelsolin-positive cells, suggesting that successful gene transfer had occurred in vivo. Tissue contained a markedly lower number of tumor cells. Fibroblastic proliferation with collagen fibers and mononuclear cell infiltration were also seen.

It has also been reported that chromosome 9q is frequently deleted in nonsmall cell lung cancers (NSCLCs).[31] Dosaka-Akita et al examined gelsolin expression in surgically resected NSCLCs by immunohistochemistry and found its reduced or undetectable levels in 51 of 91 NSCLCs (56%) compared with normal bronchial epithelial cells.[32] Gelsolin protein and RNA were also absent or markedly reduced in human breast cancer cell lines relative to mortal human mammary epithelial cells and benign, immortalized cell lines.[33] Gelsolin was also missing or greatly decreased in 70% of 30 human sporadic, invasive breast carcinomas examined by immunocytochemistry and in 100% of virally induced mouse and chemically induced rat mammary carcinomas as evaluated by Northern analysis.

All these findings suggest that partial or total loss of gelsolin expression is involved in the development of gastric, colon, urinary bladder, lung and breast cancers as one of the early events that occur during carcinogenesis, and that gelsolin may function as a tumor suppressor under these conditions (Table 9.1).

How Does Gelsolin Suppress Tumor Growth?

Several reports have suggested that actin regulatory proteins may be involved in intracellular signal transduction associated with phosphoinositide. Profilin competes efficiently with phosphoinositide-specific phospholipase C (PI-PLC) for interaction with the PIP_2 substrate and this protein thereby inhibits PIP_2 hydrolysis.[34] Phosphorylation of PLC-γ by the EGF receptor tyrosine kinase overcomes the inhibitory effect of profilin and results in the effective activation of PLC-γ.[35] Wild-type gelsolin inhibits the PIP_2 hydrolysis by the human platelet-derived PLC-γ and PLC-δ in vitro.[36] PLC-γ is the downstream mediator of both the EGF receptor's and the PDGF receptor's mitogenic signals.[37]

To investigate the functions of the mutated gelsolin His321 in mouse fibroblasts, we transfected His321 cDNA into NIH/3T3 cells and examined the effects on actin fibers, and on DNA synthesis after stimulation by growth factors.[38] Rhodamine-phalloidin staining

Table 9.1. Reduced or diminished expression of gelsolin in various human cancers

Cancers	Cell Lines	Tissues	References
Stomach	7/8 (88%)		23
Colon	4/7 (57%)	9/15 (60%)	24
Urinary bladder	6/6 (100%)	14/18 (78%)	29
Lung (Nonsmall)	12/12 (100%)	51/91	(56%) 32
Breast	5/5 (100%)	21/31 (70%)	33

revealed that two NIH/3T3 clones expressing His321 form organized actin stress fibers. The cell growth of these clones is inhibited in liquid mediums that contain both 5 and 10% serum. The doubling times of the transfectants were about 10 h longer than those of the control cell lines. The altered action of mutated gelsolin on actin might be able to stabilize the stress fibers, thereby interfering with the initiation of mitosis, which would lead in turn to the lengthening of the doubling time. To investigate the effects of His321 on the signal transduction pathway necessary for cell growth, we stimulated the cell lines by a platelet-derived growth factor (PDGF) or the epidermal growth factor (EGF). Stimulation by PDGF or EGF induced far less DNA synthesis in His321-transfectants than in neo gene-transfectants, similar to results seen with whole serum.

The amino acid sequence of the gelsolin molecule has six homologous repeats, and extensive studies involving proteolytic fragments and recombinant truncated gelsolin indicate that the various functions of gelsolin and their regulation involve cooperative interaction of the domains encoded by repeated sequences. These domains are conventionally designated as six repeated sequence segments G1-G6.[39] The mutation identified in His321 is located at a position in the middle of the sequence of segment 3 (G3). The CT28N fragment, which corresponds to G2-G3, specifically binds to actin filaments, and this binding is inhibited by polyphosphoinositides such as PIP_2.[40] Judging from the location of the mutation, we might speculate that it is the interaction of His321 with actin filaments and/or PlP_2 that is affected. An attractive working hypothesis is that, as in the case of profilin,[35] His321 may bind PIP_2 with increased affinity by amino acid substitution from proline to histidine with a positive charge, thus blocking PIP_2 hydrolysis by PLC in response to growth factor stimulation. In fact, the most basic profilin isoform found in the Acanthameba has a much higher affinity for PIP_2 and inhibits its hydrolysis by PLC more strongly than the acidic isoform.[41] Although a breakdown of polyphosphoinositides is not always associated with changes in the actin cytoskeleton,[42] such a model is supported by the findings that (a) gelsolin can inhibit PIP_2 breakdown by PLC in vitro[36] and (b) that suppression of PIP_2 turnover by microinjection of an anti-PIP_2 antibody into ras transformed cells causes a reduction in proliferation.[43] The wild-type gelsolin protein may have transformation-suppressing activity by inhibiting PIP_2 hydrolysis, and the mutation in His321 may strongly enhance this activity. Alternatively, if the mutation impairs the severing activity of the protein, His321 gelsolin might be able to compete with wild-type gelsolin for binding to actin filaments and thereby exert a stabilizing effect on the cytoskeleton; this would lead to tumor suppression.

To investigate the biochemical consequences of the amino acid substitution of His321, we expressed the His321 gelsolin and wild-type gelsolin in *Escherichia coli*, purified them, and analyzed their effects on actin, polyphosphoinositol lipids and PLC.[44] His321 gelsolin has decreased actin-filament-severing activity and increased nucleating activity compared with wild-type gelsolin in vitro. Furthermore, compared to wild-type gelsolin both nucle-

ation and severing by His321 gelsolin are inhibited more strongly by the phosphoinositol lipids phosphatidylinositol 4-phosphate (PIP) and PIP_2. In addition, # His321 gelsolin inhibits PIP_2 hydrolysis by PLC-γ more strongly than wild-type gelsolin in vitro because of its higher binding capacity for phosphoinositol lipids.

Gelsolin segment 1 and 15 residues at the N-terminus of domain 2 are minimally required for actin-filament severing,[45,46] but recent studies indicate that segment 3 which contains the His321 mutation, may influence the affinity with which segment 2 binds to the side of actin filaments.[47,48] Chou-Fasmann analysis of the protein structure has shown a reduced probability of formation of the β-turn structure in mutated gelsolin compared with wild-type gelsolin by the amino acid substitution.[17] This prediction allowed us to speculate that the activity of mutated gelsolin may be affected by this structural alteration. One explanation for inhibition of severing by His321 gelsolin would be if the mutant gelsolin binds actin monomers more readily than wild-type gelsolin, since once bound to a subunit, the gelsolin-actin subunit complex loses severing activity. The findings that His321 gelsolin has increased actin nucleation activity and loses severing activity more rapidly than wild-type gelsolin in the presence of actin monomers are consistent with that mechanism. Gelsolin's nucleation activity involves segments 2 and as yet undetermined actin-binding sites in segments 4-6. It is therefore conceivable that the alterations in tertiary structure imposed by the proline-histidine change could facilitate the interactions of these segments with actin subunits to promote nucleation at the expense of severing. The presence in a cell of high levels of a gelsolin mutant with increased actin nucleation and diminished severing activities might confer on the cell the ability to construct actin structures more efficiently in the context of a condition, transformation, that ordinarily favors actin disassembly. Previous studies have revealed that mouse fibroblasts which overexpress wild-type gelsolin disrupt actin stress fibers.[49,50] In our present study, expression of His321 in NIH/3T3 cells produced no visible actin fiber changes. This result may suggest that the severing activity of the mutated gelsolin is less powerful than that of wild-type gelsolin in vivo.

The binding of polyphosphoinositol lipids by gelsolin appears to be mediated by specific short stretches of amino acids. Such sequences have been identified in segment 1 (residues 135-149) and the junction of segments 1 and 2 (residues 150-169).[51] In addition, a polyphosphoinositol lipid-binding site has been inferred to exist in segment 3, so that the tighter binding of His321 gelsolin to polyphosphoinositol lipids could result from perturbations by the mutation in that domain. Of the various differences between His321 gelsolin and wild-type gelsolin, the most compelling, with respect to transformation, is tighter binding of polyphosphoinositol lipids, since this class of lipids has been implicated in cell growth and transformation.[52] Consistent with tighter binding to the substrate of PLC-γ, # His321 gelsolin has greater inhibitory action against this enzyme. Western and Northern blot analyzes of phosphoinositide-specific PLCs revealed elevated expression of PLC-γ 1 at both the protein and mRNA levels in most colorectal carcinomas when compared with paired adjacent normal mucosa samples.[53,54] These findings imply that PLC-γ 1 may play an important role in the proliferation of colorectal carcinoma cells and that gelsolin overexpression inhibits the function of PLC-γ 1 in colon cancer cells and leads to the suppression of the malignant phenotype.

Accumulated evidence indicates that actin-regulatory proteins can have tumor suppressive functions. For example, several transformed fibroblast and epithelial cell lines have been found to express reduced, or undetectable levels of vinculin or α-actinin. In addition, the overexpression of vinculin, α-actinin, or tropomyosin can suppress the tumorigenic and malignant metastatic ability of cells.[55-57] The F-actin capping protein NF2/Merlin has an anti-ras function, and is closely related to ezrin, radixin and moesin, members of the ERM family, which link the actin-cytoskeleton and the cell membrane.[58] Mutations of the

Table 9.2. Tumor suppressive function of actin-regulatory proteins with PIP_2 binding activity

Proteins	PIP_2 Binding	Tumor Suppression	References
vinculin	+	+	61,55
α-actinin	+	+	62,56
gelsolin	+	+	51,29

NF2 tumor suppressor gene in neurofibromatosis 2 patients generate truncated, inactive or dominant negative mutants.[59] The facts that vinculin, α-actinin, ERM and gelsolin have both tumor-suppressive[29,55,56,58,60] and phosphoinositides-binding activities[51,61-63] in common suggest that their actin-PIP_2-binding activity is responsible for their tumor suppressive function (Table 9.2).

In support of the idea that structural proteins can play roles in tumorigenesis, the *Drosophila* lethal[2] giant larvae tumor suppressor is a myosin II-binding protein.[64] Su et al have also identified two cellular proteins that associate with APC as the E-cadherin-associated proteins α- and β-catenin.[65] Some of the human colon carcinoma cell clones transfected by a full-length, wild-type APC gene suppressed tumorigenicity when grown in soft agar and prevented tumor formation in nude mice.[66] The DCC gene encodes a neural cell adhesion-like molecule that is a transmembrane protein.[67] A full-length DCC gene suppressed the tumorigenicity of nitrosomethylurea (NMU), transformed tumorigenic HPV-immortalized human epithelial cells that underwent allelic loss and reduced expression of DCC.[68] Syndecans, which are integral membrane proteoglycans, can suppress malignant growth, stimulate actin polymerization, and induce epithelioid morphology in mouse mammary tumor cells.[69] Human colon cancer cells stably transfected with decorin, which is a leucine-rich proteoglycan cDNA, exhibit a marked suppression of the transformed phenotype.[70] All these results suggest an important link between the cytoskeleton, cell adhesion and tumor growth suppression.

Recently, we also found out that gelsolin transfectants were significantly radioresistant compared with their parent cells or control clones transfected with the neo gene alone.[71] To understand the different resistance to DNA damage by UVC-irradiation between gelsolin- and neo-transfectants, we compared the cell cycle responses of transfectants by measuring the DNA content of S-phase synchronized cells by flow cytometry. Human urinary bladder cancer cells overexpressing gelsolin demonstrated an accumulation of cells in G2 and/or a protracted delay in G2 phase as compared to neo-transfected cells. Continuous treatment with caffeine, an agent that relieves the G2 delay, prevented the accumulation of cells in G2 phase, when given immediately after UVC-irradiation. Synchronized and neo-transfected-UMUC-2 cells showed the highest expression of cyclin B1 after 12 hours which rapidly fell, coincident with the completion of mitosis. However, the highest expression levels of cyclin B1 in gelsolin-overexpressing-UMUC-2 cells was delayed until 24 hours, and this cyclin was not degraded for this period. The activity of cdk1 (Histone 1) kinase in the neo-transfectants decreased after 20 hours, while gelsolin transfected-UMUC-2 cell cdk1 activity remained high during the G2 delay. These findings suggest that gelsolin may downregulate cdk1 kinase activation through a mechanism that affects the formation of cdk1/cyclin B1 complexes and that gelsolin may affect the G2 checkpoint function of cells, leading to growth suppression of various human tumors.

In searching for a common mediator elicited by the action of gelsolin, we compared the time course and the degree of production of diacylglycerol (DAG) in human urinary bladder cancer cells.[71] We observed a transient increase of DAG which activates protein kinase C in neo-transfectants, but not in gelsolin-transfectants. The time course of DAG accumulation is reported to be biphasic.[72] The two phases of DAG are due to the initial formation within one minute from PIP_2 via PI-PLC, and then PC via phospholipase D (PLD) and PAP (phosphatidate phosphohydrolase). Judging from the time course, our profile of DAG could indicate a reduced function of PLD. Recent studies indicate that PLD activities require the lipid cofactor PIP_2.[73] Interaction of gelsolin and PLD was also shown by Steed.[74] All these results indicate that gelsolin could inhibit not only the function of PLC but also that of PLD through this phosphoinositol lipid metabolism, and regulate the G2 checkpoint function of cancer cells.

There are several reports describing that protease inhibitors induce cell cycle alteration and have tumor suppressive function. Inhibitors of the chymotrypsin-like proteinase and trypsin induce arrest in G2-phase and in metaphase of HeLa cells.[75,76] In addition, cysteine protease inhibitors (for example stefin) are thought to have tumor suppressive properties,[77] and inhibition of ICE (IL-1β converting enzyme) suppresses AML blast proliferation.[78] We have recently found that activity of a protease, caspase 3 which plays an important role in apoptosis, was inhibited in a gelsolin-overexpressing human T-cell lymphoma cell line.[79] All these findings suggest that the function of gelsolin as a protease inhibitor is also related to the G2 extra-delay and tumor suppression induced by gelsolin.

Conclusion

We have shown that both mutant gelsolin His321 and human wild-type gelsolin, have a suppressive potential against the tumorigenicity of mouse ras-transformed cells (EJ-NIH/3T3). His321 inhibited PIP_2 hydrolysis by PLC-γ more strongly in vitro than did wild-type gelsolin because of its higher binding capacity to phosphoinositide. We have also demonstrated that the production of gelsolin was either lost or notably reduced in human gastric, colon, urinary bladder, and lung cancers. The gelsolin-overexpressing colon and urinary bladder cancer cells lost their tumorigenicity in nude mice. Gelsolin plays a key role as a tumor suppressor by regulating a G2 checkpoint function of cancer cells through phosphoinositol lipid and/or cysteine protease metabolism. The studies described demonstrate the potential of gene therapy using the gelsolin tumor suppressor in human urinary bladder carcinomas.

Acknowledgment

We thank Dr. Karl Riabowol for his stimulating discussions and comments on the manuscript.

References

1. Yin HL, Stossel TP. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein. Nature 1979; 281:583-6.
2. Yin HL, Albrecht JH, Fattoum A. Identification of gelsolin, a Ca^{2+}-dependent regulatory protein of actin gel-sol transformation, and its intracellular distribution in a variety of cells and tissues. J Cell Biol 1981; 91:901-6.
3. Yin HL, Kwiatkowski DJ, Mole JE et al. Structure and biosynthesis of cytoplasmic and secreted variants of gelsolin. J Biol Chem 1984; 259:5271-6.
4. Wegner A, Aktories K, Ditsch A et al. Actin-gelsolin interaction. Adv Exper Med & Biol 1994; 358:97-104.
5. Janmey PA. Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. Ann Rev Physiol 1994; 56:169-91.

6. Lassing I, Lindberg U. Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. Nature 1985; 314:472-4.
7. Janmey PA, Stossel TP. Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate. Nature 1987; 325:362-4.
8. Yu FX, Johnston PA, Sudhof TC et al. gCap39, a calcium ion- and polyphosphoinositide-regulated actin capping protein. Science 1990; 250:1413-5.
9. Yonezawa N, Homma Y, Yahara I et al. A short sequence responsible for both phosphoinositide binding and actin binding activities of cofilin. J Biol Chem 1991; 266:17218-21.
10. Kuzumaki N, Ogiso Y, Oda A et al. Resistance to oncogenic transformation in revertant R1 of human ras-transformed NIH 3T3 cells. Mol & Cell Biol 1989; 9:2258-63.
11. Pollack R, Osborn M, Weber K. Patterns of organization of actin and myosin in normal and transformed cultured cells. Proc Natl Acad Sci USA 1975; 72:994-8.
12. Vandekerckhove J, Leavitt J, Kakunaga T et al. Coexpression of a mutant β-actin and the two normal β- and γ-cytoplasmic actins in a stably transformed human cell line. Cell 1980; 22:893-9.
13. Hendricks M, Weintraub H. Tropomyosin is decreased in transformed cells. Proc Natl Acad Sci USA 1981; 78:5633-7.
14. Leavitt J, Gunning P, Kedes L et al. Smooth muscle α-actin is a transformation-sensitive marker for mouse NIH 3T3 and Rat-2 cells. Nature 1985; 316:840-2.
15. Müllauer L, Fujita H, Suzuki H et al. Elevated gelsolin and α-actin expression in a flat revertant R1 of Ha-ras oncogene-transformed NIH/3T3 cells. Biochem & Biophys Res Comm 1990; 171:852-9.
16. Fujita H, Suzuki H, Kuzumaki N et al. A specific protein, p92, detected in flat revertants derived from NIH/3T3 transformed by human activated c-Ha-ras oncogene. Exp Cell Res 1990; 186:115-21.
17. Müllauer L, Fujita H, Ishizaki A et al. Tumor-suppressive function of mutated gelsolin in ras-transformed cells. Oncogene 1993; 8:2531-6.
18. Dieffenbach CW, SenGupta DN, Krause D et al. Cloning of murine gelsolin and its regulation during differentiation of embryonal carcinoma cells. J Biol Chem 1989; 264:13281-8.
19. Vandekerckhove J, Bauw G, Vancompernolle K et al. Comparative two-dimensional gel analysis and microsequencing identifies gelsolin as one of the most prominent downregulated markers of transformed human fibroblast and epithelial cells. J Cell Biol 1990; 111:95-102.
20. Chaponnier C, Gabbiani G. Gelsolin modulation in epithelial and stromal cells of mammary carcinoma. Amer J Pathol 1989; 134:597-603.
21. Lanks KW, Kasambalides EJ. Dexamethasone induces gelsolin synthesis and altered morphology in L929 cells. J Cell Biol 1983; 96:577-81.
22. Kwiatkowski DJ. Predominant induction of gelsolin and actin-binding protein during myeloid differentiation. J Biol Chem 1988; 263:13857-62.
23. Moriya S, Yanagihara K, Fujita H et al. Differential expression of HSP90, gelsolin and GST-π in human gastric carcinoma cell lines. Internatl J Oncol 1994; 5:1347-1351.
24. Furuuchi K, Fujita H, Tanaka M et al. Gelsolin as a suppressor of malignant phenotype in human colon cancer. Tumor Targeting 1996; 2:277-283.
25. Kwiatkowski DJ, Westbrook CA, Bruns GA et al. Localization of gelsolin proximal to ABL on chromosome 9. Amer J Hum Gen 1988; 42:565-72.
26. Spruck CH 3rd, Ohneseit PF, Gonzalez-Zulueta M et al. Two molecular pathways to transitional cell carcinoma of the bladder. Cancer Res 1994; 54:784-8.
27. Orlow I, Lianes P, Lacombe L et al. Chromosome 9 allelic losses and microsatellite alterations in human bladder tumors. Cancer Res 1994; 54:2848-51.
28. Kroft SH, Oyasu R. Urinary bladder cancer: mechanisms of development and progression. Laboratory Investigation 1994; 71:158-74.
29. Tanaka M, Mullauer L, Ogiso Y et al. Gelsolin: A candidate for suppressor of human bladder cancer. Cancer Res 1995; 55:3228-32.

30. Tanaka M, Sazawa A, Shinohara N et al. Gelsolin gene therapy by retrovirus producer cells for human bladder cancer in nude mice. (submitted).
31. Merlo A, Gabrielson E, Mabry M et al. Homozygous deletion on chromosome 9p and loss of heterozygosity on 9q, 6p, and 6q in primary human small cell lung cancer. Cancer Res 1994; 54:2322-6.
32. Dosaka-Akita H, Hommura F, Fujita H et al. Frequent loss of gelsolin expression in non-small cell lung cancers of heavy smokers. Cancer Res 1998; 58:322-327.
33. Asch HL, Head K, Dong Y et al. Widespread loss of gelsolin in breast cancers of humans, mice, and rats. Cancer Res 1996; 56:4841-5.
34. Goldschmidt-Clermont PJ, Machesky LM, Baldassare JJ et al. The actin-binding protein profilin binds to PIP2 and inhibits its hydrolysis by phospholipase C. Science 1990; 247:1575-8.
35. Goldschmidt-Clermont PJ, Kim JW, Machesky LM et al. Regulation of phospholipase C-γ 1 by profilin and tyrosine phosphorylation. Science 1991; 251:1231-3.
36. Banno Y, Nakashima T, Kumada T et al. Effects of gelsolin on human platelet cytosolic phosphoinositide-phospholipase C isozymes. J Biol Chem 1992; 267:6488-94.
37. den Hartigh JC, van Bergen en Henegouwen PM, Verkleij AJ et al. The EGF receptor is an actin-binding protein. J Cell Biol 1992; 119:349-55.
38. Ishizaki A, Fujita H, Kuzumaki N. Growth-inhibitory functions of a mutated gelsolin (His321) in NIH/3T3 mouse fibroblasts. Exp Cell Res 1995; 217:448-52.
39. McGough A, Way M. Molecular model of an actin filament capped by a severing protein. J Structural Biol 1995; 115:144-50.
40. Yin HL, Iida K, Janmey PA. Identification of a polyphosphoinositide-modulated domain in gelsolin which binds to the sides of actin filaments. J Cell Biol 1988; 106:805-12.
41. Machesky LM, Goldschmidt-Clermont PJ, Pollard TD. The affinities of human platelet and Acanthamoeba profilin isoforms for polyphosphoinositides account for their relative abilities to inhibit phospholipase C. Cell Regulation 1990; 1:937-50.
42. Dadabay CY, Patton E, Cooper JA et al. Lack of correlation between changes in polyphosphoinositide levels and actin/gelsolin complexes in A431 cells treated with epidermal growth factor. J Cell Biol 1991; 112:1151-6.
43. Fukami K, Matsuoka K, Nakanishi O et al. Antibody to phosphatidylinositol 4,5-bisphosphate inhibits oncogene-induced mitogenesis. Proc Natl Acad Sci USA 1988; 85:9057-61.
44. Fujita H, Laham LE, Janmey PA et al. Functions of [His321]gelsolin isolated from a flat revertant of ras-transformed cells. European Journal of Biochemistry 1995; 229:615-20.
45. Way M, Gooch J, Pope B et al. Expression of human plasma gelsolin in *Escherichia coli* and dissection of actin binding sites by segmental deletion mutagenesis. J Cell Biol 1989; 109:593-605.
46. Kwiatkowski DJ, Janmey PA, Yin HL. Identification of critical functional and regulatory domains in gelsolin. J Cell Biol 1989; 108:1717-26.
47. Yu FX, Zhou DM, Yin HL. Chimeric and truncated gCap39 elucidate the requirements for actin filament severing and end capping by the gelsolin family of proteins. J Biol Chem 1991; 266:19269-75.
48. Sun HQ, Wooten DC, Janmey PA et al. The actin side-binding domain of gelsolin also caps actin filaments. Implications for actin filament severing. J Biol Chem 1994; 269:9473-9.
49. Cunningham CC, Stossel TP, Kwiatkowski DJ. Enhanced motility in NIH 3T3 fibroblasts that overexpress gelsolin. Science 1991; 251:1233-6.
50. Finidori J, Friederich E, Kwiatkowski DJ et al. In vivo analysis of functional domains from villin and gelsolin. J Cell Biol 1992; 116:1145-55.
51. Janmey PA, Lamb J, Allen PG et al. Phosphoinositide-binding peptides derived from the sequences of gelsolin and villin. J Biol Chem 1992; 267:11818-23.
52. Cantley LC, Auger KR, Carpenter C et al. Oncogenes and signal transduction [published erratum appears in Cell 1991 May 31; 65(5):following 914]. Cell 1991; 64:281-302.
53. Noh DY, Lee YH, Kim SS et al. Elevated content of phospholipase C-γ 1 in colorectal cancer tissues. Cancer 1994; 73:36-41.

54. Nomoto K, Tomita N, Miyake M et al. Expression of phospholipases γ1, β1, and δ1 in primary human colon carcinomas and colon carcinoma cell lines. Molecular Carcinogenesis 1995; 12:146-52.
55. Rodriguez Fernandez JL, Geiger B, Salomon D et al. Suppression of tumorigenicity in transformed cells after transfection with vinculin cDNA. J Cell Biol 1992; 119:427-38.
56. Gluck U, Kwiatkowski DJ, Ben-Ze'ev A. Suppression of tumorigenicity in simian virus 40-transformed 3T3 cells transfected with α-actinin cDNA. Proc Natl Acad Sci USA 1993; 90:383-7.
57. Prasad GL, Fuldner RA, Cooper HL. Expression of transduced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the ras oncogene. Proc Natl Acad Sci USA 1993; 90:7039-43.
58. Tikoo A, Varga M, Ramesh V et al. An anti-Ras function of neurofibromatosis type 2 gene product (NF2/Merlin). J Biol Chem 1994; 269:23387-23390.
59. Louis DN, Ramesh V, Gusella JF. Neuropathology and molecular genetics of neurofibromatosis 2 and related tumors. Brain Pathol 1995; 5:163-72.
60. Lutchman M, Rouleau GA. Neurofibromatosis type 2: A new mechanism of tumor suppression. Trends Neurosci 1996; 19:373-7.
61. Gilmore AP, Burridge K. Regulation of vinculin binding to talin and actin by phosphatidyl-inositol-4-5-bisphosphate. Nature 1996; 381:531-5.
62. Fukami K, Endo T, Imamura M et al. α-Actinin and vinculin are PIP2-binding proteins involved in signaling by tyrosine kinase. J Biol Chem 1994; 269:1518-22.
63. Hirao M, Sato N, Kondo T et al. Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: Possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. J Cell Biol 1996; 135:37-51.
64. Strand D, Jakobs R, Merdes G et al. The Drosophila lethal(2)giant larvae tumor suppressor protein forms homo-oligomers and is associated with nonmuscle myosin II heavy chain. J Cell Biol 1994; 127:1361-73.
65. Su LK, Vogelstein B, Kinzler KW. Association of the APC tumor suppressor protein with catenins. Science 1993; 262:1734-7.
66. Groden J, Joslyn G, Samowitz W et al. Response of colon cancer cell lines to the introduction of APC, a colon-specific tumor suppressor gene. Cancer Res 1995; 55:1531-9.
67. Cho KR, Fearon ER. DCC: linking tumour suppressor genes and altered cell surface interactions in cancer? Eur J Cancer 1995; 31A:1055-60.
68. Klingelhutz AJ, Hedrick L, Cho KR et al. The DCC gene suppresses the malignant phenotype of transformed human epithelial cells. Oncogene 1995; 10:1581-6.
69. Mali M, Andtfolk H, Miettinen HM et al. Suppression of tumor cell growth by syndecan-1 ectodomain. J Biol Chem 1994; 269:27795-8.
70. Santra M, Skorski T, Calabretta B et al. De novo decorin gene expression suppresses the malignant phenotype in human colon cancer cells. Proc Natl Acad Sci USA 1995; 92:7016-20.
71. Sakai N, Ohtsu M, Fujita H et al. Enhancement of G2 checkpoint function by gelsolin in human cancers. (In preparation).
72. Carsberg CJ, Ohanian J, Friedmann PS. Ultraviolet radiation stimulates a biphasic pattern of 1,2-diacylglycerol formation in cultured human melanocytes and keratinocytes by activation of phospholipases C and D. Biochem J 1995; 305:471-7.
73. Liscovitch M. Phospholipase D: Role in signal transduction and membrane traffic. J Lipid Mediators & Cell Signalling 1996; 14:215-21.
74. Steed PM, Nagar S, Wennogle LP. Phospholipase D regulation by a physical interaction with the actin-binding protein gelsolin. Biochem 1996; 35:5229-37.
75. Wojcik C, Schroeter D, Stoehr M et al. An inhibitor of the chymotrypsin-like activity of the multicatalytic proteinase complex (20S proteasome) induces arrest in G2-phase and metaphase in HeLa cells. Eur J Cell Biol 1996; 70:172-8.
76. Kozaki Y, Ariki T, Kubo M et al. trans-4-amidinocyclohexanecarboxylic acid 4-tert-butylphenyl ester, a trypsin inhibitor, blocks entry of HeLa cells from G2 phase into mitosis. Biological & Pharmaceutical Bulletin 1993; 16:829-33.

77. Calkins CC, Sloane BF. Mammalian cysteine protease inhibitors: biochemical properties and possible roles in tumor progression. Biological Chemistry Hoppe-Seyler 1995; 376:71-80.
78. Estrov Z, Black RA, Sleath PR et al. Effect of interleukin-1β converting enzyme inhibitor on acute myelogenous leukemia progenitor proliferation. Blood 1995; 86:4594-602.
79. Ohtsu M, Sakai N, Fujita H et al. Inhibition of apoptosis by the actin-regulatory protein gelsolin. EMBO J 1997; 16:4650-4656.

Chapter 10

PIP_2: A Protooncogenic Phospholipid

Hiroshi Maruta

Introduction: My Personal Link to Actin-Cytoskeleton, G Proteins and Cancer

In the summer of 1974 at Cold Spring Harbor Symposium, Klaus Weber and his colleagues reported[1] that actin stress fibers or cables rapidly disappear when normal fibroblasts (Swiss or Balb/c 3T3 cells or rat embryo REF cells) are transformed with SV40 virus (MSV). The impact that his talk created was tremendous, in particular on me. Like muscle fibers, nonmuscle actin-cytoskeleton (microfilament) is a complex of actomyosin (actin filament and myosin ATPases) and many other actin/myosin-binding proteins (AMBs). A few months later, hoping to find a molecular link(s) between actin-cytoskeleton and cancer sometime in the future, I joined Ed Korn's lab at NIH to start our very basic, biochemical study on myosin ATPases and other actin-binding proteins isolated from a soil ameba called Acanthameba. The reason why we chose this unique ameba as a protein source was very simple: at that time this ameba was the only eukaryotic nonmuscle cell source that could be grown in a huge scale in an axenic culture medium so that we can identify, purify and characterize myosins, AMBs or other proteins efficiently without any contamination of bacterial proteins, and no one was aware then that yeast also contains actin, myosins and AMBs. Although our biochemical study on the Ameba myosins and AMBs was apparently very remote from cancer research, we established the first foundation for biochemistry of nonmuscle myosin ATPases and AMBs from Acanthameba which were found later in most of mammalian nonmuscle cells, and even human cancer cells.

My major contribution to the Acanthameba actin-cytoskeleton study (1974-1980) was to establish that (i) this single ameba contains at least two distinct groups of myosins called myosin I (single-headed) and myosin II (double-headed), (ii) the actin-activation of all three myosin I Mg^{2+} ATPases (A, B and C) requires a specific phosphorylation of each myosin heavy chain by a unique Ser/Thr kinase called MIHCK, and (iii) their ATPase activity resides solely in their heavy chain.[2-4] Subsequent cloning of cDNAs for these myosin I heavy chains has revealed that all these myosin I ATPases contain the SH3 domains at the C-termini.[5] As discussed in detail later, a novel F-actin binding/ PIP_2-binding tumor suppressor called HS1 and its related protein EMS1/cortactin share 50% sequence identity with these myosin I ATPases in the C-terminal SH3 domains.[6] Furthermore, recent cloning of the MIHCK from another ameba called Dictyostelium has revealed that MIHCK is closely related to the mammalian CDC42/Rac-activated Ser/Thr kinase PAK, and it is indeed activated by both CDC42 and Rac GTPases.[7] Realization of the direct link between this myosin I heavy chain kinase and these Rho family GTPases took nearly two decades. For detail of MIHCK/ PAK family, see the preceding chapter by John Hammer and Graham Cote.

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

Unfortunately, genetic analysis of these proteins' physiological function in Acanthameba is very difficult as no haploidal cell line of this ameba is available. Thus, I then decided to switch my subject to the slime molds called Dictyostelium and Physarum with which genetic manipulation of genes encoding these actin-cytoskeletal proteins is possible, and worked at Max-Planck-Institute in Martinsried near Munich (l980-1984). My major contribution there was to establish that (i) fragmin, a Ca^{2+}-dependent F-actin severing protein from Physarum, acts as an F-actin plus-end capper when it forms a stable 1:1 complex with actin monomer (G-actin) in a Ca^{2+}-dependent manner, and that (ii) its capper activity is regulated by a specific phosphorylation of G-actin in the complex.[8-10] As discussed in detail by Helen Yin and her colleagues (chapter 2), fragmin is a prototype of the first mammalian Ca^{2+}-dependent F-actin severer called gelsolin which, unlike fragmin, contains a pair of actin-binding domains.[10,11] Furthermore, as discussed in detail by Noboru Kuzumaki and his colleagues (chapter 9), a mutant of gelsolin which caps the F-actin plus-end and also binds PIP_2 turns out to be a tumor suppressor that blocks oncogenic action of Ras mutants.[12] However, it still remains to be determined whether either F-actin capping/severing or PIP_2-binding or both are required for its anti-Ras action.

In the fall of 1987, another great impact was stirred by Frank McCormick and his colleague. They reported that the oncogenic Ras mutants are no longer susceptible to the action of Ras GAPs that attenuate the mitogenic signaling of the normal Ras GTPases.[13] Then I realized the time was just ripe for me to tackle the oncogenic Ras directly, and decided to switch swiftly my major subject to Ras in mammalian cells. I crossed the equator to join the Melbourne Branch of Ludwig Institute for Cancer Research. However, this sudden change in the field was not so drastic as it sounds. For I spent a few years at both Max-Planck-Institute and Yale University to work on the cytoskeletal GTPases called α- and β-tubulins/microtubules,[14,15] and at University of California (San Diego) to work on Dictyostelium Ras genes. As discussed in chapter 11, the "ultimate" goal of our Ras research has been (and still is) to design/ create the "Super GAP", a special Ras GAP mutant or drug, that can stimulate the intrinsic GTPase activity of these oncogenic Ras mutants, thereby converting the Ras mutants to the inactive GDP-bound forms. A decade ago, many people used to call this task a "mission impossible". However, since the 3D structure of a Ras GAP complex has been determined this year as discussed in detail by Klaus Scheffzek and Fred Wittinghofer (chapter 13), the "impossible" has begun to look like a "possible" at least in a few experts' eyes...

The major contribution of my group to the Ras field during the last several years were (i) the structure-function relationship study on Ras GAPs and other effectors of Ras, in particular the tumor suppressor NF1 and the effector Raf through the site-directed mutational analysis, and (ii) the development of several distinct anti-Ras tumor suppressors including the short Ras-binding fragments of NF1 and Raf[16,17] as well as cytoskeletal tumor suppressors including NF2/Merlin, the plus-end F-actin cappers (tensin and cytochalasins), the PIP_2-binders (HS1 and cofilin mutants) and the large GTPase p190-A.[18-21]

Finally our recent work on HS1 and EMS1, F-actin cross-linking SH3 proteins, led us to a surprise discovery of the first clue to our understanding of how Ras causes a dramatic change in actin-cytoskeleton during malignant transformation, without any suppression of genes encoding myosins or other components of actin-cytoskeleton. PIP_2, which is overproduced upon Ras-transformation, blocks the EMS1-actomyosin II interaction by inhibiting the F-actin cross-linking activity of EMS1. HS1 reverses Ras-induced malignancy, presumably by sequestering PIP_2, restoring the ability of EMS1 to bind actomyosin II and inducing the plus-end F-actin capping. In this chapter I will discuss mainly a few PIP_2-binding/F-actin capping tumor suppressors that block the oncogenic Ras/Rac pathways, providing the first evidence supporting a new concept that PIP_2 is a unique acidic phospholipid of "oncogenic" potential. For details of two other PIP_2-binding tumor suppressors

(vinculin and α-actinin) that block the oncogenic action of SV40 virus, see the preceding chapters 7 and 8.

Ras Signaling Network Controlling Actin-Cytoskeleton

Not only SV40 virus, but also a variety of oncogenes including Ras and Src cause the dramatic changes in the actin-cytoskeleton of fibroblasts and epithelial cells.[22] The previous finding that Src requires Ras for oncogenic action indicates that Ras acts downstream of Src.[23] Both Ras and Src downregulate the expression of fibronectin,[24,25] and a specific fibronectin-integrin (α5) interaction is required for actin stress fiber formation.[26] Thus, suppression of fibronectin gene appears to be one of the Ras/Src pathways that lead to the disruption of stress fibers upon malignant transformation. At least two distinct drugs, azatyrosine and oxamflatin, that reverse Ras-transformation can reactivate fibronectin gene.[24,27] However, how Ras/Src suppress this gene still remains to be determined. Moreover, it was also shown previously that (i) both Ras and SV40 virus downregulate several other genes encoding F-actin binding proteins such as α-actinin, vinculin and gelsolin which are associated with stress fibers or focal adhesion plagues where cells adhere to the substratum,[28] and that (ii) overexpression of these genes suppresses SV40/Ras-induced malignancy.[12,29,30] These findings indicate that these F-actin binding proteins play the critical role in both the formation of actin stress fibers/focal adhesions and prevention of normal cells from malignant transformation. However, how Ras/ SV40 downregulates these genes, and how overexpression of one of these AMBs alone is sufficient to block the oncogenic action of Ras or SV40 virus still remain in a big mystery. We have demonstrated that the N-terminal SH2/SH3/SH2 domain of GAP1, a Ras GAP of 120 kDa, is capable of transforming the normal NIH 3T3 cells, indicating that GAP1 is an oncogenic effector of Ras.[31] Furthermore, the N-terminal domain of GAP1 disrupts the actin stress fibers and cell adhesion to fibronectin.[32] However, how this domain causes the disassembly of the actin cytoskeleton still remains basically unknown. At least the involvement of Tyr-phosphorylated p190-A which binds the SH2 domains of GAP1 has been excluded, simply because we found that p190-A is an anti-Ras tumor suppressor, reverting Ras-transformed NIH 3T3 cells to flat fibroblasts which show massive stress fibers.[21] Shortly after microinjection of oncogenic Ras mutants into normal fibroblasts, a rapid disruption of the stress fibers takes place and is followed by induction of membrane ruffling.[33] These observations clearly indicate that Ras-induced suppression of the AMB genes is not absolutely required for the rapid changes in actin-cytoskeleton. How does Ras cause such a re-arrangement of actin-cytoskeleton?

The recent finding that oncogenic Ras mutants require both Rac and Raf for their oncogenicity indicates that both Rac and Raf act downstream of Ras.[17,34,35] Although oncogenic mutants of Raf such as v-Raf cause a significant change in actin cytoskeleton organization, the pathway responsible for this change still remains unknown. However, it does not appear to involve RhoB at least, because unlike Ras-induced cytoskeletal transformation, Raf-induced transformation cannot be reversed by farnesyltransferase inhibitors which block the farnesylation of both RhoB and Ras.[36] Unlike other Rho GTPases which are required for stress fiber formation,[37] RhoB appears to be involved in the disassembly of stress fibers.[36] An oncogenic mutant (V12) of Rac induces membrane ruffling and a few other changes in actin-cytoskeleton.[38] A constitutively active mutant of PAK, a Rac/CDC42-activated Ser/Thr kinase, causes a disruption of stress fibers in the same manner as do the oncogenic Rac mutants.[39] These observations indicate that the PAK pathway is involved in the disassembly of stress fibers. However, the substrate of PAK that is responsible for the regulation of stress fiber formation has not been identified as yet. As discussed in detail by Chandra Kumar (chapter 20), the drug SCH51344 which reverses Ras transformation can block Ras/Rac-induced membrane ruffling, but not the JNK activation by Ras/Rac.[40,41] These

observations suggest the critical role of RhoB/Rac-induced disruption of stress fibers and Ras/Rac-dependent induction of membrane ruffling in Ras-induced transformation. A novel Rac-binding protein called POR1 appears to be responsible for membrane ruffling.[42] However, how POR1 induces membrane ruffling still remains to be clarified. Interestingly, as discussed in chapter 2, Rac induces uncapping of actin filaments (F-actin) at the plus-ends by activating PI-4/PI-5 kinases which produce PIP_2.[43]

Cytoskeletal Tumor Suppressors

The critical question is then whether the plus-end uncapping of F-actin is essential for the oncogenicity of Ras/Rac, or whether the plus-end capping is sufficient for suppressing the Ras/Rac-induced malignant transformation. Since gelsolin mutant caps the plus-ends of actin filaments and binds PIP_2, it is conceivable that the gelsolin could exert its anti-Ras tumor suppressor action by both capping the plus-ends directly and sequestering PIP_2, which is essential for Ras/Rac-induced uncapping. However, since gelsolin has at least an additional unique biological activity, that is to say, F-actin severing activity, it is not clear as yet which function of gelsolin actually contributes to its anti-tumor action. Besides, so far no experimental evidence has been provided indicating that its actin-binding is essential for the anti-Ras action of the gelsolin mutant.

To clarify these two points, and further understand the molecular mechanism underlying the tumor suppression by actin-binding proteins, we have investigated the possible anti-Ras suppressor activity of functionally much simpler molecules which have no F-actin severing activity.

NF2/Merlin

One of such molecules is NF2/Merlin of 595 amino acids which shares more than 50% sequence identity with the plus-end capper radixin.[44,45] In fact, like radixin, NF2 is colocalized with the actin-contractile ring during mitosis, and the cell-cell adhesion site. In addition, NF2 is localized in leading edges and membrane ruffles as well as cell/substratum adhesions, but not associated with stress fibers of fibroblasts.[46,47] NF2 belongs to an actin-binding protein family called ERM which includes ezrin, radixin and moesin, and binds a hyaluronate-receptor called CD44 in a Rho-dependent manner.[48] CD44 splice variants are responsible for metastasis of pancreatic cancers and many other carcinomas.[49] Dysfunction or deletion of NF2 is tightly associated with neurofibromatosis type 2, the development of CNS tumors including schwannomas and meningiomas. Since dysfunction or deletion of NF1 also causes the development of schwannomas, and the Ras-binding fragments of NF1 reverse Ras-induced malignant transformation,[16,17] it is quite conceivable that NF2 is also a good candidate tumor suppressor that blocks oncogenic Ras signaling. As discussed in detail later, NF2 binds F-actin, but unlike gelsolin, it does not sever F-actin. Thus, we have overexpressed the full-length NF2, its N-terminal half (NF2-N, residues 1-359) and C-terminal half (NF2-C, residues 354-595) in v-Ha-Ras-transformed NIH 3T3 cells, and found that either NF2-N or NF2-C alone suppresses Ras-induced malignant transformation, but the anti-Ras tumor suppressor activity of the full-length NF2 is significantly higher than that of either NF2-N or NF2-C alone.[18] These observations indicate that (i) the F-actin binder NF2 is indeed able to suppress Ras transformation without severing F-actin, and that (ii) it contains at least two separable tumor suppressor domains, i.e., the N- and C-terminal halves. It should be noted that the expression level of endogenous NF2 is not suppressed by Ras.[18] This is a sharp contrast to the expression levels of the three cytoskeletal tumor suppressors (α-actinin, vinculin and gelsolin) which are markedly reduced by Ras or SV40 virus as discussed before. How do these two distinct NF2 domains independently block the oncogenic Ras signaling?

The N-terminal half of NF2 is responsible for its binding to CD44 as are the corresponding domains of other ERM family proteins.[48] Recently the N-terminal half of ezrin (residues 1-333) alone was shown to bind F-actin, although the full-length ezrin requires at least both the C-terminal residues (534-586) and the N-terminal residues[13-30] for its binding to F-actin.[50] We have also found that the NF2-N alone is able to bind F-actin.[18] Currently, using a series of deletion mutants of NF2, we are identifying the F-actin binding motifs in the NF2-N in an attempt to determine whether the F-actin binding is essential for the anti-Ras action. Furthermore, since the N-terminal F-actin binding domain of NF2 appears to be highly homologous to the corresponding domain of the F-actin capper radixin and other EMR family proteins, it would be of great interest to determine whether NF2 is also a plus-end F-actin capper or not. It has recently been revealed that the binding of the three full-length ERM family proteins (ezrin, radixin and moesin) to CD44 depends on either PIP_2 or the Rho-GTP complex which activates a PIP_2 producing enzyme, PI-5 kinase, although their N-terminal halves alone are able to bind CD44 directly, without PIP_2 or the Rho-GTP complex, indicating that these three ERM family proteins are PIP_2-binders, and PIP_2 unmasks their N-terminal CD44-binding domains by unfolding these molecules.[48] We recently found that at least the NF2-N binds PIP_2 (Tikoo A, Maruta H, unpublished observation), and are currently identifying the PIP_2-binding domain(s) of NF2 and other ERM family proteins. It is conceivable that the PIP_2-binding activity also contributes to the anti-Ras action of NF2. Needless to say, it would be of great interest to see whether other three ERM family proteins also display any tumor suppressor activity.

Interestingly, the NF2-C contains two overlapping Pro-rich motifs (residues 478-495) which are supposed to bind the SH3 domain of a certain protein(s). A lymphoblastic cell line called GUS5722 derived from a NF2 patient carries a NF2 mutant that lacks a 78 amino acid segment (residues 447-524) which includes the Pro-rich motifs in the C-terminal half,[44] suggesting that this missing segment of NF2-C plays a critical role in its tumor suppressor activity. Thus, using a yeast two-hybrid system, we are currently identifying another binding partner(s) of NF2-C among the SH3 protein family. It would be of great interest to examine whether an NF2-C mutant which lacks the Pro-rich motifs is still active as an anti-Ras tumor suppressor. Disruption of NF2 gene (homozygosity) in mice (and the nematode *C. elegans*) causes an embryonic lethality as does that of NF1,[51] indicating that NF2 is involved not only in the growth regulation, but also in the differentiation events of a certain type(s) of cells during the embryogenesis of these animals.

Tensin

Like radixin, tensin is a plus-end F-actin capper.[52] Chicken tensin is a large actin-binding protein of 1744 amino acids,[53] and has an SH2 domain (residues 1471-1580) towards the C-terminus that determines the localization in focal adhesion plaque.[54] The tensin contains three distinct actin-binding domains (I, II and III). The domain III (residues 888-989) is responsible for capping the plus-end of actin filament.[52] Two other actin-binding domains I (residues 1-263) and II (residues 264-463) appear to bind the side of actin filament, cross-linking actin filaments, but do not affect the kinetics of actin polymerization at all.[52] We found that overexpression of full-length tensin from chicken strongly suppresses v-Ha-Ras-induced malignant transformation.[19] Again, the expression level of the endogenous tensin is not affected at all by Ras, indicating that like NF2, tensin blocks the oncogenic Ras signaling by a novel mechanism. Which domains of tensin are involved in the anti-oncogenic action? To see whether the plus-end capping domain III of tensin alone is sufficient for its anti-Ras tumor suppressor activity, we are currently examining the effect of this domain alone on Ras transformation. Interestingly, a novel candidate tumor suppressor called P-TEN is not only a protein phosphatase, but also related to tensin in the

domain I,[55-55b] suggesting the possibility that the domain I of tensin might also contribute to the anti-oncogenicity of tensin. Needles to say, it would be of great interest to determine whether P-TEN can also suppress Ras-induced malignancy. Mutations of P-TEN were frequently found in prostate, brain and breast cancers.[55,55a] I recently realized that both the chicken tensin domain I and P-TEN carry a similar "putative" PIP_2-binding motif (tensin residues 166-173, and P-TEN residues 123-130). In fact, we found recently that the tensin domain I binds PIP_2[19] and are currently testing the possible PIP_2-binding activity of P-TEN. Interestingly, mutations within this "putative" PIP_2-binding motif of P-TEN have been shown to abolish its protein phosphatase activity as well as PIP3 phosphatase acitivity.[55c]

Cofilin Mutants

Avri Ben-Ze'ev's group first showed that overexpression of either vinculin or α-actinin suppresses SV40-induced malignant transformation of NIH 3T3 cells.[29,30] Although these two F-actin cross-linking proteins do not cap the plus-ends of actin filaments, both not only bind PIP_2 but also their F-actin binding activity depends on PIP_2.[56-58] Thus, indirectly they could block uncapping of the plus-ends of actin filaments by sequestering PIP_2. Three other cytoskeletal proteins, i.e., the gelsolin mutant, NF2 and tensin cap directly the plus-ends, and bind PIP_2.[11,18,19] These findings altogether tend to lead us to a rather "provocative" conclusion that if there is any common mechanism underlying the suppression of SV40/Ras-induced malignant transformation by these five distinct F-actin binding proteins, it must be either capping the plus-ends directly, or blocking the uncapping of the plus-ends indirectly by sequestering PIP_2.

To test this hypothesis, we examined a possible effect of two cofilin mutants which still bind PIP_2, but whose actin-binding is partially or completely impaired. Cofilin is an ubiquitous phosphoprotein of 166 amino acids which is present in all eukaryotes from yeast to human.[59,60] Like gelsolin, cofilin is an F-actin severing protein, but its severing activity is regulated by H^+, instead of Ca^{2+}.[59] When cofilin is phosphorylated at Ser 3, it no longer binds F-actin, and stays in either cytosol or nuclei.[61] However, when it is dephosphorylated, it is translocated into the plasma membrane area, forming ruffles, and is also associated with actin-contractile ring during cytokinesis.[61] Recently a Rac-dependent cofilin kinase was identified as a member of the LIM kinase/Kiz-1 family (refs. 62, 63). When the Ser 3 of cofilin is replaced by an acidic amino acid such as Asp, such a mutant mimics the phosphorylated form and no longer binds F-actin.[61] The residues 104-115 of cofilin are also involved in the actin-binding.[64] When both Lys 112 and Lys 114 are replaced by Gln residues, this double-mutant no longer binds actin, but still binds PIP_2.[64] The replacement of Lys114 by a Gln residue alone impairs partially the actin-binding, but not the PIP_2-binding.[64] We found that overexpression of both mutants of cofilin suppresses v-Ha-Ras-induced malignancy, but the tumor suppressing activity of the single-mutant is significantly stronger than that of the double-mutant.[20] These observations have proved for the first time that PIP_2-binding/sequestering is indeed sufficient for the suppression of Ras transformation, and also suggested that the F-actin severing/capping activity appears to enhance the tumor suppressor activity. Which pathways or events that PIP_2 could mediate are responsible for the Ras-induced malignant transformation? At least one PIP_2-induced event, i.e., uncapping of the actin filament plus-ends, seems most likely to be involved. PIP_2 inactivates several distinct F-actin capping proteins such as profilin and CapG which cap the plus-ends of actin filaments, and releases these cappers from this end.[43] The uncapping leads to a rapid actin polymerization at this fast-growing end for the elongation of actin filaments.[43] This notion has begun to refocus our attention to a variety of F-actin plus-end cappers (proteins or chemicals) that I used to handle more than a decade ago.

Cytochalasins

There is a large family of antibiotics called "cytochalasins", of which at least 24 distinct members are known to cap the plus-end of actin filament and subsequently block membrane ruffling.[65] Among them, cytochalasin D (CD) is so far the most potent plus-end capper. 0.2 μM CD is sufficient to cap almost completely the plus-ends of actin filaments (25 μM) in vitro and block membrane ruffling in vivo.[65] We found that CD at the same concentration is sufficient to suppress v-Ha-Ras-induced malignant transformation.[19] These observations have almost proved that (i) capping at the plus-ends is sufficient for the suppression of Ras-induced malignancy, and that (ii) the Ras/Rac/PIP_2-induced uncapping at the plus-ends is required for the oncogenicity of Ras.

However, the careful comparison of biological properties between CD and another cytochalasin called Chetoglobosin K (CK), has revealed that CD must exert its variety of biological functions, not only through its capping of the pluds-ends, but also through another as yet uncharacterized action.[65] For, unlike CD and most of other cytochalasins, CK does not cause either the rounding-up of cells or the contraction of actin cables, although CK caps the plus-ends, and blocks membrane ruffling.[65] Thus, we could not entirely exclude a rather remote possibility that CD suppresses Ras transformation through some unknown action(s) other than the plus-end capping. Thus, we examined the tumor suppressing effect of CK as well. In support of our "cappers are tumor suppressors" hypothesis, CK (0.2 to 0.6 μM) is indeed able to suppress v-Ha-Ras-induced malignant transformation as is CD.[19] Thus, it is now more likely that the plus-end capping is sufficient for the suppression of Ras transformation.

CK is a plant growth inhibitor and toxin isolated from Diplodia macrospore by Horace Cutler and his colleagues in 1980.[66] This fungus is a pathogen that causes ear rot and stalk rot of corn (Zea mays L.). His group subsequently produced a metabolite that acts as both a potent inhibitor of wheat coleoptile growth and a mycotoxin. Further work indicated that the metabolite is a cytochalasin, and the physical and chemical data identified the metabolite CK, a cytochalasin possessing an indol-3-yl attached group.[66] The chemical structure of CK is shown in Figure 10.1.

Unfortunately, however, CD or CK alone shows an undesirable "side" effect that causes apoptosis of both normal and Ras-transformed NIH 3T3 cells, although normal cells appear to be more resistant to these drugs than Ras-transformants.[19] Since overexpression of the F-actin capper tensin does not cause any apoptosis, it is clear that this side effect is not simply a consequence of the plus-end capping of actin filament, but is due to other as yet uncharacterized action of CD/CK. To our great relief, however, such an apoptosis can be completely abolished by a specific inhibitor of ICE/Ced3 protease family called Z-Asp-CH_2-DCB which also blocks the apoptosis caused by serum-starvation, but not the TSA-induced apoptosis.[19] As discussed in detail by Minoru Yoshida and his colleague (chapter 22), TSA (Trichostatin A) is a specific inhibitor of histone deacetylase, and upregulates gelsolin gene,[67] thereby suppressing both Ras-induced cytoskeletal transformation and malignant growth (ref. 68; Tikoo A and Maruta H, unpublished observation). Thus, for the treatment of Ras-associated cancers, these cytochalasins have to be used in the combination with this ICE/Ced3 inhibitor or other compounds that block the cytochalasin-induced apoptosis.

PIP_2: A Second Messenger Mediating Ras-Induced Disruption of EMS1-Actomyosin II Complex

Both HS1 (Hematopoiesis Specific) and EMS1/cortactin belong to a growing family of cytoskeletal SH3 proteins.[6] Most of the proteins in this family, except for α-spectrin, each contains a single SH3 domain at the C-terminus and an F-actin binding domain(s) at the N-terminus. In α-spectrin, the SH3 domain is localized in the center of this molecule, and

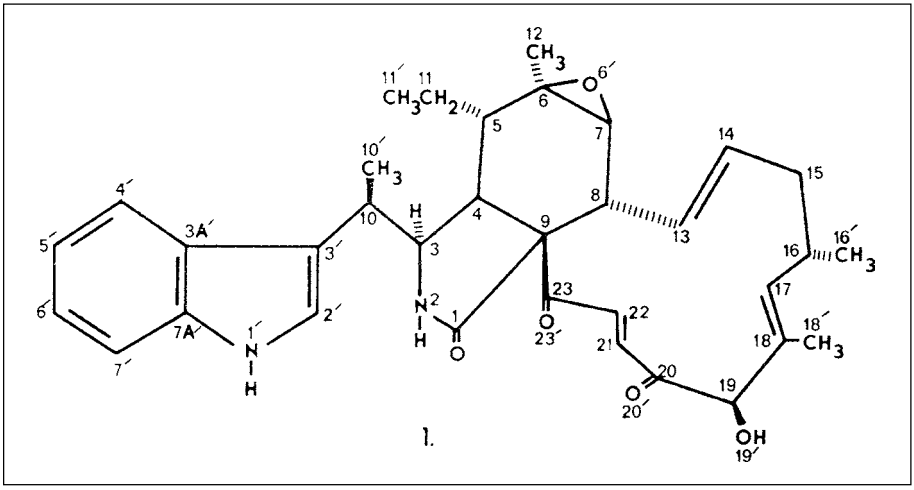


Fig. 10.1. Structure and numbering system of chetoglobosin K.

the actin-binding domains are localized in β-spectrin which forms a tight heterodimer with α-spectrin.[69] In addition to the above three proteins, this family includes a unique single-headed myosin (myosin I) subfamily, yeast actin-binding protein called ABP1, RVS167 and CDC15.[6,70] So far the actin-binding domain of myosin I, which is the actin-activated Mg^{2+}-ATPase head, has been best characterized biochemically,[3-6] as discussed in detail by John Hammer and Graham Cote (chapter 3).

The most intriguing feature that HS1 and EMS1 share in common is that both contain tandem repeats of a unique 37-amino acid helix-turn-helix (HTH) motif in their N-terminal halves.[6,71,72] One HTH motif shares 60-70% sequence identity with another in both HS1 and EMS1 (see Fig. 10.2). HS1 has three repeats, whereas EMS1 has six repeats. We found that (i) all three HS1 HTH motifs bind F-actin, whereas only 4th EMS1 HTH motif binds F-actin,[6] and that (ii) both HS1 and EMS1 cross-link F-actin through these HTH motifs, although the F-actin cross-linking activity of HS1 is much weaker than that of EMS1.[6,20,73] EMS1 appears to cross-link F-actin by forming an EMS1 homodimer.[73] More interestingly, as illustrated in Figure 10.2, each of all these four F-actin binding HTH repeats of HS1 or EMS1 contains a "putative" PIP_2-binding motif (R/KYGV/I R/KDR/K). We then found that HS1 shows a much higher affinity for PIP_2 than EMS1, although both bind PIP_2 significantly in vitro.[20] Furthermore, PIP_2 inhibits almost completely the F-actin cross-linking activity of EMS1 (0.1 μM), whereas HS1 at the same concentration does not cross-link F-actin at all, but reverses completely the PIP_2 effect on the F-actin cross-linking activity of EMS1 by sequestering PIP_2.[20] These observations indicate that HS1 can restore the ability of EMS1 to cross-link F-actin which is inhibited by PIP_2 that is overproduced during Ras-induced malignant transformation. As summarized in Figure 10.3, Ras activates PI-3 kinase which in turn activates Rac through a Rac GDS,[74] and Rac then activates PI-4/PI-5 kinase that produce PIP_2.[43] Does Ras disrupt the interaction of EMS1 with F-actin or actin-cytoskeleton by overproducing PIP_2 during malignant transformation? If so, can an ectopic expression of HS1 in Ras transformed cells reverse both the PIP_2 effect on EMS1 and the malignancy by sequestering PIP_2?

EMS1 is a ubiquitous protein expressed in many mammalian cells including both normal and Ras-transformed NIT 3T3 cells, whereas HS1 is expressed only in hematopoietic

			* * *			
HS1-1	82-118	GYGG	*RFGVERDR*	MDKSAVGH	EYVA EVEKHS	SQTDA
HS1-2	119-155	GFGG	*KYGVERDR*	ADKSAVGF	DYKGEVEKHT	SQKDY
HS1-3	156-192	GFGG	*RYGVERDK*	WDKAALGY	DYKGETEKHE	SQRDY
			* * *			
EMS1-1	83-119	GYGG	KFGVEQDR	MDKSAVGH	EYQS KLSKHC	SQVDS
EMS1-2	120-156	GFGG	KFGVQMDR	VDQSAVGF	EYQGKTEKHA	SQKDY
EMS1-3	157-193	GFGG	KYGVQADR	VDKSAVGF	DYQGKTEKHE	SQRDY
EMS1-4	194-230	GFGG	*KYG I D KDK*	VDKSAVGF	EYQGKTEKHE	SQKDY
EMS1-5	231-267	GFGG	KFGVQTDR	QDKCALGW	DHQEKLQLHE	SQKDY
EMS1-6	268-304	GFGG	KFGVQ SER	QDSAAVGF	DYKEKLALHE	SQQDY

Fig. 10.2. "Putative" PIP_2-binding motifs in the actin-binding domains of HS1 and EMS1.

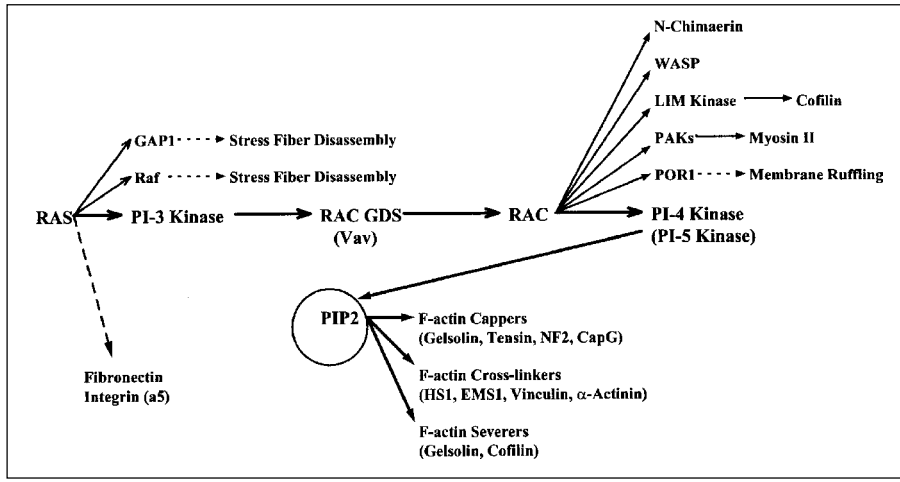


Fig. 10.3. RAS/RAC signaling network controlling actin-cytoskeleton.

cells and not in fibroblasts such as NIH 3T3 cells.[71,72] First of all, we found that overexpression of full-length HS1 of 486 amino acids in v-Ha-Ras-transformed NIH 3T3 cells suppresses their malignancy, i.e., anchorage-independent growth in soft agar.[20] Second, in normal NIH 3T3 cells, we found a stable complex of EMS1 with myosin II through F-actin.[20] Surprisingly, in Ras-transformed cells, this EMS1-actomyosin II complex disappears completely, although the expression levels of EMS1, actin and myosin II are not affected by Ras-transformation at all.[20] Lastly, in the HS1 overexpressing revertants derived from Ras-transformants, we found again the stable complex of EMS1 and actomyosin II.[20] These observations indicate clearly that (i) the PIP_2-binder HS1 is an anti-Ras tumor suppressor as is the cofilin mutants, and that (ii) Ras causes a disruption of the EMS1-actomyosin II complex that can be restored by HS1. Thus, the F-actin cross-linking/PIP_2-binding protein EMS1 became the first actin-cytoskeletal protein whose interaction with the actomyosin complex/micro-filament has been shown to be blocked by the oncogenic Ras signaling, most

likely through the PI-3 kinase/Rac-induced overproduction of PIP_2. In support of the notion that Ras exerts this effect on the actin-cytoskeleton through PIP_2 at least in part, we found that the EMS1-actomyosin II complex can be restored by the treatment of Ras transformants with drug SCH51344, which blocks the Rac-induced membrane ruffling.[20,41,74,75]

HS1 contains several distinct functional domains: (i) The N-terminal three actin-binding HTH repeats (residues 82-192), each carrying a PIP_2-binding motif, (ii) the nuclear localization sequence (NLS, residues 263-274), (iii) the Pro-rich motif (PLLP) which binds the SH3 domain of the Tyr kinase Lck,[76] (iv) the two critical Tyr residues at positions 378 and 397 whose phosphorylation by the Tyr kinase Syk is required for the B-cell antigen receptor (BCR)-mediated apoptosis of B-lymphocytes,[77] and (v) the C-terminal SH3 domain. This SH3 domain shares 80% sequence identity with the corresponding EMS1 SH3 domain at the C-terminus, and both bind a novel cytoplasmic protein of 45 kDa called Pash3.[78] We found that dynamin GTPase also binds the SH3 domain of EMS1, but not HS1 in normal NIH 3T3 cells, whereas the EMS1-dynamin interaction is greatly reduced upon the Ras transformation (He H, Liu JP and Maruta H, unpublished observations). Thus, in the cytoplasm of the normal fibroblasts, EMS1 forms a large complex consisting of at least actin filaments, myosin II, Pash3 and dynamin, and upon Ras transformation this EMS1 complex is disassembled. In the HS1 overexpressing revertant cells, the same EMS1 complex is reassembled, whereas HS1 forms a complex only with PIP_2 and Pash3.

To determine which domain(s) of HS1 are required for the anti-Ras action, we have generated a series of deletion/substitution mutants of HS1. The deletion of the SH3 domain translocates HS1 from the cytoplasm to the nucleus, and completely abolishes its tumor suppressor activity,[20] indicating that (i) the SH3 domain somehow masks the NLS to prevent full-length HS1 from the nuclear translocation, and (ii) the SH3 domain is required for HS1 to suppress Ras transformation. The deletion of all three actin-binding HTH repeats also abolishes the anti-Ras tumor suppressor activity of HS1, although this mutant remains in the cytoplasm.[20] The corresponding first three HTH repeats of EMS1 (residues 83-193) bind neither F-actin nor PIP_2. Replacement of the HS1 first actin-binding HTH repeats by the EMS1 first three HTH repeats also abolishes the anti-Ras action of HS1. This HS1/EMS1 chimera remains in the cytoplasm but fails to suppress Ras transformation.[20] These observations clearly indicate that these three actin/PIP_2-binding HTH repeats are also essential for HS1 to suppress Ras transfomation, although unlike the SH3 domain they are not required for the cytoplasmic localization of HS1 at all. However, it still remains to be clarified which function of these HTH repeats, the F-actin binding or PIP_2-binding alone, or both are required for the anti-oncogenicity of HS1. To solve the above puzzle, we need to generate the more specific HS1 mutants that, like the cofilin mutant, no longer bind F-actin, but still bind PIP_2, or vice versa if any.

Since the full-length EMS1 of 550 amino acids also contains both the N-terminal actin/ PIP_2-binding motif and the C-terminal SH3 domain, it is possible that EMS1 is an intrinsic tumor suppressor in the normal fibroblasts, if these two motifs/domains alone are sufficient for the tumor suppressor activity. The F-actin cross-linking activity of EMS1 is strongly inhibited not only by PIP_2, but also through the Tyr phosphorylation by Src in vitro.[73] These observations suggest that the addition of an extra negative charge(s) in the actin-binding site by either PIP_2-binding or phosphorylation is a common mechanism by which its F-actin cross-linking is blocked. Interestingly, the cytoskeletal tumor suppressor HUGL, a human homolog of Drosophila l(2)lg/p127, binds the C-terminal tail of myosin II,[79] and the HUGL-myosin II interaction is inhibited by the Ser/Thr phosphorylation of either myosin II or HUGL.[80] More interestingly, HUGL also contains a "putative" PIP_2-

binding motif within the phosphorylation site which regulates the HUGL-myosin II interaction. Thus, it is of great interest to see if Ras could disrupt the HUGL-myosin II through PIP_2. For detail, see the chapter by Dennis Strand.

To test more directly the notion that the PIP_2-binding alone is sufficient for the anti-oncogenicity, we are currently examining the anti-Ras action of PIP_2-binding antibiotics such as neomycin and its analog G418 which inhibit both thrombin-induced actin polymerization and cell proliferation,[75,81] presumably by blocking PIP_2-induced uncapping of actin filament plud-ends.[43] If these PIP_2-binding drugs prove to have a potent anti-cancer activity, the screening for various other PIP_2-binding compounds or inhibitors of PI-4/PI-5 kinases such as ribofuranosyl derivatives of echiguanine analogs[82] could lead to the development of novel anti-cancer therapeutics useful for the treatment of Ras-associated tumors which represent more than 30% of all human cancers. The above provocative hypothesis has been supported by a previous observation that micro-injection of an antibody specific for PIP_2 into Ras transformed NIH 3T3 cells inhibits their proliferation and reverses their morphology to the normal flat phenotype.[83]

In conclusion, we have demonstrated the first biochemically defined pathway in which, without changing the expression levels of any genes, Ras induces the disassembly of an actomyosin-EMS1 complex through PIP_2. As illustrated in Figure 10.3, PIP_2 is produced by PI-4/PI-5 kinases which are activated by Rac, that is activated by PI-3 kinase or its products (D3 phosphoinositides) through a Rac GDS, and PI-3 kinase is directly activated by Ras. PIP_2 binds several distinct F-actin binding proteins the majority of which have been proven to be tumor suppressors. We have provided the first evidence supporting a new concept that the PIP_2-binding of various cytoskeletal tumor suppressors including HS1 and cofilin mutants plays the critical role in their anti-Ras action. In other words, PIP_2 has a potential as a "protooncolipid" whose overproduction could cause or promote malignant transformation as do many protooncoproteins such as Src, Ras, Raf, PI-3 kinase and Rac. The first precedents for such protooncolipids are the tumor promoting phorbol esters such as TPA, which mimic functionally diacylglycerol (DAG) and activate several members of protein kinase C (PKC) family that activate the kinase Raf.[84] Oncogenic Ras mutants or TPA alone cannot develop any tumors in normal mice, but the combination of these two agents can cause papillomas or carcinomas in the experimental animals.[85] Similarly a constitutively active mutant of Raf or Rac alone is hardly able to transform normal fibroblasts, but the combination of these two mutants causes malignant transformation as do oncogenic Ras mutants.[34] Perhaps the combination of the DAG analog TPA and PIP_2 might cause malignant transformation by activating the oncogenic Raf/ MAPK- and PI-3 kinase/Rac-dependent pathways, respectively. Thus, if PIP_2-binding proteins or drugs could sequester PIP_2 as well as block the hydrolysis of PIP_2 by phospholipase C isozymes that generates both DAG and IP-3, they might serve as highly potent anti-cancer reagents.

Acknowledgment

The author is very grateful to his colleagues, in particular Hong He and Anjali Tikoo, for their unpublished data, and also to Tony Burgess for his consistent support and encouragement. This work has been supported in part by a grant from the Anti-Cancer Council of Victoria.

References

1. Weber K, Lazarides E, Goldman RD et al. Localization and distribution of actin fibers in normal, transformed and revertant cells. Cold Spring Harbor Symp Quant Biol 1974; 39:363-369.
2. Maruta H, Korn ED. Acanthamoeba myosin II. J Biol Chem 1977; 252:6501-6509.

3. Maruta H, Korn ED. Acanthamoeba cofactor protein is a heavy chain kinase required for actin-activation of the Mg^{2+}-ATPase activity of Acanthamoeba myosin I. J Biol Chem 1977; 252:8329-8332.
4. Maruta H, Korn ED. The isolated heavy chain of an Acanthamoeba myosin contains full enzymatic activity. J Biol Chem 1978; 253:6297-6300.
5. Pollard TD, Doberstein SK, Zot HG. Myosin I. Annu Rev Physiol 1991; 53:653-681.
6. Maruta H, He H. Cytoskeletal SH3 proteins. Biochemistry (Tokyo) 1995; 10:1210-1217.
7. Lee SF, Egelhoff TT, Mahasneh A et al. Cloning and characterization of a Dictyo-stelium myosin I heavy chain kinase activated by CDC42 and Rac. J Biol Chem 1996; 271: 27044-27048.
8. Maruta H, Isenberg G. Ca^{2+}-dependent actin-binding phosphoprotein in Physarum. II. Ca^{2+}-dependent F-actin capping activity of subunit a and its regulation by phosphorylation of subunit b. J Biol Chem 1983; 258:10151-10158.
9. Maruta H, Isenberg G. Ca^{2+}-dependent actin-binding phosphoprotein in Physarum. III. Subunit b is a DNase I-binding and F-actin capping protein. J Biol Chem 1984; 259:5208-5213.
10. Ampe C, Vandekerckhove J. The F-actin capping proteins of Physarum: Cap42(a) is very similar, if not identical, to fragmin and is structurally very homologous to gelsolin; Cap42(b) is Physarum actin. EMBO J 1987; 13:4149-4157.
11. Yin HL. Gelsolin: Ca^{2+}- and PIP2-regulated actin-modulating protein. BioEssays 1987; 7:176-178.
12. Muellauer L, Fujita H, Ishizaki A et al. Tumor suppressive function of mutated gelsolin in Ras-transformed cells. Oncogene 1993; 8:2531-2536.
13. Trahey M, McCormick F. A cytoplasmic protein stimulates normal Ras GTPase, but does not affect oncogenic mutants. Science 1987; 238:542-545.
14. Hesse J, Maruta H, Isenberg G. Monoclonal antibodies localize the exchangeable GTP-binding site in β- and not α-tubulins. FEBS Lett 1985; 179:91-95.
15. Maruta H, Greer K, Rosenbaum JL. A-tubulin acetylation and microtubule assembly and disassembly. J Cell Biol 1986; 103:571-579.
16. Nur-E-Kamal MSA, Varga M, Maruta H. The GTPase-activating NF1 fragment of 91 amino acids reverses v-Ha-Ras-induced malignant phenotype. J Biol Chem 1993; 268:22331-22337.
17. Fridman M, Tikoo A, Varga M et al. The minimal fragments of Raf and NF1 that can suppress a v-Ha-Ras-induced malignant phenotype. J Biol Chem 1994; 269:30105-30108.
18. Tikoo A, Varga M, Ramesh V et al. An Anti-Ras function of neurofibromatosis type 2 gene product (NF2/Merlin). J Biol Chem 1994; 269:23387-23390.
19. Tikoo A, Lo SH, Chen LB et al. PIP2-induced uncapping of actin filaments at the plus-ends is required for v-Ha-Ras-induced malignant transformation: the effects of tensin, cytochalasins and PIP2-binding mutants of cofilin. Nature 1997; (submitted).
20. He H, Watanabe T, Zhan X et al. Role of PIP2 in the Ras-induced disruption of an EMS1-actomyosin II complex, and the suppression of Ras-induced malignancy by the PIP2-binding SH3 protein HS1. Mol Cell Biol 1998; 18:3829-3837.
21. Wang DZM, Nur-E-Kamal MSA, Tikoo A et al. The GTPase and GAP domains of p190, a tumor suppressor that binds a Ras GAP of 120 kDa (GAP1), independently function as anti-Ras tumor suppressors. Cancer Res 1997; 57:2478-2484.
22. Prendergast GC, Gibbs JB. Pathways of Ras function: Connections to the actin cytoskeleton. Adv Cancer Res 1993; 62:19-64.
23. Smith MR, DeGudicibus SJ, Stacey DW. Requirement for Ras during viral oncogene (v-Src) transformation. Nature 1986; 320:540-543.
24. Krzyzosiak WJ, Shindo-Okada N, Teshima H et al. Isolation of genes specifically expressed in flat revertant cells derived from Ras-transformed NIH 3T3 cells by treatment with azatyrosine. Proc Natl Acad Sci USA 1992; 89:4879-4883.
25. Inoue H, Tavoloni N, Hanafusa H. Suppression of v-Src transformation in primary rat embryo fibroblasts. Oncogene 1995; 11:231-238.

26. Defilippi P, Olivo c, Tarone G et al. Actin polymerization in DBL-transformed NIH 3T3 fibroblasts is dependent on cell adhesion to specific extracellular matrix proteins. Oncogene 1997; 14:1933-1943.
27. Sonoda H, Nishida K, Yoshida T et al. Oxamflatin: a novel compound which reverses malignant phenotype to nomal one via induction of JunD. Oncogene 1996; 13:143-149.
28. Vandekerhove J, Bauw G, Vancompernolle A et al. Comparative two-dimentional gel analysis and microsequencing identifies gelsolin as one of the most prominent down-regulated markers of transformed human fibroblast and epithelial cells. J Cell Biol 1990; 111:95-102.
29. Fernandez JLR, Geiger B, Salomon D et al. Suppression of tumorigenicity in SV40-transformed cells after transfection with vinculin cDNA. J Cell Biol 1992; 119:427-438.
30. Glueck U, Kwiatkowski D, Ben-Zen'ev A. Suppression of tumorigenicity in SV40-transformed 3T3 cells transfected with α-actinin cDNA. Proc Natl Acad Sci USA 1993; 90:383-387.
31. Chang JS, Kobayashi M, Wang DZM et al. Two regions with differential growth-modulating activity in the N-terminal domain of GAP1: SH2/SH3 and Gly-Ala-Pro-rich regions. Eur J Biochem 1995; 232:691-699.
32. McGlade J, Brunkhorst B, Anderson D et al. The N-terminal region of GAP1 regulates cytoskeleton structure and cell adhesion. EMBO J 1993; 12:3073-3081.
33. Bar-Sagi D, Feramisco J. Induction of membrane ruffling and fluid phase pinocytosis in quiescent fibroblasts by Ras proteins. Science 1986; 233:1061-1065.
34. Qiu RG, Chen J, Kirn D et al. An essential role for Rac in Ras transformation. Nature 1995; 374:457-459.
35. Prendergast GC, Davide JP, DeSolms SJ et al. Farnesyltransferase inhibition causes morphological reversion of Ras-transformed cells by a complex mechanism that involves the regulation of actin cytoskeleton. Mol Cell Biol 1994; 14:4193-4202.
36. Ridley AJ, Hall A. Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992; 70:389-399.
37. Lebowitz PF, Davide JP, Prendergast GC. Evidence that farnysyltransferase inhibitors suppress Ras transformation by interfering with Rho activity. Mol Cell Biol 1995; 15:6613-6622.
38. Ridley AJ, Paterson HF, Johnson CL et al. Rac regulates growth factor-induced membrane ruffling. Cell 1992; 70:401-410.
39. Manser E, Huang HY, Loo TH et al. Expression of constitutively active α-PAK reveals effects of the kinase on actin and focal complexes. Mol Cell Biol 1997; 17:1129-1143.
40. Kumar CC, Prorock-Rogers C, Kelley J et al. SCH51344 inhibits Ras transformation by a novel mechanism. Cancer Res 1995; 55:5106-5117.
41. Kumar CC. Inhibition of Ras transformation by SCH51344. Jpn J Cancer & Chemotherapy 1997; 24:1503-1511.
42. Van Aelst L, Joneson T, Bar-Sagi D. Identification of a novel Rac-interacting protein involved in membrane ruffling. EMBO J 1996; 15:3778-3786.
43. Hartwig JH, Bokoch, GM, Carpenter Cl et al. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell 1995; 82:643-653.
44. Trofatter JA, MacCollin MM, Rutter JL et al. A novel moesin/ezrin/radixin-like protein is a candidate for the NF2 tumor suppressor. Cell 1993; 72:791-800.
45. Rouleau GA, Merel P, Lutchman M et al. Alteration in a new gene encoding a putative membrane-organizing protein causes NF2. Nature 1993; 363:515-521.
46. Gonzalez-Agosti C, Xu L, Pinney D et al. The tumor suppressor merlin/NF2 localizes preferentially in membrane ruffles. Oncogene 1996; 13:1239-1247.
47. Schmucker B, Ballhausen WG, Kressel M. Subcellular localization and expression pattern of NF2/merlin/schwannomin. Eur J Cell Biol 1997; 72:46-53.
48. Tsukita S, Oishi K, Sato N et al. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J Cell Biol 1994; 126:391-401.
49. Guenthert U, Hofmann M, Rudy W et al. A new variant of glycoprotein CD44 conferes metastatic potential to rat carcinoma cells. Cell 1991; 65:13-24.

50. Roy C, Martin M, Mangeat P. A dual involvement of the amino terminal domain of ezrin in F- and G-actin binding. J Biol Chem 1997; 272:20088-20095.
51. McClathchey AI, Saotome I, Ramesh V et al. The NF2 tumor suppressor gene product is essential for extraembryonic development immediately prior to gastrulation. Genes Develop 1997; 11:1253-1265.
52. Lo SH, Janmey PA, Hartwig JH et al. Interactions of tensin with actin and identification of its distinct actin-binding domains. J Cell Biol 1994; 125:1067-1075.
53. Lo SH, An Q, Bao S et al. Molecular cloning of chick cardiac muscle tensin: full-length cDNA sequence, expression and characterization. J Biol Chem 1994; 269:22310-22319.
54. Lo SH, Weisberg E, Chen LB. Tensin: A potential link between the cytoskeleton and signal transduction. BioEssays 1994; 16:817-823.
55. Li J, Yen C, Liaw D et al. P-TEN, a putative Tyr phosphatase gene mutated in human brain, breast and prostate cancer. Science 1997; 275:1943-1947.
55a. Steck PA, Pershouse MA, Jasser SA et al. Identification of a candidate tumor suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nature Genet 1997; 15:356-362.
55b. Myers MP, Stolarov JP, Eng C et al. P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificicity phosphatase. Proc Natl Acad Sci 1997; 94: 9052-9057.
55c. Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, PIP3. J Biol Chem 1998; 272:13375-13378.
56. Menkel AR, Kroemker M, Bubeck P et al. Characterization of an F-actin binding domain in the cytoskeletal protein vinculin. J Cell Biol 1994; 126:1231-1240.
57. Jockusch BM, Isenberg G. Interaction of α-actinin and vinculin with actin: opposite effects on filament network formation. Proc Natl Acad Sci USA 1981; 78:3005-3008.
58. Fukami K, Endo T, Imamura M et al. Alapha-actinin and vinclulin are PIP2-binding proteins involved in signaling by Tyr kinase. J Biol Chem 1994; 269:1518-1522.
59. Matsuzaki F, Matsumoto F, Yahara I et al. Cloning and characterization of porcine brain cofilin cDNA: cofilin contains the nuclear transport signal sequence. J Biol Chem 1988; 263:11564-11568.
60. Moon AL, Janmey PA, Louie KA et al. Cofilin is essential component of yeast cortical cytoskeleton. J Cell Biol 1993; 120:421-435.
61. Nagaoka R, Abe H, Obinata T. Site-directed mutagenesis of the phosphorylation site of cofilin: its role in cofilin-actin interaction and cytoplasmic localization. Cell Motil Cytoskeleton 1996; 35:200-209.
62. Arber S, Barbayannis FA, Hanser H et al. Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature 1998; 393:805-809.
63. Yang N, Higuchi O, Ohashi K et al. Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature 1998; 393:809-312.
64. Moriyama K, Yonezawa N, Sakai H et al. Mutational analysis of an actin-binding site of cofilin and characterization of cofilin/destrin chimeras. J Biol Chem 1992; 267:7240-7244.
65. Yahara I, Harada F, Sekita S et al. Correlation between effects of 24 different cytochalasins on cellular structures/events and those on actin in vitro. J Cell Biol 1982; 92:69-78.
66. Cutler HG, Crumley FG, Cox RH et al. Chaetoglobosin K: A new plant growth inhibitor and toxin from Diplodia macrospora. J Agric Food Chem 1980; 28:139-142.
67. Yoshida M, Horinouchi S, Beppu T. Trichostatin A and trapoxin: Novel chemical probes for the role of histone acetylation in chromatin structure and function. BioEssays 1995; 17:423-430.
68. Futamura M, Monden Y, Okabe T et al. Trichostatin A inhibits both Ras-induced neurite outgrowth of PC12 cells and morphological transformation of NIH 3T3 cells. Oncogene 1995; 10:1119-1123.
69. Wasenius VM, Saraste M, Salven P et al. Primary structure of the brain α-spectrin. J Cell Biol 1988; 108:79-93.
70. Fanhauser C, Reymond A, Cerutti L et al. The S. Pombe CDC15 gene is a key element in the reorganization of F-actin at mitosis. Cell 1995; 82:435-444.

71. Schuuring E, Verhoeven E, Litvinov S et al. The product of the EMS1 gene, amplified in human carcinomas, is homologous to a v-Src substrate and is located in cell-substratum contact sites. Mol Cell Biol 1993; 13:2891-2898.
72. Kitamura D, Kaneko H, Miyagoe Y et al. Isolation and characterization of a novel human gene expressed specially in the cells of hematopoietic lineage. Nucleic Acids Res 1989; 17:9367-9379.
73. Huang C, Ni Y, Wang T et al. Down-regulation of F-actin cross-linking activity of cortactin/EMS1 by Src-mediated Tyr phosphorylation. J Biol Chem 1997; 272:13911-13915.
74. Rodriguez P, Warne P, Khwaja A et al. Role of PI-3 kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell 1997; 89:457-467.
75. Walsh AB, Dhanasekaran M, Bar-Sagi D et al. SCH51344-induced reversal of RAS-transformation is accompanied by the specific inhibition of the RAS/RAC-dependent cell morphology pathway. Oncogene 1997; 15:2553-2560.
76. Takemoto Y, Furuta M, Li XK et al. LckBP1/HS1, expressed in hematopoietic lineage cells, directly associates with the SH3 domain of Lck. EMBO J 1995; 14:3403-3414.
77. Yamanashi Y, Fukuda T, Nishizumi H et al. Role of Tyr phosphorylation of HS1 in B-cell antigen receptor-mediated apoptosis. J Exp Med 1997; 185:1387-1392.
78. Maruta H. Downstream of RAS and actin-cytoskeleton. In: Maruta H, Burgess AW, eds. Regulation of the RAS Signaling Network. Austin and Heidelberg: RG Landes and Springer-Verlag, 1996:139-180.
79. Strand D, Unger S, Corvi R et al. A human homologue of the Drosophila tumor suppressor gene l(2)gl maps to 17p11.2-12 and codes for a cytoskeletal protein that associates with non-muscle myosin II heavy chain. Oncogene 1995; 11:291-301.
80. Kalmes A, Merdes G, Neumann B et al. A Ser kinase associated to the p127/l(2)gl tumor suppressor of Drosophila may regulate the binding of p127 to non-muscle myosin II heavy chain and attachment to the plasma membrane. J Cell Sci 1996; 109:1359-1368.
81. Lassing I, Lindberg U. Evidence that the phosphatidylinositol cycle is linked to cell motility. Exp Cell Res 1988; 174:1-15.
82. Saito Y, Umezawa K. Synthesis of echiguanine analogs and their ribofuranosyl glycosides that inhibit PI-4 kinase. Bioorg Med Chem Lett 1997; 7:861-864.
83. Cai H, Smola U, Wixler V et al. Role of diacylglycerol-regulated protein kinase C isotypes in growth factor activation of the kinase Raf-1. Mol Cell Biol 1997; 17:732-741.
84. Leder A, Kuo A, Cardiff RD et al. v-Ha-Ras transgene abrogates the initiation step in mouse skin tumorigenesis: Effects of phorbol esters and retinoic acid. Proc Natl Acad Sci USA 1990; 87:9178-9182.

Section II
Ras/Rho Family GTPases

CHAPTER 11

GTPase Regulators: GAPs, GDSs and GDIs

Hiroshi Maruta

Introduction

The most characteristic feature of G proteins, GTP-dependent signal transducers, is that these unique GTPases cycle between their active GTP-bound forms and inactive GDP-bound forms. Each G protein has the intrinsic GTPase activity that hydrolyzes the bound GTP to GDP. The bound GDP then dissociates from the G protein. In the cytoplasm GTP is much more abundant than GDP. Thus, GTP preferentially binds the G protein to reactivate this signal transducer. However, without any other stimulating proteins, both GTP hydrolysis and GDP dissociation of each G protein takes place very slowly. Each G protein serves as a molecular switch to activate its specific effector proteins which bind only the GTP-bound form of the corresponding G protein. Thus, it switches "on" in the GTP-bound form and switches "off" in the GDP-bound form.

Several distinct and specific proteins bind either the GTP-bound form or GDP-bound form of each G protein and stimulate or inhibit either the intrinsic GTPase activity or GDP dissociation. GTPase activating proteins (GAPs) stimulate GTP hydrolysis by each G protein and therefore accelerate its switching "off". GDP dissociating stimulators (GDSs) bind selectively the GDP-bound form of each G protein, and reduce its affinity for GDP, thereby accelerating the replacement of GDP by GTP and its switching "on". For this reason, GDSs are also called GDP/GTP exchange factor (GEFs) or proteins (GEPs). GDP dissociation inhibitors (GDIs), on the other hand, increase its affinity for GDP, and therefore delay its switching "on". Thus, each G protein is not only a switch, but also serves as a highly sophisticated molecular "timer".[1] GAPs could speed up this "natural" timer for its switching "off", whereas GDSs and GDIs could regulate the same timer for its switching "on", in positive and negative manners, respectively.

A small monomeric G protein family called Ras has been studied most extensively by molecular oncologists since its discovery in early 1980s, simply because several mutants of Ras are highly oncogenic, and contribute to the development of more than 30% of all human carcinomas, notably more than 90% of pancreatic cancers and 50% of colon cancers.[2] Why are these Ras mutants so oncogenic? These oncogenic Ras mutants are "broken" timers which are kept switched "on", basically all the time. This important concept was first introduced by Frank McCormick and his colleague Meg Trahey in late October 1987 through their exciting finding of a Ras GAP activity in extracts of both mammalian cells and Xenopus oocytes.[3]

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

They found that a cytosolic protein(s) can highly stimulate the very low intrinsic GTPase activity of normal Ras, but not oncogenic Ras mutants. In other words, in the presence of these GAPs, normal Ras is rapidly converted to the inactive GDP-bound form, whereas oncogenic Ras mutants remain in the active-GTP-bound form. In that "unforgettable" fall, I was still working on the transcriptional regulation of Dictyostelium Ras genes at UCSD (San Diego). As soon as I saw this "historic" paper that appeared in *Science*, I decided to study molecular biology of oncogenic Ras mutants, using mammalian cells. A few months later, I left San Diego, flew across the equator and joined the Melbourne Branch of Ludwig Institute for Cancer Research (LICR).

I thought then, since normal Ras is rapidly converted by GAPs from the active GTP-bound form (T-RAS) to the inactive GDP-bound form (D-RAS), there must be a protein(s) in the cytoplasm that can re-convert rapidly the inactive D-RAS to the active T-RAS by either stimulating the replacement of GDP by GTP (GDSs), or alternatively phosphorylating the bound GDP to GTP (GDP kinase). In fact, even several months before the discovery of Ras GAPs, the first GDS gene was cloned from yeast by Michael Wigler and his colleagues.[4] This GDS was called CDC25. A loss-of-function mutation of CDC25 impairs the function of normal Ras to activate its effector called adenylate cyclase in yeast. However, this CDC25 mutation can be suppressed by a mutation of Ras which is equivalent to an oncogenic mutation of Ras. Clearly these findings suggest that CDC25 is responsible for the activation of Ras, i.e., the D-RAS to T-RAS conversion. Some years later the GDS activity of CDC25 was experimentally confirmed.[5] Thus, the year 1987 was one of the most critical turning points in the history of Ras research.

Although no GDI specific for Ras has been isolated as yet, a few distinct GDIs specific for other small monomeric G proteins such as Rho and Rab family GTPases began to be isolated, cloned and extensively characterized by Yoshimi Takai and his colleagues since 1990.[6] In this chapter, I will summarize the rapid progress of research in this field during the last decade, regarding these three groups of GTPase regulators, i.e., GAPs, GDSs and GDIs, specific for Ras or Rho family G proteins, and discuss any potential of these proteins in terms of the regulation of actin-cytoskeleton and cancer.

GTPase Activating Proteins (GAPs)

Ras GAPs

As soon as McCormick's group found a Ras GAP activity in mammalian extracts,[3] several groups started a fierce race to identify and isolate the first Ras GAP, which we call GAP1, from mammalian tissues. Jay Gibbs and his colleagues won this so-called "GAP1" race by purifying the GAP1 of 120 kDa from bovine brain.[7] Subsequently Gibbs' group cloned a cDNA encoding the bovine GAP1.[8] It consists of 1044 amino acids and its minimum GAP domain which stimulates the Ras GTPase activity is localized within the C-terminal 320 amino acids.[9,10] As revealed later, GAP1 turns out to be the first effector of Ras as well. The first evidence suggesting that GAP1 is not only an attenuator of normal Ras, but also serves as an effector of oncogenic Ras mutants such as v-Ha-Ras is the finding that a mutation of v-Ha-Ras in its effector domain I (residues 32 to 40) abolishes not only the oncogenicity of v-Ha-Ras,[11] but also the interaction between v-Ha-Ras and GAP1.[12,13] Furthermore, we found that replacement of Tyr64 in the effector domain II of v-Ha-Ras by any other amino acids, except for Phe and Leu, abolishes not only its oncogenicity, but also the interaction of Ras with GAP1.[14] Interestingly, the full-length GAP1 inhibits a muscarinic K^+-ion channel in a Ras-dependent manner, whereas the N-terminal half of GAP1 alone which lacks the Ras GAP domain can block the same ion channel even without Ras.[15] These observations indicate that the GAP domain blocks the effector function of the N-terminal half of GAP1, and Ras releases this intramolecular lock by interacting with the GAP do-

main. However, the possible connection between blocking the ion channel and malignant transformation still remains unclear. Finally, we found that the N-terminal fragment of GAP1 which contains only the first SH2, SH3 and second SH2 domains (SH232) can cause malignant transformation of normal fibroblasts in the presence of a phorbol ester,[16] confirming that this N-terminal fragment alone is oncogenic, while the full-length GAP1 has to be activated by oncogenic Ras mutants for its oncogenic action. In further support of this notion, Bruno Tocque and his colleagues found that the SH3 domain of GAP1 alone can bind a novel cytoplasmic protein of 466 amino acids called p68/G3BP, whereas the full-length GAP1 requires Ras for its binding to p68.[17] His group also has demonstrated that the SH3 domain of GAP1 is required for the Ras signaling leading to the maturation of Xenopus oocytes.[18] In other words, if one can create a small molecule that blocks the interaction of oncogenic Ras mutants with GAP1, one could suppress the Ras-induced malignant transformation.

In November 1990, three groups independently reported in the journal *Cell* that a tumor suppressor called NF1 (neurofibromatosis type 1) contains a GAP1-related domain (GRD) that also stimulates the intrinsic GTPase activity of normal Ras, but not oncogenic Ras mutants.[19-21] Thus, NF1 is a second mammalian Ras GAP. It consists of 2818 amino acids,[22] and we localized the minimum GAP domain in the 78-amino acid fragment (NF78, residues 1441-1518) of this GRD.[23] Rather surprisingly, NF78 shares only 19% sequence identity with the corresponding short fragment within the C-terminal GAP domain of GAP1 which alone has no detectable GAP activity. These observations suggest the possibility that GAP1 and NF1 may not bind the exactly same site of Ras GTPase. Nevertheless, like GAP1, NF1 requires both the effector domains I and II of Ras for its GAP activity, indicating that these two GAPs bind the same "general" area of the Ras molecule including these two effector domains.[24] In fact, NF1-GRD, the C-terminal GAP domain of GAP1 (GAP1C), and the N-terminal half of the Ser/Thr kinase Raf (Raf-N) compete each other for the binding to the Ras/GTP complex (T-RAS).[25] Raf is the so far best characterized oncogenic effector of T-RAS and also requires both effector domains I and II of Ras for its binding to the T-RAS.[26]

In order to test whether a small Ras-binding fragment(s) derived from NF1-GRD, GAP1 or Raf-N can suppress Ras-induced malignant transformation by blocking the Ras interaction with its effectors such as GAP1 and Raf, we created a series of Ras-binding peptides from these three proteins. The smallest Ras-binding peptide of NF1 called NF56 corresponds to the residues 1441-1496 within the GRD domain, and that of Raf called Raf81 corresponds to the residues 51-131 within the Raf-N.[27] We found that overexpression of either NF56 or Raf81 is indeed sufficient to suppress v-Ha-Ras-induced malignant transformation of NIH 3T3 cells.[27] Thus, it would be worthwhile to develop a novel group of drugs that functionally mimic these Ras-binding NF1/Raf peptides in order to treat effectively a variety of Ras-associated tumors which represent more than 30% of human carcinomas.

Either deletion or dysfunction of NF1 leads to the type 1 neurofibromatosis, the development of tumors in the central nervous system (CNS) including schwannomas. Many schwannoma cells which carry NF1 mutants, but normal GAP1, and show a much higher level of T-RAS (GTP/Ras complex) than the normal Schwann cells,[28,29] clearly indicating that in these cells NF1 plays the major role in attenuating normal Ras signal. However, the minimum Ras GAP domain (residues 1441-1518) of NF1 represents less than 3% of the full-length NF1. What is the function of the remaining 97% of this molecule in mammalian cells? If the sole physiological role of NF1 is to attenuate the normal Ras signaling, it is expected that the phenotype of NF1-deficient mice should be basically identical to that of transgenic mice carrying oncogenic Ras mutants such as v-Ha-Ras. However, disruption of NF1 gene (homozygosity) in mice was found to result in mid-gestational embryonic lethality, primarily due to a heart failure,[30,31] whereas v-Ha-Ras transgenic mice develop normally.[32] Clearly NF1 is required not only as a GAP to attenuate the normal Ras signaling,

but also as an effector to control the normal development of the heart. Interestingly, disruption of GAP1 gene (homozygosity) also results in a similar but more severe embryonic lethality due to abnormal cardiac development and extensive neuronal apoptosis.[33] This finding also strongly supports the notion that GAP1 acts not only as an attenuator of Ras, but also as an effector.

Unlike the NF1 null mutation in mice, homozygous NF1 null mutations in Drosophila are not embryonically lethal, but reduce the size of larvae, pupae and adults by blocking a cAMP synthesis pathway.[33a,33b] The neuropeptide PACAP38 (pituitary adenylate cyclase-activating polypeptide) at the neuromuscular junction induces a 100-fold enhancement of K^+-currents by activating both Ras-Raf and adenylate cyclase pathways.[33c] In the NF1 null flies, PACAP38 fails to activate an adenylate cyclase, and therefore no PACAP38-induced enhancement of K^+-currents takes place. However, cAMP or the catalytic subunit of protein kinase A (PKA) can restore the NF1 null phenotype, indicating that NF1 is essential for the cAMP production. Thus, it would be of great interest to examine whether in mammalian cells, NF1 is involved in cAMP production.

In addition to Ras, two other GTPases bind NF1:[20,26,34,35] (i) Rapl family G proteins; and (ii) microtubules. Although GTPases in Ras and Rapl families share only 50% overall sequence identity, their effector domains I are 100% identical, and domains II are also very similar to each other.[36] At least the effector domain I is required for the interaction of Rapl GTPases with either NF1 or GAP1, although their intrinsic GTPase activity is not stimulated by either NF1 or GAP1 at all.[24,26,37,38] Furthermore, Rapl competes with Ras for binding to both NF1 and GAP1.[26,37,38] However, unlike Ras, Rapl never becomes oncogenic even if it is mutated in the exact same manner as Ras, such as replacement of Gly12/Gly13 by other amino acids, Ala59 by Thr, or Gln61/Thy61 by Leu.[39] Instead, overexpression of Rap1 was reported to suppress malignant transformation caused by v-Ki-Ras, an oncogenic mutant of c-Ki-Ras,[40] although its anti-oncogenicity appears to be very weak if any.[41] Microtubules are polymers of tubulin heterodimers consisting of α- and β-tubulins.[42] Both α- and β-tubulins are GTPases, sharing 40% sequence identity.[42-44] NF1 appears to bind both tubulin heterodimers and microtubules.[34] FurthermoreNF1 and microtubules are colocalized within the cytoplasm.[35] Interestingly, the tubulin-binding domain (TBD) of NF1 (residues 1300-1456) partially overlaps the minimum Ras-binding domain (NF56, residues 1441-1496) whose overexpression suppresses Ras-induced malignant transformation.[27,34] In fact, tubulin inhibits the Ras GAP activity of NF1, probably by blocking the Ras/NF1 interaction.[34] However, the physiological role that the NF1-microtubules interaction might play still remains to be clarified.

In 1993 a third mammalian Ras GAP of 100 kDa was purified from rat brain,[45] and its cDNA was subsequently cloned by Seisuke Hattori and his colleagues.[46] This new GAP of 847 amino acids shares 40% sequence identity with Drosophila Gap1 and therefore was named $Gap1^m$ (m for mammalian). However, to avoid any possible confusion between the first GAP (GAP1) and a third GAP ($Gap1^m$), we shall call the latter p100 GAP here. p100 GAP contains two Lys-rich phospholipid-binding sites and a BTK homology region.[46] Like GAP1 and NF1, p100 GAP is highly specific for Ras GTPases, and does not stimulate the intrinsic GTPase activity of any other GTPases at all.[45,46] Nevertheless, the sequence homology in the GAP domains between these three distinct GAPs is limited only to 25%.[46] Although the Drosophila homolog of p100 GAP is known to be responsible for the negative regulation of Ras signaling during the development of photoreceptor cells in the fly's eyes,[47] the exact role of this GAP in mammals still remains to be determined.

In the cellular slime mold Dictyostelium, two distinct members of p100 GAP family were identified recently.[48,49] The GAP of 841 amino acids called DdRasGAP1 stimulates the intrinsic GTPase activity of a Dictyostelium Ras (RasD) and is required for both cytokinesis and development of fruiting bodies.[48] For the following two reasons, it is most likely that

Table 11.1. Mammalian GAPs for Ras/Rho family GTPases

Ras/Rap1 GAP	Targets	Rho GAPs	Targets
GAP1 (120 kDa)	Ras	GAP2 (50 kDa)	Rho, Rac, CDC42
NF1	Ras	p190-A	Rho, Rac, CDC42
p100 GAP	Ras	p190-B	Rho, Rac, CDC42
		Myr 5	Rho, Rac, CDC42
GAP/IP_4	Ras, Rap1	3 BP-1	Rho, Rac, CDC42
R-Ras GAP	R-Ras	PARG1	Rho, Rac, CDC42
		ABR	Rac, CDC42
GAP3	Rap1 & 2, Rsr1	BCR	Rac, CDC42
Spa-1	Rap1 & 2, Rsr1	N-Chimerin	Rac
Tsc2	Rab5, Rapl (?)	Graf	Rho, CDC42
		p122 ARP	Rho

this GAP (and a mammalian homolog) serves as an effector of Ras for cytokinesis: (i) constitutive expression of activated Ras mutant does not cause highly multinucleated cells, and (ii) Dictyostelium RasG is also required for cytokinesis.[49a] The other Dictyostelium "GAP" of 860 amino acids called GAPA is also required for the cytokinesis, but not for the development of fruiting bodies.[49] However, it still remains to be determined whether GAPA is a Ras GAP as is Dd Ras GAP1, or binds Rac and CDC42 as do the human IQGAPs which are related to p100 GAP family (for detail of IQGAP family, see chapter 18 by Andre Bernards).

In 1995 a fourth "unique" Ras GAP was cloned by Robin Irvine's group.[50] Since this GAP of 829 amino acids binds inositol$^{1,3-5}$ tetra-kisphosphate (IP_4), it is called GAP/IP_4. Like p100 GAP, it contains two Lys-rich phospholipid-binding sites, in addition to the central GAP domain and a pleckstrin-homology (PH) domain. The most unique feature of this GAP is that it stimulates the intrinsic GTPase activity of not only Ras but also Rapl. So far GAP/IP_4 is the only GAP that acts on both Ras and Rapl GTPases (see Table 11.1). Interestingly, IP_4 stimulates its GAP activity towards Ras GTPases but not towards Rapl GTPases, whereas phospholipids inhibit only its Ras GAP activity and not Rapl GAP activity. Thus, in the presence of phospholipids, GAP/IP_4 becomes highly specific for Rapl GTPases, whereas in the presence of IP_4, it becomes relatively specific for Ras GTPases.

Interestingly, GAP1, p100 GAP and GAP/IP_4 share 25-30% sequence identity in their Ca^{2+}-dependent lipid-binding domain of 43 amino acids called CaLB.[51] The CaLB of GAP1 was recently shown to bind a Ca^{2+}-dependent phospholipid-binding protein of 70 kDa called annexin VI.[51] Annexin VI is a tumor suppressor which reverses the EGF-dependent malignant transformation of A431 squamous epithelial carcinoma cells.[52] Thus, GAP1 binds two distinct tumor suppressors, i.e., p190 and annexin VI through the SH2 and CaLB domains, respectively.[26,51,52,68] Annexin VI is an inhibitor of protein kinase C, and is required for budding of clathrin-coated pits.[53,54] However, the exact molecular mechanism underlying the tumor suppression by annexin VI still remains to be determined.

More recently Kozo Kaibuchi and his colleagues cloned a fifth Ras GAP from bovine brain cytosol which is highly specific for R-Ras, and therefore called R-Ras GAP.[55] This GAP of 834 amino acids is also closely related to p100 GAP. Unlike three other members of Ras family (Ha-Ras, Ki-Ras and N-Ras), R-Ras is responsible for the apoptotic cell death during growth factor withdrawal.[56] Bcl-2 binds the C-terminal domain (residues 159-218) of R-Ras, but no other members of Ras family, and blocks R-Ras-induced apoptosis.[57] Thus, it would be of great interest to examine whether this R-Ras GAP blocks the apoptosis by attenuating R-Ras signaling or is required for the R-Ras-dependent apoptosis as an effector.

Unfortunately, none of these five distinct Ras GAPs from mammals or their short fragments stimulates the intrinsic GTPase activity of any oncogenic Ras mutants. A specific point mutation of the normal Ras converts this molecule to an oncoprotein. Conversely, is it possible for us to convert one of these Ras GAPs by a specific point mutation(s) to an anti-oncogenic protein? If one could create or design a Ras GAP mutant or drug which can stimulate the intrinsic GTPase activity of these oncogenic Ras mutants, such a GAP mutant or drug would be an ideal molecule for either genotherapy or chemotherapy of Ras-associated cancer. Such a molecule, which we might call a "Super GAP", has been the "Holy Grail" of mine since our Ras GAP research commenced in Melbourne a decade ago. Recently both the C-terminal GAP domain of GAP1 (GRD) alone and its complex with Ras were crystallized, and their three-dimensional (3D) structures have been determined by Alfred Wittinghofer and his colleagues.[58,59] Thus, it might no longer be a mere dream for us to figure out how the native GAPs activate normal Ras GTPases, and why they fail to activate oncogenic Ras GTPases. Based on these data and our insight, perhaps we might be able to model a "Super GAP" molecule that stimulates the intrinsic GTPase activity of oncogenic Ras mutants. Such a challenge will be our mission in Ras GAP research towards the coming new century. For detail of the Ras-GRD/Ras-Raf 3D structures, see chapter 13.

GAPs for Rho Family GTPases

Mammalian Rho family consists of at least seven distinct monomeric G proteins called RhoA, RhoB, RhoC, RhoG, Rac1, Rac2, and CDC42. These Rho family GTPases share only 30% sequence identity with mammalian Ras family GTPases (Ha-Ras, Ki-Ras, N-Ras, and R-Ras). None of the five distinct Ras GAPs from mammals that I have discussed previously stimulates the intrinsic GTPase activity of any Rho family G proteins. As discussed in chapter 12 and elsewhere,[26] Rho family GTPases, in particular Rac, RhoB and CDC42, are required for malignant transformation by oncogenic Ras mutants. Thus, it would be important to understand precisely how the signal transducing activity of these Rho family G proteins are regulated by specific GAPs, GDSs and GDIs, and how these regulators are linked to Ras effectors.

In 1989, shortly after the first Ras GAP (GAP1) was cloned, a second mammalian GAP was isolated by Alan Hall and his colleagues.[60] This GAP of 28 kDa, which we call GAP2, activates only Rho family GTPases, and neither Ras nor any other GTPases. Five years later the cDNA for GAP2 was cloned by the same group, and its sequence has revealed that the initially purified GAP2 molecule is just the C-terminal GAP domain of the full-length GAP2 which is a 50 kDa protein.[61] The N-terminal Pro-rich domain of GAP2 binds the SH3 domain of c-Src and p85, the regulatory subunit of PI-3 kinase.[62] Besides GAP2, several distinct GAPs specific for Rho family GTPases were cloned from mammals (see Table 11.1). One of the largest is p190-A which was cloned by Bob Weinberg and his colleagues in 1992.[63] p190-A binds the SH2 domains of GAP1, when the former is Tyr-phosphorylated at positions 1087 and 1105.[64] p190-A interacts with Rho family GTPases through its C-terminal GAP domain (residues 1186-1513).[63,65] Since Ras GTPases interact with GAP1 through its C-terminal GAP domain, Ras and Rho family GTPases could be linked through these two distinct GAPs, i.e., GAP1 and p190-A, only when p190-A is Tyr-phosphorylated. Interestingly, the N-terminal domain of p190-A (residues 1-251) is a GTPase which is highly activated by an as yet unidentified GAP (GAPX) present in mammalian cell extracts.[66] Thus, at least three distinct GAPs (GAP1, p190-A and GAPX) could form a complex, once p190-A is Tyr-phosphorylated. Recently a protein related to p190-A was cloned and called p190-B.[67] These two p190 isoforms share 50% sequence identity. The C-terminal domain of p190-B also stimulates Rho, Rac and CDC42 GTPase activity, and both the two phosphorylatable Tyr residues and the N-terminal GTPase domain also are conserved in p190-B. Both isoforms of p190 are ubiquitously expressed in a variety of mammalian tissues. However, as dis-

cussed in the following paragraph, these two isoforms are not functionally redundant at least for the cell growth regulation.

In an attempt to understand the possible role that p190-A might play in the regulation of oncogenic Ras signaling network, we have performed a series of experiments, overexpressing the following four distinct p190-A constructs in either normal or v-Ha-Ras-transformed NIH 3T3 cells:[68] (i) p190-A antisense RNA (p190-NA), which blocks de novo synthesis of endogenous p190-A; (ii) a dominant negative mutant of p190-A GTPase domain (p190-NN), in which Ser36 is replaced by Asn, sequestering the GDSs for p190-A to keep the endogenous p190-A in the inactive GDP-bound form; (iii) the C-terminal GAP domain of p190-A (p190-C); and (iv) the N-terminal GTPase domain of p190-A (p190-N). First of all, overexpression of p190-NA completely blocks de novo synthesis of p190-A in the normal fibroblasts, and transforms them into malignant cells, clearly indicating that (a) p190-A is a tumor suppressor, and (b) the endogenous p190-B cannot overcome the loss of p190-A function. Secondly, overexpression of the dominant negative mutant which blocks only the function of the GTPase domain also transforms the normal cells, but less efficiently than the antisense RNA, indicating that the GTPase domain alone is a tumor suppressor, but another domain of p190-A also contributes to the tumor suppressor activity of the full-length p190-A. Thirdly, overexpression of either p190-C or p190-N alone suppresses malignant transformation caused by v-Ha-Ras, indicating that both the GTPase and GAP domains of p190-A are anti-Ras tumor suppressors. Lastly, overexpression of p190-C, which attenuates the signal transducing activity of Rho family G proteins, downregulates c-Fos gene which has been activated by the oncogenic Ras mutant through Rho family GTPases. Thus, although p190-A binds the Ras effector GAP1, the former is not an oncogenic effector of Ras or GAP1, but instead can block the oncogenic Ras signaling network, not only through its GAP domain, an attenuator of Rho family G proteins, but also through its GTPase domain by a novel mechanism.

The biological function of the large central piece of p190-A molecule (residues 252-1185) flanked by both the GTPase and GAP domains still remains to be identified. Interestingly, however, the GRF-1 cDNA previously cloned from the human breast cancer cell line MCF-7 turns out to encode a protein of 835 amino acids which is highly homologous to this central piece of p190-A.[69] DNA sequence comparison between the GRF-1 and rat p190-A cDNAs has revealed that GRF-1 is a truncated variant or mutant of human p190-A. Because GRF-1 cDNA lacks both the third base (G) of the 388th codon and the first base (C) of the 1167th codon of full-length p190-A cDNA, the gene product contains only residues 389-1166 of p190-A and the extra 55 frame-shifted amino acids, and lacks entirely both the GTPase and GAP domains that are tumor suppressors.[68,69] Although GRF-1 was reported to bind the glucocorticoid receptor gene and repress its expression,[69] it still remains to be clarified whether the extra 55 amino acids or the p190-A residues 389-1166 are responsible for the gene repressor activity of GRF-1. More importantly, it is possible that the deletion of both GTPase and GAP domains of p190-A contributes to malignant transformation of these breast cells. GRF-1 could serve as a dominant negative mutant of the tumor suppressor p190-A, or simply be an inactive form of the tumor suppressor. We recently cloned from this tumor cell line the full-length wild-type p190-A cDNA, in addition to the truncated variant/mutant GRF-1 (Tikoo A, Maruta H, unpublished data), and its chromosomal localization and mapping are under way. It also still remains to be determined whether p190-B has any tumor suppressor activity as does p190-A.

In 1995 Martin Baehler and his colleagues cloned a very unique single-headed myosin of 1980 amino acids called Myr5 (myosin from rat 5). This myosin contains a GAP domain towards the C-terminus which, like p190-A, stimulates the intrinsic GTPase activity of all members of Rho family.[70] Like other myosins, Myr5 contains the myosin head at the N-terminus whose Mg^{2+}-ATPase activity is activated by actin filament (F-actin). In other

words, through Myr5, F-actin and Rho family GTPases can be linked together. In a sense Myr5 is a cousin of p190s: the two proteins share the so-called Rho GAP domain at the C-terminus, and the N-terminal GTPase domain of p190s is replaced by the myosin ATPase domain in Myr5. Since p190-A exerts its anti-Ras tumor suppressor activity, in part through the C-terminal GAP domain, it is quite conceivable that Myr5 also could serve as an anti-Ras tumor suppressor, at least by attenuating the essential signal transducing activity of Rho family through the common GAP domain.

In 1997 Carl Heldin and his colleagues cloned another unique GAP for Rho family GTPases, called PARG1.[70a] It is a 150 kDa protein that binds a Tyr phosphatase called PTPL1. The last four aliphatic amino acids (Pro-Gln-Phe-Val) of PARG1 are responsible for binding to the fourth PDZ domain pf PTPL1. Interestingly, PTPL1 shares with ERM protein family (Ezrin, Radixin, Moesin, NF2/Merlin and Band 4.1) the domain which binds both F-actin and PIP_2. For at least two reasons, PTPL1 could be a potential tumor suppressor: (i) The N-terminal half of NF2 alone, which is shared with PTPL1 and other ERM proteins, is sufficient for suppressing Ras transformation;[70b] and (ii) a novel Tyr phosphatase called P-TEN is a tumor suppressor.[70c] Also it is quite possible that PARG1 serves as a tumor suppressor as it is a Rho GAP as is the tumor suppressor p190-A.[68] In other words, PARG1 and PTPL1 could form a powerful tumor suppressive complex.

The above five GAPs (GAP2, p190-A, p190-B, Myr5 and PARG1) serve as GAPs for all members of Rho family. However, the GAP activity of five additional mammalian GAPs for Rho family GTPases is more specific for a selected member(s) of Rho family.[62] A unique PLase C-δ activator called p122ARP contains a GAP domain which is highly specific for Rho GTPase and does not activate either Rac or CDC42 GTPases.[71] Both N-chimerin and β-chimerin, which are expressed in brain and testes, respectively, are highly specific for Rac GTPases and do not activate either Rho or CDC42 GTPases. Both BCR and ABR activate Rac and CDC42 GTPases, but not Rho GTPases. BCR of 143 kDa contains a Ser/Thr kinase domain in the N-terminal half, in addition to the C-terminal GAP domain. ABR shares 68% sequence identity with BCR, but lacks the Ser/Thr kinase domain. More recently, a FAK-binding protein of 584 amino acids was cloned.[72] This protein called Graf contains at least two functional domains. The C-terminal SH3 domain binds a pro-rich motif (residues 875-884) of FAK. The other domain (residues 213-385) of Graf serves as a GAP for Rho and CDC42, but not for Rac. Interestingly, Graf colocalizes with actin stress fibers. Since Rho is required for both the stress fiber formation and activation of the focal adhesion kinase FAK, it is possible that Graf plays the critical role in the Rho-mediated activation of FAK.

The physiological role of these so-called Rho GAPs still remains to be determined by either antisense RNA or gene knock-out approaches. Nevertheless, since Rho family GTPases are required for oncogenic action of Ras, it would be no big surprise if one finds that, like p190-A, some of these GAPs for Rho family serve as anti-Ras tumor suppressors.

However, it is also possible that some of these Rho GAPs act not only as attenuators for Rho family G proteins, but also as an effector(s). For example, it was shown recently that the full-length N-chimerin, a GAP for Rac GTPases, induces membrane ruffling (MR, lamellipodia) in both fibroblasts and neuroblastoma cells as does Rac alone, whereas the GAP domain of N-chimerin alone blocks the Rac-induced MR.[73] Furthermore, N-chimerin's effect is inhibited by a dominant negative mutant of Rac (Asn17) or GDI for Rho family GTPases, indicating that N-chimerin exerts its effect through Rac. More interestingly, although a mutant of N-chimerin which lacks both GAP activity and Rac-binding is no longer effective, a mutant of N-chimerin which lacks GAP activity but still binds Rac effectively induces MR,[73] indicating that its Rac-binding, but not GAP activity, is responsible for its MR induction. Clearly, N-chimerin is an effector of Rac, as is GAP1 an oncogenic effector of

Ras. Thus, it would be of great interest to identify the downstream effector(s) of N-chimerin involved in the induction of MR.

Rapl/Rap2/Rsr1 GAPs

Rapl family consists of only two members called RaplA and RaplB which are 95% identical.[36] This family shares 50% sequence identity with Ras family and was reported to suppress malignant transformation caused by Ras mutants,[40] probably through the competition between Ras and Rapl for binding to several effectors of Ras. Since Ras and Rapl family GTPases are identical in their effector domain I (residues 32 to 40), all of the mammalian proteins including Ras GAPs and effectors of Ras that bind the Ras/GTP complex also bind the Rapl/GTP complex.[26] However, none of Ras GAPs, except for GAP/IP_4, stimulates the intrinsic GTPase activity of Rapl family. Thus, to identify a mammalian GAP(s) specific for Rapl family, a series of the Rapl GAP purification attempts commenced in the late 1980s.

In 1989, shortly after the discovery of GAP2 (a Rho GAP of 28 kDa), Takai's group found that the intrinsic GTPase activity of Rapl is stimulated by two separable protein fractions from the cytosol of bovine brain,[74] which we call collectively "GAP3". A few years later we purified the first mammalian GAP3 of 55 kDa (GAP3c) from the cytosol which is highly specific for Rapl family and does not stimulate the intrinsic GTPase activity of either Ras or Rho families.[10,75,76] Interestingly, GAP3c stimulates the intrinsic GTPase activity of an yeast G protein of 272 amino acids called Rsrl or Bud1 which shares 50% sequence identity with both Ras and Rapl families.[75] Rsr1 is required for the bud-site selection in yeast.[77] Independently, McCormick's group purified and cloned a human membrane-bound GAP3 of 88 kDa (GAP3m) from HL60 cells.[78,79] The enzymatic properties of GAP3c and GAP3m are almost indistinguishable from each other, and the partial sequence of GAP3c is very similar to that of GAP3m, if not identical. Thus, these two GAP3s are very closely related. GAP3m also activates the yeast Rsr1 GTPase.[80]

In 1993, Ira Herskowitz and his colleagues found that a yeast gene called Bud2 encodes a Rsrl GAP of 1104 amino acids.[81] However, Bud2 does not activate Rapl GTPases, and shows little sequence homology to these mammalian GAP3s. This is not a surprise, as yeast NF1-related Ras GAPs called IRA-1 and IRA-2 activate only yeast Ras GTPases, and not mammalian Ras GTPases, although NF1 activates both yeast and mammalian Ras GTPases.[19,82] It appears that yeast GAPs are active only towards yeast GTPases, and not towards mammalian GTPases. Whether any homolog of the yeast Rsr1 is present in mammalian cells still remains to be clarified.

A series of GAP3m deletion analysis has revealed that the GAP domain of GAP3m is located within the N-terminal half (residues 75-407).[83,84] Interestingly, the almost entire GAP domain shares 38% sequence identity with the corresponding N-terminal domain (residues 198-529) of a novel 130 kDa protein called Spa-1 from both mouse and human.[80,85] The full-length Spa-1 of around 1040 amino acids is localized in the cytoplasm, whereas the C-terminal deletion translocates the remaining domain of Spa-1 into the nucleus. Both Spa-1 and GAP3s activate Rapl and Rap2 GTPases, which share the almost identical effector domains I and II, but both GAPs have no effect on either Ras or Rho GTPases.[80,85] Interestingly, however, expression of Spa-1 and GAP3m appears to be rather exclusive and complementary to each other. Spa-1 is expressed predominantly in lymphoid tissues, but not in brain, whereas GAP3m is expressed in brain and other tissues such as kidney and pancreas, but not in lymphoid tissues.[85] In HL60 cells, TPA which induces granulocytic differentiation, downregulates Spa-1 expression, whereas it upregulates GAP3m expression.[85] Furthermore, expression of Spa-1 is normally restricted to G_0 phase. An ectopic overexpression of Spa-1 causes an apoptotic cell death of NIH 3T3 cells.[80]

Independently, a novel tumor suppressor called Tsc2/Tuberin was found to share a 39-amino acid motif with both Spa-1 and GAP3m.[87] This new motif, which we call "Tuberin-homology" (TH) motif, corresponds to the residues 403-441 of human Spa-1, 263-301 of human GAP3m and 1593-1631 of human Tsc2. The dysfunction of Tsc2 is associated with tuberous sclerosis, a human hereditary disease developing tumors mainly in kidneys.[87] Interestingly, the full-length Tsc2 of 1784 amino acids is associated with Golgi-apparatus as is Rapl GTPase.[88,89] A C-terminal domain of human Tsc2 (residues 1387-1784) containing the TH motif was reported to interact with Rapl GTPases in vitro,[88] suggesting the possibility that Rapl is involved in the transport of Tsc2 to the Golgi-membranes. More recently Tsc2 was identified as a GAP specific for Rab5 GTPase.[90] Furthermore, Okio Hino and his colleagues[91] found in the C-terminus of rat Tsc2 two transcriptional activation domains (AD1, residues 1163-1259; AD2, residues 1690-1743). The C-terminal half of Tsc2 (residues 1006-1743) containing AD1, TH motif and AD2 is localized in the nucleus.[91] Thus, the TH motif alone is not sufficient for the Golgi-localization. Although the precise role of this TH motif still remains to be determined, these distinct "TH" family GAPs would be potentially fascinating targets of our future study in order to understand the physiological role of Rapl/Rap2 GTPases in mammalian cells.

GDP Dissociation Stimulators (GDSs)

Ras GDSs

The approach for Ras GDSs' discovery is historically a great contrast to that for GAPs' discovery. The former is mainly a genetic approach, whereas the latter is mainly a biochemical one, with a few exceptions such as NF1. As I mentioned before, the gene encoding the first Ras GDS called CDC25 was cloned from yeast,[4] far before the corresponding protein of 1589 amino acids was eventually isolated from yeast, and biochemically characterized. Furthermore, it took 15 years since the CDC25 cloning until the first mammalian Ras GDS called SOS1 was cloned from mouse by David Bowtell and his colleagues in 1992,[92] and subsequently characterized biochemically. The most crucial factor which would eventually led to the identification of this first mammalian GDS was the cloning of a Drosophila gene called SOS (son of sevenless) by Gerald Rubin and his colleagues in 1991.[93] The sequence of the Drosophila SOS of 1596 amino acids has revealed that it contains a domain which shares 45% sequence identity with the GDS domain of yeast CDC25, suggesting that SOS might be a Ras GDS in Drosophila.

The genetic analysis of Drosophila eye development by Rubin's group and others has identified several distinct genes that are required for the differentiation of a specific photoreceptor cell called R7.[94] These genes include Boss (bride of sevenless), Sevenless, SOS, D-Ras1 and D-Raf. Boss is an unusual ligand, a seven-transmembrane protein of 892 amino acids, which is expressed in R8 cells and activates the receptor Sevenless expressed in the immediately adjacent R7 cells. Thus, R8 cells are responsible for the induction of R7 cell differentiation. Sevenless is a large receptor Tyr-kinase that eventually activates D-Ras1 through SOS. Then D-Ras1 activates D-Raf, a Ser/Thr kinase, which in turn activates MEK, a Tyr/Ser/Thr kinase, which phosphorylates and activates MAP kinase (MAPK). This Drosophila D-Ras1/D-Raf/MEK/MAPK kinase cascade leading to the R7 cell differentiation is quite similar to the mammalian Ras/ Raf/MEK/MAPK cascade leading to the cell proliferation (fibroblasts) or differentiation (neuronal cells).[94,95]

How about a signaling cascade upstream of Ras? As soon as the GDS domain of mammalian SOS1 was experimentally confirmed to stimulate the GDP dissociation from Ras, a signal transduction pathway from EGF receptor, an EGF-activated transmembrane Tyr-kinase, to Ras was quickly established by several groups.[95] First of all, it was shown that an adaptor protein called Grb-2 which contains two SH3 domains flanking an SH2 domain

binds the C-terminal Pro-rich motifs of SOS1 through the two SH3 domains to form a heterodimer in the cytosol. Secondly, the Sh2 domain of Grb-2 binds EGF receptor (EGFR), as soon as EGFR is activated by its ligands such as EGF and TGF-α, and subsequently autophosphorylated at Tyr residues. The EGFR/Grb-2 docking leads to the recruitment of the cytosolic SOS1 to the plasma membrane where the inactive Ras/GDP complex (D-RAS) is localized. There SOS1 converts D-RAS to the active Ras/GTP complex (T-RAS). Alternatively, another SH2 protein called SHC also binds the autophosphorylated EGFR through its SH2 domains and then is Tyr-phosphorylated by EGFR. The phosphorylated SHC then binds the SH2 domain of the Grb-2/SOS1 complex and recruits this cytosolic complex to the plasma membrane.[96]

In addition to the Ras GDS domain, SOS1 contains two other domains in the N-terminal half: (i) the DBL-homology (DH) domain and (ii) the pleckstrin-homology (PH) domain. The DH domain corresponds to the GDS domain of DBL which stimulates the GDP-dissociation from CDC42 and Rho.[97] DBL is highly oncogenic.[98] However, so far there is no experimental evidence indicating that the DH domain of SOS1 stimulates the GDP/GTP exchange of any G proteins. The PH domain was first recognized as a repeated motif in pleckstrin, a substrate for protein kinase C (PKC), and thought to be involved in specific protein-protein interactions or phospholipid-binding. Interestingly, an in vivo experiment using Drosophila has revealed that deletion of either the DH or PH domains of SOS impairs the function of SOS, whereas deletion of the C-terminal Grb-2 binding domain (Pro-rich motifs) of SOS causes no effect on the SOS function.[99] These observations suggest that the C-terminal domain of SOS negatively regulates SOS activity and that the role of Grb-2 is to suppress this negative effect. Thus, although there is no doubt that SOS1 is a Ras GDS, how SOS1 is activated still remains to be determined.

From mammalian tissues at least two additional Ras GDSs have been cloned. One is a cousin (isoform) of SOS1 called SOS2. SOS1 of 1336 amino acids and SOS2 of 1297 amino acids share 67% sequence identity.[92] Thus, it is predicted that SOS2 is also a Ras GDS, although this notion has not been experimentally confirmed as yet. Interestingly, the homozygous null mutation of SOS1 gene causes cardiovascular and yolk sac defects in mouse embryos which leads to death at mid-gestation,[100] indicating that SOS2 cannot functionally replace SOS1. These two SOSs are ubiquitously expressed in most mammalian tissues.[92] The other is Ras GRF/CDC25Mm of 1244 amino acids cloned by Larry Feig and his colleagues.[101] The full-length Ras GRF expression is limited strictly to the brain.[101] In primary culture of newborn rat cortical neurons, Ras is activated by either brain-derived neurotrophic factor (BDNF) or Ca^{2+}-influx induced by a membrane de-polarization.[102] BDNF acts through the Tyr-kinase receptor TrkB which eventually activates SOS as well.[95,96] Feig's group found that the Ras activation by the Ca^{2+}-influx requires an IQ (Ca^{2+}/calmodulin-binding) motif near the DH domain in the N-terminal half of the Ras GRF.[101]

Ian Macara and his colleagues found recently, using mouse NIH 3T3 fibroblasts, that Ras GRF, but not SOS1, is responsible for muscarinic receptor-mediated activation of Ras, and that the agonist carbachol induces the phosphorylation of Ras GRF which is required for the activation of Ras GRF.[104] In this system, transducin α-subunits inhibit the Ras GRF activation, whereas G protein βγ subunits cause a constitutive activation. In NIH 3T3 fibroblasts, there are at least four isoforms of Ras GRF, consisting of 666, 836, 1120 and 1260 amino acids, which are generated by differential N-terminal deletions. In 1990, just before SOS1 was cloned, Julain Downward and his colleagues reported that Ras GDS activity is associated with a cytosolic protein of around 60 kDa in human placenta, and that its GDS activity is dramatically reduced by a mutation at position 61 of Ras, in particular the Lys substitution.[105] However, whether this GDS is a proteolytic product/isoform of SOSs or Ras GRF, or an unrelated novel GDS still remains to be clarified.

GDSs for Rho Family GTPases

In 1990, Takai's group isolated a GDS of 61 kDa from bovine brains.[106] This GDS of 558 amino acids was later called Smg GDS (GDS for small GTPases) as its specificity turns out to be rather broad, and it stimulates GDP-dissociation from not only Rho family GTPases but also Ki-Ras and Rapl GTPases.[107,108] However, it does not affect the GDP/GTP exchange of H-Ras. Furthermore, it requires the lipid modification (prenylation) at the C-terminus of its target GTPases to stimulate the GDP/GTP exchange. Therefore, it clearly differs from the Ras GDS of around 60 kDa isolated from human placenta which stimulates the GDP/GTP exchange of H-Ras, even without any C-terminal prenylation.[105] The sequence of Smg GDS cDNA revealed that this GDS contains 11 repeats of 42-amino acid Armadillo (ARM) motif.[107,109] A similar ARM motif has been found in several other proteins such as the tumor suppressors APC and importin/SRP1 as well as an APC-binding protein called β-catenin/Armadillo and SMAP, an Smg GDS-binding protein.[109-112]

β-Catenin is a cytoplasmic oncoprotein which directly binds c-ErbB-2, an oncogenic receptor Tyr-kinase, through its ARM motifs.[113] An N-terminally deleted mutant of β-catenin which no longer binds the tumor suppressor cadherin, but still binds c-ErbB-2, blocks the c-ErbB-2 dependent invasion and metastasis of gastric cancer cells.[113] This mutant blocks the interaction between the endogenous β-catenin and c-ErbB-2.[113] Three other proteins, APC, E-cadherin and an F-actin bundler of 55 kDa called fascin, also bind β-catenin competitively through these ARM motifs.[114] Furthermore, an oncogenic cytokine called Wnt-1/wingless activates free β-catenin somehow through Fz and Dsh, allowing β-catenin to form a complex with transcription factors of Tcf/Lef family and translocates it into the nucleus, causing malignant transformation.[115] However, both the nuclear localization and Tcf/Lef activation of β-catenin are blocked by its binding to the tumor suppressor APC which is mediated by a protein kinase called ZW3/GSK3-β.[110,112,115] By analogy with APC and β-catenin, it is also conceivable that SMAP is a tumor suppressor that sequesters Smg GDS which is potentially an oncoprotein, as the latter activates Ki-Ras. In fact, Tyr-phosphorylation of SMAP by v-Src significantly reduces its affinity for Smg GDS.[112] Interestingly, SMAP is closely related to an accessory subunit of sea urchin kinesin II, a motor ATPase translocating vesicles along microtubules.

Three other unique features of Smg GDS distinguish this GDS from any other GDSs:[116] (i) unlike other GDSs which bind only the GDP-bound forms (and not the GTP-bound forms) of their target GTPases, Smg GDS binds both the GDP-bound and the GTP-bound forms of its target GTPases; (ii) Smg GDS no longer stimulates the GDP/GTP exchange, when its target GTPases are bound to the plasma membranes, whereas other GDSs can stimulate the GDP/GTP exchange whether their target GTPases are on the membrane or not; and (iii) Smg GDS releases its target GTPases from the membrane. These observations led Takai's group to a very provocative conclusion: the major physiological function of Smg GDS is the translocation of its target GTPases from the membrane to the cytosol, rather than the stimulation of their GDP/GTP exchange per se.[116]

Subsequently, DBL and several other GDSs which are highly specific for Rho family GTPases were isolated or cloned (see Table 11.2). Most of these so-called Rho GDSs are oncogenic and share with DBL both DH and PH domains which are located immediately adjacent to each other. It is the DH domain that is responsible for stimulation of the GDP/GTP exchange.[97] Two distinct oncoproteins DBL and Ost stimulate the GDP/GTP exchange of both CDC42 and RhoA.[97,117] Interestingly, the CDC42 GDS activity is stimulated by a neuropeptide called bradykinin through its cell surface receptor,[118] and this receptor is overproduced around 50 times more over the normal level upon Ras-induced malignant transformation.[119] Since both RhoB and CDC42 are required for oncogenicity of Ras,[26,120] it is conceivable that these Rho family G proteins could be activated by Ras through DBL or Ost. The PH domain of DBL alone is not sufficient, but required, for malignant transforma-

tion.[121] Furthermore, the PH domain is not required for the in vitro GDS activity, but is both required and sufficient for the association of DBL with actin-cytoskeleton.[121] Interestingly, unlike normal fibroblasts, DBL-transformed fibroblasts require fibronectin to form actin stress fibers,[122] suggesting that, like Ras and Src, DBL downregulates fibronectin gene. However, unlike Ras and Src, DBL upregulates its receptor (α5 integrin) gene. Ost binds Rac, but in the GTP-bound form, without any GAP or GDS activity towards Rac, suggesting that Ost could be an effector of Rac. However, so far no effect of Rac has been shown on the GDS activity of Ost. Both DBL and Ost activate Jun-kinase (JNK) through CDC42, and this activation is inhibited by either the C-terminal GAP domain of p190-A or a dominant negative mutant of the CDC42/Rac-activated kinase PAK.[123] For details of the PAK kinase family, see chapter 16.

Two additional oncogenic members of the DH family called LBC and p115 are GDSs specific for Rho GTPases.[124,125] The faciogenital dysplasia gene product FGD1 of 961 amino acids is a GDS highly specific for CDC42, and mutations in the DH domain cosegregate with the developmental disease Aarskog-Scott syndrome.[126] Interestingly, an FGD1-related protein of 766 amino acids called frabin shares 71 sequence identity with FGD1 in the DH domain, and cross-links actin filaments through the N-terminal domain. Its overexpression leads to the activiation of JNK, presumably through CDC42.[126a] The oncoprotein VAV serves as a GDS for Rac, only when Vav is Tyr-phosphorylated.[127] Furthermore, the phosphorylation of Vav by Lck is inhibited by PIP_2 (a substrate of PI-3 kinase), but enhanced by PIP_3 (a product of PI-3 kinase), clearly indicating that Vav mediates the Ras/PI-3 kinase-induced Rac activation.[127a] The oncogenicity of the Rho GDSs (LBC and p115) and Rac GDS (Vav) supports the notion that both Rho and Rac are required for Ras transformation. However, the role of either CDC42 or FGD1 in the transformation process still remains to be determined. The oncoprotein Tiam-1 which is responsible for the Rac-dependent invasiveness of T-lymphoma cells is also a member of DH family GDS which activates not only Rac, but also Rho and CDC42.[128] Interestingly, Tiam-1 contains an extra PH domain at the N-terminus. This N-terminal PH domain, but not the DH-adjacent PH domain, is essential for Tiam-1 to be membrane-localized and activate Rac.[129] The N-terminal PH domain can be functionally replaced by the myristoylation signal.

Rather surprisingly, Richard Cerione and his colleagues reported[130] that GDP dissociation of Rho family GTPases, in particular CDC42 and Rho, is stimulated in vitro by a unique acidic phospholipid, phosphatidylinositol 4,5 bisphosphate (PIP_2). The action of PIP_2 requires the C-terminal basic tail of Rho family GTPases, and it binds preferentially the GDP/GTP-free form of these GTPases. Ras, Rapl and Ran GTPases are insensitive to the action of PIP_2. Since Ras induces the production of this phospholipid through the PI-3 kinase/Rac pathway, it is possible that Ras activates both Rho and CDC42 through PIP_2, in addition to the pathway involving the bradykinin receptor and Rho/CDC42 GDSs.[26] However, whether PIP_2 plays any physiological role in the activation of these Rho family G proteins in vivo still remains to be determined.

Rapl/Ras GDSs

Initially Smg GDS was identified as the first GDS for Rapl family. However, it is not specific for Rapl and activates even Ki-Ras and Rho family as well. In 1994, another GDS called C3G was cloned by Michiyuki Matsuda and his colleagues that, like SOS, activate Ras family.[131] C3G binds Crk, an oncogenic Grb-2 -related SH2/SH3 protein, through an interaction between the SH3 domain of Crk and the Pro-rich motif of C3G. However, it was realized later that the GDS activity of C3G is much higher towards Rapl family than Ras family.[132] Thus, C3G could be considered as a Rap1 GDS. Since overexpression of Rapl GTPases was reported to suppress v-Ki-Ras-induced malignant transformation,[40] one might expect that its activator C3G serves as an anti-Ras tumor suppressor. However, it was found

Table 11.2. Mammalian GDSs for Ras/Rho family GTPases

Ras GDSs	Targets	Rho GDSs	Targets
SOS	Ras	DBL	Rho, CDC42
		OST	Rho, CDC42
Ras-GRF	Ras	Tiam-1	Rac, Rho, CDC42
		VAV	Rac
C3G	Rapl, Ras	LCB	Rho
		p115	Rho
Smg GDS	Rapl, Ki-Ras, Rho, Rac	FGD1	CDC42

recently that C3G is required for both Crk-induced JNK activation and malignant transformation in NIH 3T3 fibroblasts, whereas Rapl suppresses Crk-induced transformation.[133] Thus, it is clear that (i) C3G serves as an oncogenic effector of Crk, and that (ii) Rapl is not an effector of C3G at least for malignant transformation. Crk induces neurite outgrowth of PC12 cells in a Ras-dependent manner.[134] However, Ras is not essential for C3G-induced JNK activation (Tanaka S, personal communication). Thus, it appears that Crk activates another Ras-related G protein through C3G. In this context, it is of interest to note that Rheb, a novel Ras-related G protein, also binds Raf, and induces neurite outgrowth of PC12 cells strongly, in particular in the presence of both NGF and a cAMP derivative.[135] Since the phosphorylation of Raf at Ser43 by cAMP-dependent protein kinase (PKA) blocks the Ras-Raf interaction, whereas it increases the affinity of Raf for Rheb.[135] Rheb could serve as an alternative effector of C3G for both fibroblast transformation and neuronal differentiation when cAMP blocks the Ras-Raf interaction.

GDP Dissociation Inhibitors (GDIs)

So far no GDI has been identified that is specific for either Ras or Rapl family GTPases. Thus, here I will discuss only the GDIs specific for Rho family GTPases. The majority of work on Rho GDIs was done by Takai's group since 1990. The first GDI of 27 kDa was isolated from the cytosol of bovine brains[6] and subsequently its cDNA was cloned.[136] It binds preferentially the GDP-bound form of all Rho family GTPases (Rho, Rac and CDC42), but neither Ras nor Rapl family GTPases. This GDI inhibits the GDP dissociation from its target GTPases only when they are prenylated at the C-termini. Furthermore, GDI releases these GTPases in their GDP-bound forms (D-GTPases) from the plasma membranes. Thus, in the nonstimulated cells, D-GTPases form a complex with GDI and remain in the cytosol. Upon the activation of GDSs for these GTPases, D-GTPases are converted to their GTP-bound forms (T-GTPases), and subsequently the T-GTPases are released from GDI. As a consequence, T-GTPases are translocated to the plasma membranes and interact with their effectors. When T-GTPases are converted to D-GTPases by various GAPs, GDI binds D-GTPases and this complex is released from the plasma membranes.

Interestingly, however, GDI blocks the GDP-dissociatiating activity of DBL and other GDSs, probably due to the competition between GDI and GDSs for binding to D-GTPases. This implies that for D-GTPases to be activated, either the affinity for GDI for D-GTPases must be reduced or that of GDSs be increased by some factor(s). In fact, a novel membrane-bound protein that has such a function was detected in mammalian cells.[137] It is called GDF (GDI displacement factor). The GDF is considered to form a complex with GDI, reducing the affinity for D-GTPases, and thereby allowing GDSs to bind D-GTPases. However, the

detailed mode of action of GDF should await its purification and further characterization in the future.

Acknowledgment

The author is very grateful to all of his colleagues, in particular Alam Nur-E-Kamal for his work on both Ras and GAPs, Dennis Wang for his work on the tumor suppressor p190-A, and Tony Burgess for his consistent support and encouragement during the last decade. This work has been supported in part by a grant from the Anti-Cancer Council of Victoria.

References

1. Rybin V, Ullrich O, Rubino M et al. GTPase activity of Rab5 acts as a timer for endocytic membrane fusion. Nature 1996; 383:266-269.
2. Bos JL. Ras oncogenes in human cancer: a review. Cancer Res 1989; 49:4682-4689.
3. Trahey M, McCormick F. A cytoplasmic protein stimulates normal Ras GTPase, but does not affect oncogenic mutants. Science 1987; 238:542-545.
4. Broek D, Toda T, Michaeli T et al. The S. cerevisiae CDC25 gene product regulates the Ras/adenylate cyclase pathway. Cell 1987; 48:789-799.
5. Jones S, Vignais ML, Broach JR. The CDC25 protein of S. cerevisiae promotes GDP/GTP exchange of Ras. Mol Cell Biol 1991; 11:2641-2646.
6. Ueda T, Kikuchi A, Ohga N et al. Purification and characterization from bovine brain cytosol of a novel protein inhibiting the GDP dissociation from RhoB. J Biol Chem 1990; 265:9373-9380.
7. Gibbs JB, Schaber MD, Allard WJ et al. Purification of Ras GAP from bovine brain. Proc Natl Acad Sci USA 1988; 85:5026-5030.
8. Vogel US, Dixon RAF, Schaber MD et al. Clong of bovine GAP and its interaction with oncogenic Ras. Nature 1988; 335:90-93.
9. Marshall MS, Hill WS, Ng AS et al. C-terminal domain of GAP is sufficient to stimulate Ras GTPase activity. EMBO J 1989; 8:1105-1110.
10. Maruta H, Holden J, Sizeland A et al. The residues of Ras and Rap proteins that determine their GAP specificityies. J Biol Chem 1991; 266:11661-11668.
11. Sigal IS, Gibbs JB, D'Alonzo JS et al. Identification of effector residues and a neutralizing epitope of Ha-Ras. Proc Natl Acad Sci USA 1986; 83:4725-4729.
12. Adari H, Lowy DR, Willumsen BM et al. GAP interacts with the Ras effector domain. Science 1988; 240:518-521.
13. Cales C, Hancock JF, Marshall CJ et al. The GAP is implicated as the target for regulation by Ras. Nature 1988; 332:548-551.
14. Nur-E-Kamal MSA, Sizeland A, D'Abaco G et al. Asn26, Glu31, Val45 and Tyr64 of Ras proteins are required for their oncogenicity. J Biol Chem 1992; 267:1415-1418.
15. Martin G, Yatani A, Clark R et al. GAP domains responsible for Ras-dependent inhibition of muscarinic atrial K^+ channel currents. Science 1992; 255:192-194.
16. Chang JS, Kobayashi M, Wang DZM et al. Two regions with differential growth-modulating activity in the N-terminal half of Ras GAP: SH2/SH3 and Gly-Ala-Pro-rich regions. Eur J Biochem 1995; 232:691-699.
17. Parker F, Maurier F, Delumeau I et al. A Ras GAP SH3 binding protein (p68/G3BP). Mol Cell Biol 1996; 16:2561-2569.
18. Duchesne M, Schweighoffer F, Parker F et al. Identification of the SH3 domain of GAP as an essential sequence for Ras-GAP-mediated signaling. Science 1993; 259:525-528.
19. Xu G, Lin B, Tanaka K et al. The catalytic domain of the NF1 stimulates Ras GTPase and complements IRA mutants of S. cerevisiae. Cell 1990; 63:835-841.
20. Martin G, Viskochil D, Bollag G et al. The GAP-related domain of the NF1 interacts with Ras. Cell 1990; 63:843-849.
21. Ballester R, Marchuk D, Boguski M et al. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA. Cell 1990; 63:851-859.

22. Marchuk D, Saulino AM, Tavakkol R et al. cDNA clong of the NF1: complete sequence of NF1. Genomics 1991; 11:931-940.
23. Nur-E-Kamal MSA, Varga M, Maruta H. The GTPase-activating NF1 fragment of 91 amino acids reverses v-Ha-Ras-induced malignant phenotype. J Biol Chem 1993; 268:22331-22337.
24. Nur-E-Kamal MSA, Maruta H. The role of Gln61 and Gln63 of Ras GTPases in their activation by NF1 and Ras GAP. Mol Biol Cell 1992; 3:1437-1442.
25. Warne PH, Viciana PR, Downward J. Direct interaction of Ras and the N-terminal region of Raf in vitro. Nature 1993; 364:352-355.
26. Maruta H. Downstream of RAS and actin-cytoskeleton. In: Maruta H, Burgess AW, eds. Regulation of the RAS Signaling Network. Austin, Heidelberg: RG Landes, Springer-Verlag, 1996:139-180.
27. Fridman M, Tikoo A, Varga M et al. The minimal fragments of Raf and NF1 that can suppress v-Ha-Ras-induced malignant phenotype. J Biol Chem 1994; 269; 30105-30108.
28. Basu TN, Gutman DH, Fletcher JA et al. Aberrant regulation of Ras proteins in malignant tumors from NF1 patients. Nature 1992; 356:713-715.
29. DeClue JE, Papageorge AG, Fletcher JA et al. Abnormal regulation of mammalian Ras contributes to malignant tumor growth in NF1. Cell 1992; 69:265-273.
30. Brannan C, Perkins AS, Vogel KS et al. Targeted disruption of the NF1 gene leads to developmental abnormalities in heart and various neural crest-derived tissues. Genes & Develop 1994; 8:1019-1029.
31. Jacks T, Shih TS, Schmitt EM et al. Tumor predisposition in mice heterozygous for a targeted mutation in NF1. Nature Genet 1994; 7:353-361.
32. Leder A, Kuo A, Cardiff RD et al. v-Ha-Ras transgene abrogates the initiation step in mouse skin tumorigenesis: effects of phorbol esters and retinoic acid. Proc Natl Acad Sci USA 1990; 87:9178-9182.
33. Henkemeyer M, Rossi DJ, Holmyard DP et al. Vascular system defects and neuronal apoptosis in mice lacking Ras GAP. Nature 1995; 377:695-701.
33a. The I, Hannigan GE, Cowley GS et al. Rescue of a Drosophila NF1 mutant phenotype by protein kinase A. Science 1997; 276:791-794.
33b. Guo HF, The I, Hannan F et al. Requirement of Drosophila NF1 for activation of adenylate cyclase by PACAP38-like neuropeptides. Science 1997; 276:795-798.
33c. Zhong Y. Mediation of PACAP-like neuropeptide transmission by coactivation of Ras/Raf and cAMP signal transduction pathways in Drosophila. Nature 1995; 375:588-592.
34. Bollag G, McCormick F, Clark R. Characterization of full-length NF1: tubulin inhibits Ras GAP activity. EMBO J 1993; 12:1923-1927.
35. Gregory PE, Gutman Dh, Mitchell A et al. NF1 associates with microtubules. Somat Cell Mol Genet 1993; 19:265-274.
36. Pizon V, Chardin P, Lerosey I et al. Human cDNAs Rapl and Rap2 homologous to the Drosophila D-Ras3 encode proteins closely related to Ras in the "effector" region. Oncogene 1988; 3:201-204.
37. Frech M, John J, Pizon V et al. Inhibition of GAP stimulation of Ras GTPase by Rapl. Science 1990; 249:169-171.
38. Hata Y, Kikuchi A, Sasaki T et al. Inhibition of GAP-stimulated Ras GTPase activity by Rapl. J Biol Chem 1990; 265:7104-7107.
39. Kitayama H, Matsuzaki T, Ikawa et al. Genetic analysis of Rapl: Potentiation of its tumor suppressor activity by specific point mutations. Proc Natl Acad Sci USA 1990; 87:4284-4288.
40. Kitayama H, Sugimoto Y, Matsuzaki T et al. A Ras-related gene with transformation suppressor activity. Cell 1989; 56:77-84.
41. Sato KY, Polakis PG, Haubruck H et al. Analysis of the tumor suppressor activity of the Rapl/Krev-1 in human tumor cell lines. Cancer Res 1994; 54:552-559.
42. Hesse J, Maruta H Isenberg I. Monoclonal antibodies localize the exchangeable GTP-binding site in β- and not α-tubulins. FEBS Lett 1985; 179:91-95.
43. Maruta H, Greer K, Rosenberg JL. The acetylation of α-tubulin and its relationship to the assembly and disassembly of microtubules. J Cell Biol 1986; 103:571-579.
44. Stearns T, Evans L, Kirschner M. γ-Tubulin is a highly conserved component of the centrosome. Cell 1991; 65:825-836.

45. Maekawa M, Nakamura S, Hattori S. Purification of a novel GAP from rat brain. J Biol Chem 1993; 268; 22948-2952.
46. Maekawa M, Li S, Iwamatsu A et al. A novel Ras GAP which has phospholipid-binding and BTK homology regions. Mol Cell Biol 1994; 14:6879-6885.
47. Gaul U, Mardon G, Rubin GM. A putative Ras GAP acts a negative regulator of signalling by the sevenless receptor Tyr-kinase. Cell 1992; 68:1007-1019.
48. Lee S, Escaante R, Firtel R. A Ras GAP is essential for cytokinesis and spacial patterning in Dictyostelium. Develop 1997; 124:983-996.
49. Adachi H, Takahashi Y, Hasebe T et al. Dictyostelium IQGAP-related protein specifically involved in the completion of cytokinesis. J Cell Biol 1997; 137:891-898.
49a. Tuxworth RI, Cheetham JL, Machesky LM et al. Dictyosytelium Ras G is required for normal motility and cytokinesis, but not growth. J Cell Biol 1997; 138:605-614.
50. Cullen PJ, Hsuan JJ, Truong O et al. Identification of a specific Ins (1,3,4,5) P_4-binding protein as a member of the GAP1 family. Nature 1995; 376:527-530.
51. Davis AJ, Butt JT, Walker JH et al. The Ca^{2+}-dependent lipid binding domain of GAP1 mediates protein-protein interactions with Ca^{2+}-dependent membrane-binding proteins.: evidence for a direct interaction between annexin VI and GAP1. J Biol Chem 1996; 271:24333-24336.
52. Theobald J, Hanby A, Patel K et al. Annexin VI has tumor suppressor activity in human A431 squamous epithelial carcinoma cells. Brit J Cancer 1995; 71:786-788.
53. Shibata S, Sato S, Maki M et al. Placental annexins inhibit protein kinase C. J Biochem 1992; 112:552-556.
54. Lin HC, Sudof TC, Anderson RGW. Annexin VI is required for budding of clathrin-coated pits. Cell 1992; 70:283-291.
55. Yamamoto T, Matsui T, Nakafuku M et al. A novel GAP for R-Ras. J Biol Chem 1995; 270:30557-30561.
56. Wang HG, Millan JA, Cox AD et al. R-Ras promotes apoptosis caused by growth factor deprivation via a Bcl-2 suppressible mechanism. J Cell Biol 1995; 129:1103-1114.
57. Fernandez-Sarabia MJ, Bischoff JR. Bcl-2 associates with the R-Ras. Nature 1993; 366:274-275.
58. Scheffzek K, Lautwein A, Kabsch W et al. Crystal structure of the GAP domain of the human GAP1 and implications for the interaction with Ras. Nature 1996; 384:591-596.
59. Scheffzek K, Ahmadian M, Kabsch W et al. The Ras-GAP1 complex: Structural basis for GTPase activation and its loss in oncogenic Ras mutants. Science 1997; 277:333-338.
60. Garret MD, Self AJ, Van Oers C et al. Indentification of distinct cytoplasmic targets for Ras and Rho regulatory proteins. J Biol Chem 1989; 264:10-13.
61. Lancaster CA, Taylor-Harris PM, Self AJ et al. Characterization of Rho GAP: a GAP for Rho-related GTPases. J Biol Chem 1994; 269:1137-1142.
62. Lamarche N, Hall A. GAPs for Rho-related GTPases. TIG 1994; 10:436-440.
63. Settleman J, Albright CF, Foster LC et al. Association between GTPases activators for Rho and Ras families. Nature 1992; 359:153-154.
64. Hu KQ, Settleman J. Tandem SH2 binding sites mediate the Ras GAP-p190 interaction: a conformational mechanism for SH3 domain regulation. EMBO J 1997; 16:473-483.
65. Morii N, Kumagai N, Nur-E-Kamal MSA et al. Rho GAP of 28 kDa (GAP2), but not of 190 kDa (p190), requires Asp65 and Asp67 of Rho GTPase for its activation. J Biol Chem 1993; 268:27160-27163.
66. Foster R, Hu KQ, Shaywitz DA et al. p190 Rho GAP, the major Ras Gap-associated protein, bind GTP directly. Mol Cell Biol 1994; 14:7173-7181.
67. Burbelo PD, Miyamato S, Utami A et al. p190-B, a new member of the Rho GAP family, and Rho are induced to cluster after integrin cross-linking. J Biol Chem 1995; 270:30919-30926.
68. Wang DZM, Nur-E-Kamal MSA, Tikoo A et al. The GTPase and Rho GAP domains of p190, a tumor suppressor that binds Ras GAP of 120 kDa (GAP1), independently function as anti-Ras tumor suppressors. Cancer Res 1997; 57:2478-2484.

69. LeClerc S, Pathaniswami R, Xie B et al. Molecular cloning and characterization of a factor that binds the human glucocorticoid receptor gene and represses its expression. J Biol Chem 1991; 266:17333-17340.
70. Reinhard J, Scheel AA, Diekmann D et al. A novel type of myosin implicated in signaling by Rho family GTPases. EMBO J 1995; 14:697-704.
70a. Saras J, Franzen P, Aspenstrom P et al. A novel GAP for Rho interacts with a PDZ domain of the protein Tyr phosphatase PTPL1. J Biol Chem 1997; 272:24333-24338.
70b. Tikoo A, Varga M, Ramesh V et al. An anti-Ras function of neurofibromatosis type 2 gene product (NF2/Merlin). J Biol Chem 1994; 269:23387-23390.
70c. Myers M, Stolarov J, Eng C et al. P-TEN, the tumor suppressor from human chromasome 10q23, is a dual-specificity phosphatase. Proc Natl Acad Sci USA 1997; 94:9052-9057.
71. Homma Y, Emory Y. A dual functional signal mediator showing Rho GAP and phospholipase C-deta stimulating activities. EMBO J 1995; 14:286-291.
72. Hildebrand JD, Taylor JM, Parsons JT. An SH3 domain-containing GAP for Rho and CDC42 associates with FAK. Mol Cell Biol 1996; 16:3169-3178.
73. Kozma R, Ahmed S, Best A et al. The GAP N-chimaerin cooperates with Rac1 and CDC42 to induce the formation of lamellipodia and filopodia. Mol Cell Biol 1996; 16:5069-5080.
74. Kikuchi A, Sasaki T, Araki S et al. Purification and characterization from bovine brain cytosol of two GAPs specific for Smg/Rapl. J Biol Chem 1989; 264:9133-9136.
75. Holden J, Nur-E-Kamal MSA, Fabri L et al. Rsr1 and Rapl GTPases are activated by the same GAP and require Thr65 for their activation. J Biol Chem 1991; 266:16992-16995.
76. Nice E, Fabri L, Hammacher A et al. The purification of a Rapl GAP from bovine brain cytosol. J Biol Chem 1992; 267:1546-1550.
77. Bender A, Pringle J, Multicopy suppression of the CDC24 budding defect in yeast by CDC42 and three newly identified genes including the Ras-related gene RSR1. Proc Natl Acad Sci USA 1989; 86:9976-9980.
78. Polakis PG, Rubinfeld B, Evans T et al. Purification of plasma membrane-associated GAP specific for Rapl from HL60 cells. Proc Natl Acad Sci USA 1991; 88:239-243.
79. Rubinfeld B, Munemitsu S, Clark L et al. Molecular cloning of a GAP specific for the Rapl. Cell 1991; 65:1033-1042.
80. Hattori M, Tsukamoto, Nur-E-Kamal MSA et al. Molecular cloning of a novel mitogen-inducible nuclear protein with a Ran GTPase activating domain that affects cell cycle progression. Mol Cell Biol 1995; 15:552-560.
81. Park HO, Chant J, Herskowitz I. BUD2 encodes a GAP for BUD1/Rsrl necessary for proper bud-site selection in yeast. Nature 1993; 365:269-274.
82. Parrini MC, Bernardi A, Parmeggiani A. Determinants of Ras GTPases specifying the sensitivity to yeast IRA2 and human GAP1. EMBO J 1996; 15:1007-1111.
83. Rubinfeld B, Crosier WJ, Albert I et al. Localization of the Rapl GAP catalytic domain and sites of phosphorylation by mutational analysis. Mol Cell Biol 1992; 12:4634-4642.
84. Nur-E-Kamal MSA, Maruta H. unpublished observation.
85. Kurachi H, Wada Y, Tsukamoto N et al. Human Spa-1 gene product selectively expressed in lymphoid tissues in a GAP specific for Rapl and Rap2 GTPases. J Biol Chem 1997; 272:28081-28088.
86. Morianu J, Blobel G. Protein export from the nucleus requires the GTPase Ran and GTP hydrolysis. Proc Natl Acad Sci USA 1995; 92:4318-4322.
87. European Chromosome 16 Tuberous Sclerosis Consortium. Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell 1993; 75:1305-1315.
88. Wienecke R, Koenig A, DeClue JE. Identification of tuberin/Tsc2: Tuberin possesses specific Rapl GAP activity. J Biol Chem 1995; 270:16409-14414.
89. Wienecke R, Maize JC, Shoarinejad F et al. Co-localization of the Tsc2/ tuberin with its target Rapl in the Golgi apparatus. Oncogene 1996; 13:913-923.
90. Xiao GH, Shoarinejad F, Jin F et al. The Tsc2 gene product, tuberin, functions as a Rab5 GAP in modulating endocytosis. J Biol Chem 1997; 272:6097-6100.
91. Tsuchiya H, Orimoto K, Kobayashi T et al. Peresence of potent transcriptional activation domains in the predisposing tuberous sclerosis (Tsc2) gene product of the Ekker rat model. Cancer Res 1996; 56:429-433.

92. Bowtell D, Fu P, Simon M et al. Identification of murine homologues of the Drosophila SOS gene: potential activation of Ras. Proc Natl Acad Sci USA 1992; 89:6511-6515.
93. Simon M, Bowtell D, Dodson GS et al. Ras1 and a putative GDS perform crucial steps in signaling by the Tyr-kinase sevenless. Cell 1991; 67:701-716.
94. Bowtell D. Genetics of RAS signaling in Drosophila. In: Maruta H, Burgess AW, eds. Regulation of the RAS Signaling Network. Austin, Heidelberg: RG Landes, Springer-Verlag, 1996:1-45.
95. Maruta H, Burgess AW. Regulation of the RAS signaling network. BioEssays 1994; 16:489-496.
96. Burgess AW. Regulation of RAS activation. In: Maruta H, Burgess AW, eds. Regulation of the RAS Signaling Network. Austin, Heidelberg: RG Landes, Springer-Verlag, 1996:73-91.
97. Hart MJ, Eva A, Zangrilli D et al. Cellular transformation and GDS activity are catalyzed by a common domain on the DBL. J Biol Chem 1994; 269:62-65.
98. Eva A, Vecchio G, Rao CD et al. The predicted DBL oncogene product defines a distinct class of transforming proteins. Proc Natl Acad Sci USA 1988; 85:2061-2065.
99. Karlovich CA, Bonfini l, McCollam L et al. In vitro functional analysis of the Ras GDS SOS. Science 1995; 268:576-579.
100. Wang DZM, Hammond V, Abud H et al. Mutation in SOS1 dominantly enhances a weak allele of the EGFR, demonstrating a requirement for SOS1 in EGFR signaling and development. Gene & Develop 1997; 11:309-320.
101. Shou C, Farnworth CL, Neel BG et al. Molecular cloning of cDNA encoding a GRF (GDP releasing factor) for Ras. Nature 1992; 358:351-354.
102. Rosen LB, Ginty DD, Weber MJ et al. Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. Neuron 1994; 12:1207-1221.
103. Farnworth CL, Freshney NW, Rosen LB et al. Calcium activation of Ras mediated by neuronal exchange factor Ras GRF. Nature 1995; 376:524-527.
104. Mattingly RR, Macara IG. Phosphorylation-dependent activation of the Ras GRF by muscarinic receptors and G protein βγ subunits. Nature 1996; 382:268-272.
105. Downward J, Riehl R, Wu L et al. Identification of a GDS activity for Ras. Proc Natl Acad Sci USA 1990; 87:5998-6002.
106. Yamamoto T, Kaibuchi K, Mizuno T et al. Purification and characterization from bovine brain cytosol of proteins that regulate the GDP/GTP exchange of Smg/Rapl proteins. J Biol Chem 1990; 265:16626-16634.
107. Kaibuchi K, Mizuno T, Fujioka H et al. Molecular cloning of the cDNA for Smg GDS and characterization of the GDS. Mol Cell Biol 1991; 11:2873-2880.
108. Mizuno T, Kaibuchi K, Yamamoto T et al. Smg GDS is active on the post-translationally processed form of Ki-Ras and RhoA. Proc Natl Acad Sci USA 1991; 88:6442-6446.
109. Peifer M, Berg S, Reynolds AB. A repeating amino acid motif shared by proteins with diverse cellular roles. Cell 1994; 76:789-791.
110. Rubinfeld B, Souza B, Albert I et al. Association of the APC gene product with B-catenin. Science 1993; 262:1731-1734.
111. Toeroek I, Strand D, Schmidt R et al. The overgrown hematopoietic organs-31 tumor suppressor gene of Drosophila encodes an importin-like protein accumulating in the nucleus at the onset of mitosis. J Cell Biol 1995; 129:1473-1489.
112. Shimizu K, Takai Y. Smg GDS. Exp Med (Tokyo) 1996; 14:251-253.
113. Shibata T, Ochiai A, Kanai Y et al. Dominant negative inhibition of the association between β-catenin and c-ErbB-2 by N-terminally deleted β-catenin suppresses the invation and metastasis of cancer cells. Oncogene 1996; 13:883-889.
114. Tao YS, Edwards RA, Tubb B et al. β-Catenin associates with the actin-bundling protein fascin in a non-cadherin complex. J Cell Biol 1996; 134:1271-1281.
115. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996; 87:159-170.
116. Takai Y, Sasaki T, Tanaka K et al. Rho as a regulator of the cytoskeleton. TIBS 1995; 20:227-231.
117. Horii Y, Beeler JF, Sakaguchi K et al. A novel oncogene, Ost, encodes a GDS that potentially links Rho and Rac signaling pathways. EMBO J 1994; 13:4776-4786.

118. Kozma R, Ahmed S, Best A et al. The CDC42 and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 1995; 15:1942-1952.
119. Downward J, de Gunzburg J, Riehl R et al. Ras-induced responsiveness of phosphatidylinositol turnover to bradykinin is a receptor number effect. Proc Natl Acad Sci USA 1988; 85:5774-5778.
120. Qui RG, Abo A, McCormick F et al. CDC42 regulates anchorage-independet growth and is necessary for Ras transformation. Mol Cell Biol 1997; 17:3449-3458.
121. Zheng Y, Zangrilli D, Cerione R et al. The PH domain mediates transformation by oncogenic DBL through specific intracellular targeting. J Biol Chem 1996; 271:19017-19020.
122. Defilippi P, Livo C, Tarone G et al. Actin cytoskeleton polymerization in DBL-transformed NIH 3T3 fibroblasts is dependent on cell adhesion to specific extracellular matrix proteins. Oncogene 1997; 14:1933-1943.
123. Coso OA, Chiariello M, Yu JC et al. The Rac1 and CDC42 regulate the activity of the JNK/SAPK signaling pathway. Cell 1995; 81:1137-1146.
124. Zheng Y, Olson MF, Hall A et al. Direct involvement of Rho in LBC oncogene function. J Biol Chem 1995; 270:9031-9034.
125. Hart MJ, Sharma S, elMasry N et al. Identification of a novel GDS for the Rho GTPase. J Biol Chem 1996; 271:25452-25458.
126. Zheng Y, Fischer DJ, Santos MF et al. The faciogenital dysplasia gene product FGD1 functions as a CDC42-specific GDS. J Biol Chem 1996; 271:33169-33172.
126a. Obaishi H, Nakanishi H, Mandai K et al. Frabin, a novel FGD1-related F-actin binder capable of changing cell shape and activating JNK. J Biol Chem 1998; 272:18697-18700.
127. Crespo P, Schebel KE, Ostrom AA et al. Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the Vav. Nature 1997; 385:169-172.
127a. Han J, Luby-Phelps K, Das B et al. Role of substrates and products of PI-3 kinase in regulating activation of Rac GTPases by Vav. Science 1998; 279:558-560.
128. Michiels F, Habets GG, Stam JC et al. A role for Rac in Tiam1-induced membrane ruffling and invasion. Nature 1995; 375:338-340.
129. Michiels F, Stam JC, Hordijk PL et al. Regulated membrane localization of Tiam1, mediated by the N-terminal PH domain, is required for Rac-dependent membrane ruffling and JNK activation. J Cell Biol 1997; 137:387-398.
130. Zheng Y, Glaven J, Wu WJ et al. PIP2 provides an alternative to GDSs by stimulating the dissociation of GDP from CDC42. J Biol Chem 1996; 271:23815-23819.
131. Tanaka S, Morishita T, Hashimoto Y et al. C3G, a GDS expressed ubiquitously, binds the SH3 domains of Crk and Grb-2. Proc Natl Acad Sci USA 1994; 91:3443-3447.
132. Gotoh T, Hattori S, Nakamura S et al. Identification of Rapl as a target for the Crk SH3 domain-binding GDS C3G. Mol Cell Biol 1995; 15:6746-6753.
133. Tanaka S, Ouchi T, Hanafusa H. Downstream of Crk adaptor signaling pathway: activation of Jun kinase by v-Crk through the GDS C3G. Proc Natl Acad Sci USA 1997; 94:2356-2361.
134. Tanaka S, Hattori s, Kurata T et al. Both the SH2 and Sh3 domains of human Crk are required for neuronal differentiation of PC12 cells. Mol Cell Biol 1993; 13:4409-4415.
135. Yee WM, Worley PF. Rheb interacts with Raf-1 and may function to integrate growth factor- and protein kinase A-dependent signals. Mol Cell Biol 1997; 17:921-933.
136. Fukumoto Y, Kaibuchi K, Hori Y et al. Molecular cloning and characterization of a novel GDI for the Rho proteins. Oncogene 1990; 5:1321-1328.
137. Bokoch GM, Bohl BP, Chuang TH. GDP/GTP exchange regulates membrane translocation of Rac/Rho proteins. J Biol Chem 1994; 269:31674-31679.

Chapter 12

Oncogenic Ras Signaling Network

Julian Downward

Introduction

The *ras* genes were first discovered in the 1960s as the oncogenes of the acutely transforming Harvey and Kirsten rat sarcoma viruses, which were capable of causing tumors in rodents and cellular transformation in vitro. They were subsequently found to have been acquired by the retroviruses from cellular genes, and these *ras* protooncogenes have now been shown to be activated by point mutation in about 25% of all cases of human malignancies, making them numerically the most significant dominantly acting oncogenes in human cancer.[1] They code for closely related monomeric G proteins of 21 kDa which have proven to be the founding members of a very large superfamily of related proteins with diverse function. Ras and related proteins slowly hydrolyze bound GTP to GDP, a process that is stimulated by GTPase activating proteins (GAPs). Several lines of evidence indicate that Ras proteins are active in promoting cellular growth and transformation when they are bound to GTP and inactive when they are bound to GDP. Transforming mutants of Ras are defective in their GTPase activity, in particular its ability to be stimulated by GAPs, and hence remain in the active GTP bound state.[2]

In the past few years, very significant advances have been made in understanding the regulation and function of Ras in the control of cellular growth. It is now possible to trace every component in one of the growth signaling pathways triggered by an extracellular mitogen such as epidermal growth factor (EGF) to regulators of gene transcription within the nucleus. Upstream of Ras, stimulation of the epidermal growth factor receptor tyrosine kinase leads to the stimulation of GDP/GTP exchange activity on Ras by translocation of a GDP dissociation stimulator (GDS) for Ras, Sos, to the plasma membrane where it has access to the membrane localized Ras.[3] This translocation is achieved by the binding of an adaptor protein, Grb2, to the autophosphorylated carboxy terminal tail of the receptor, or to an intermediate tyrosine phosphorylated adaptor protein, Shc.[4] Grb2 contains one SH2 domain, which binds to phosphotyrosine residues, and two SH3 domains which are capable of binding to proline rich regions in the carboxy terminal region of the exchange factor Sos. Ras proteins respond to a very wide variety of extracellular stimuli; it is likely that many use a variation of the mechanism described above. In addition, in certain cases it has been found that the activity of GAPs is regulated by factors that activate Ras.[5]

Downstream of Ras, a number of proteins that interact directly with GTP-bound, active Ras, but not the GDP-bound, inactive form, have been identified. These are known as "effectors" of Ras, and mediate the consequences of Ras activation on the behavior of the cell. They bind to regions of Ras that alter in conformation between the GTP- and GDP-bound states, in particular the "effector loop", amino acids 32-40, which was originally

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

identified as an area in which mutations would destroy the biological activity of Ras without affecting its ability to bind to GDP/GTP.[6] This chapter will principally focus on the effects that activated Ras has on cellular behavior, and in particular will consider the various effector proteins acting downstream of Ras and their role in contributing signals that together make up the phenotype of Ras transformed cells.

Ras Effectors

Raf

The best characterized Ras effector is the product of the *raf-1* protooncogene. Raf-1 interacts directly with Ras when it is GTP-bound, but not when it is GDP-bound, both in vitro using purified proteins and in the yeast two-hybrid system.[7-10] Raf-1 and Ras have also been shown to form a complex in response to physiological Ras activating stimuli in intact cells.[11,12] Raf-1 is a serine/threonine kinase: two close relatives exist in mammals, A-Raf and B-Raf. The proteins are composed of three conserved regions: CR1 contains a cysteine rich zinc finger preceded by the Ras binding domain (RBD). CR2 contains several serine and threonine residues which can be phosphorylated. CR3 encompasses the kinase domain.

A likely model for the activation of endogenous Raf-1 in response to extracellular stimulation is that growth factor induced increases in the amount of GTP-bound Ras leads to recruitment of Raf to the plasma membrane where Ras is located: artificial localization of Raf to the plasma membrane by the addition of a CAAX isoprenylation motif at the carboxy terminus results in its constitutive activation.[13,14] The interaction with Ras does not cause activation of Raf by itself; it appears that other events are also involved, very likely including tyrosine phosphorylation of two residues on Raf, Y340 and Y341.[15] In the case of B-Raf there is evidence that interaction with Ras.GTP alone is sufficient to cause increased kinase activity.[16] In addition to these aspects of Raf regulation, Raf also binds to 14-3-3, in particular through the phosphorylated serine 259 in the CR2 domain of Raf.[17] 14.3.3 may play a role in Raf regulation, but details of exactly how this works, and how important this is in the normal mitogen regulation of Raf, are still lacking.

Moving downstream in the signaling cascade, the Raf proteins control the MAP kinase, or ERK, signaling pathway by phosphorylating and activating MEK1 and MEK2, which are MAP kinase kinases.[18] The Raf/MAP kinase pathway is essential for fibroblast proliferation[19] and is likely to be important in the transformation of fibroblasts and other cell types by Ras oncogenes (see later). The Raf/MAP kinase pathway has been identified genetically as an effector of Ras in the development of the *Drosophila* eye and *C. elegans* vulva. Clear evidence for other Ras effector pathways has not yet been identified in these organisms, although it has been suggested that the Raf related kinase Ksr might play an effector role downstream of Ras.[20]

Phosphatidylinositol-3 Kinase

In addition to Raf, a number of other possible direct targets for Ras are known. Phosphatidylinositol-3 kinase (PI-3 kinase) is an enzyme that phosphorylates the lipids phosphatidylinositol (PI), phosphatidylinositol (4) phosphate (PI(4)P) and phosphatidylinositol (4,5) bisphosphate (PI(4,5)P_2) to yield phosphatidylinositol (3) phosphate (PI(3)P), phosphatidylinositol (3,4) bisphosphate (PI(3,4)P_2) and phosphatidylinositol (3,4,5) trisphosphate (PI(3,4,5)P_3) respectively. PI(3,4)P_2 and PI(3,4,5)P_3 are second messenger molecules that are not found in unstimulated cells, but rapidly accumulate after growth factor stimulation.[21] PI-3 kinase is composed of a catalytic p110 subunit and a regulatory p85 subunit: the regulatory subunit has been reported to interact through its SH2 domains with a number of tyrosine phosphorylated proteins including autophosphorylated growth

factor receptors, such as the platelet-derived growth factor (PDGF) receptor, and adaptor molecules, such as the insulin receptor substrate 1 (IRS-1). In addition the proline rich motifs on p85 have been found to interact with a number of SH3 containing proteins including Src family kinases. The catalytic p110 subunit of PI-3 kinase has been shown to interact specifically with GTP-bound Ras.[22] This interaction results in stimulation of the lipid kinase activity of PI-3 kinase both in intact cells,[22] in a purified in vitro reconstitution system[23] and in a yeast model system.[24] Furthermore, inhibition of endogenous Ras function through the expression of dominant negative Ras results in inhibition of nerve growth factor (NGF) and epidermal growth factor (EGF) induced stimulation of PI-3 kinase activity in intact PC12 pheochromocytoma cells,[22] and of PDGF induced stimulation of PI-3 kinase activity in NIH 3T3 fibroblasts.[25]

Ras interacts with the α, β, γ and δ isoforms of p110,[22,23,26,27] but not more distantly related members of the family. A likely model for the regulation of PI-3 kinase activity in response to growth factors is that there is a synergistic activation through Ras interaction with the p110 domain and tyrosine phosphoprotein interaction with the p85 domain. Both these interactions appear to have the effect of translocating PI-3 kinase to the plasma membrane where its substrate is to be found, and also inducing its activation through inducing conformational change. Since Ras is also activated in response to growth factor treatment of cells, growth factors cause activation of PI-3 kinase both through p85/tyrosine phosphoprotein interaction and through Ras activation. Systems such as this in which multiple distinct synergistic signaling mechanisms are activated by the same growth factors, and then act on the same target enzymes in different ways, are likely to give very great sensitivity of the enzyme to the extracellular stimuli. This may explain why many mitogen stimulated enzymes are activated at very low concentrations of ligand which might only occupy a tiny fraction of the receptors at the cell surface.

As a result of PI-3 kinase activation, a number of target enzymes are switched on, in particular the serine/threonine kinases Akt/PKB and $p70^{S6K}$, and the small GTPase Rac (see later).

Ral-GDS

Use of the yeast two hybrid interaction screen revealed that activated Ras interacts with Ral-GDS, the founder member of a GDS family for the Ral GTPases[28-32] that also includes the related proteins RGL and RLF. Activated Ras interacts with the regulatory domain of Ral-GDS and is able to stimulate the enzymatic activity of its catalytic domain, which has homology to Ras GDSs such as CDC25, but acts only on RalA and RalB and not Ras.[33] In this way Ras is able to initiate a GTPase cascade resulting in Ral activation. Ral is about 50% identical to Ras, but its function is clearly distinct and not well understood. It has been implicated in the control of phospholipase D[34] which leads to the generation of lipid second messengers such as phosphatidic acid. Some Ral binding proteins have been identified in the yeast two hybrid system: Ral binding protein 1 (Ral-BP1) and RLIP76 are related proteins that possess GAP activity for the Rho family GTPases Rac and CDC42.[35,36] It has been postulated that this could be the means by which Ras controls Rac activity in cells, although definitive support for this is lacking.

GTPase Activating Proteins

The Ras specific GAPs include $p120^{GAP}$, Gap1 and neurofibromin, all of which interact with Ras.GTP through the effector loop, but not with Ras.GDP, could possibly have some function downstream of Ras, in addition to their negative regulatory function.[37,38] $p120^{GAP}$ contains SH2, SH3 and pleckstrin homology (PH) domains which could connect to other signaling proteins. Overexpression of the SH2 and SH3 domains of $p120^{GAP}$ can regulate

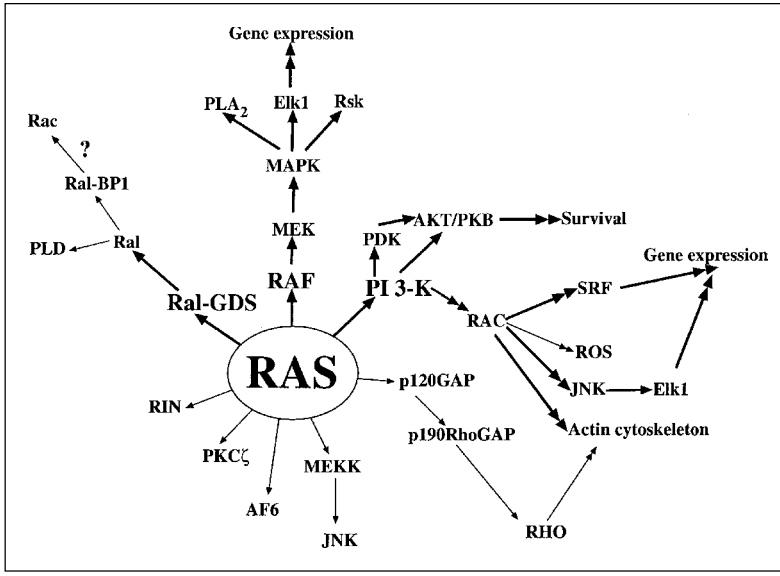


Fig. 12.1. Ras signals through multiple effector pathways to influence cell proliferation. Heavy arrows represent well-established pathways, while signaling via light arrows is less well proven. Double-headed arrows involve multiple steps.

transcription in a Ras-independent manner,[39] and $p120^{GAP}$ interacts with p190 Rho GAP,[40] hence providing possible mechanisms whereby $p120^{GAP}$ could act as an effector of Ras. It has been proposed that Ras could control the Rho GAP activity of p190 through $p120^{GAP}$, and thereby exert its effects on the actin cytoskeleton.[38] However, despite much investigation, no definitive proof that $p120^{GAP}$ significantly contributes to Ras downstream function under normal circumstances has emerged; indeed, deletion of both alleles of $p120^{GAP}$ does not affect the ability of Ras to transform mouse embryo fibroblasts.[41] The other Ras GAPs, neurofibromin and Gap1 and close relatives, could also conceivably have downstream function, though in these cases possible pathways that might be involved are less obvious. Neurofibromin may be involved in regulation of a cyclic AMP dependent pathway, but this appears to be independent of Ras.[42,43]

Other Mammalian Ras Targets

In addition to these Ras effectors, a number of other mammalian proteins have been described which interact with Ras in a GTP-dependent manner but for which further confirmation of their role in Ras signaling is not yet available. Ras has been reported to interact with MEKK1,[44] a MAP kinase kinase kinase type enzyme that was originally thought to activate the ERK pathway, but more recently has been shown to control the stress activated protein kinase JNK.[45] GTP, but not GDP, bound Ras interacts directly with the carboxy-terminal kinase domain of MEKK1, but it is not clear whether this interaction is sufficient to activate the kinase activity, or whether Ras directly affects MEKK under physiological conditions.

Ras also interacts directly with an atypical protein kinase C isoform, PKCζ in vitro.[46] The significance of this interaction is also not completely clear, but it has been shown that functional PKCζ is important in both the proliferation of fibroblasts and germinal vesicle

breakdown in *Xenopus* oocytes, a Ras induced response.[47] The atypical PKCs may also be involved in regulation of apoptosis: the product of the par-4 gene, whose expression has been shown to correlate with growth inhibition and apoptosis, specifically interacts with the regulatory domains of PKCζ and PKCι/λ, inhibiting their enzymatic activity.[48]

A yeast two hybrid screen identified another possible Ras effector, AF6.[49] This protein was also found in brain extract as a major Ras.GTP affinity column binding protein.[50] AF6 is homologous to the *Drosophila* Canoe protein, which is believed to function in the Notch developmental pathway, and has been identified as a fusion partner of ALL-1 in some acute lymphoblastic leukemias. It has been suggested that AF6 may be involved in regulating cell-cell junctions.

Rin was isolated as a human cDNA able to suppress the heat-shock sensitivity conferred by activated RAS2 in *S. cerevisiae* and was subsequently shown to bind to Ras.GTP, but not Rap1, in vitro.[51] Rin contains an SH2 domain and a proline-rich motif which interacts with the SH3 domain of Abl;[52] however, little is known of the biological significance of the Rin-Ras interaction.

As well as these putative effectors, several other proteins have been reported that interact with Ras in vitro. For example, both Jun and Jun N-terminal kinase (JNK) have been reported to bind to Ras.[53,54] It seems likely that yet more Ras binding proteins will be found in the future. The biological significance of these interactions remains open to speculation. Some of the interactions occur with very low affinity and might therefore be suspected to be less significant than high affinity interactions. However, it is clear that some extremely low affinity interactions, such as that of Ras with $p120^{GAP}$, have great biological significance, so affinity itself may not be a good guide to physiological relevance. There are circumstances where very high affinity interactions would not be advantageous in a signaling pathway: they would reduce the speed with which signals could be terminated. The ability of a protein to interact with Ras in a GTP-dependent manner is not sufficient to guarantee that it has effector function; its downstream function must be demonstrated in a whole cell, or whole organism, context.

Effectors of Ras in Yeast

Before any effectors for Ras were discovered in mammalian systems, *S. cerevisiae* Ras was found to interact with, and regulate the activity of, adenylyl cyclase.[55] However, this regulatory pathway does not operate in other systems, including other yeasts. In fission yeast, Ras has been shown to directly regulate two effectors. One is a MAP kinase kinase kinase, Byr2, the other is Scd1, a GDS for the Rho family protein CDC42, which is involved in regulation of cell morphology.[56] There is therefore precedent for Ras directly controlling a GDS for Rho family GTPases, although no such connection has yet emerged in mammalian systems. In budding yeast, CDC24, the homolog of Scd1, is not controlled by Ras but by the closely related protein Rsr1, whose closest mammalian relative is Rap1.[57] The Ras subfamily has at least eleven members in mammals, any of which, by analogy with yeast, might be capable of controlling the GDSs for Rho family. However, yeast does not always accurately mirror mammalian pathways, as is seen with Ras regulation of adenylyl cyclase in budding yeast.

Ras Effects on Cell Mitogenesis, Transformation and Other Behavior

The ability of Ras to interact with effector proteins leads to the propagation of signals that ultimately affect the progression of the cell through the cell cycle, and in cases of continuously activated signaling can cause cellular transformation. This section considers the signaling pathways acting downstream from the direct effectors of Ras, and how they contribute to mitogenesis, transformation and other events. It also examines the methodology

used to determine the roles played by the various Ras effector pathways in the control of different downstream events, including the powerful use of partial loss of function mutations in the Ras effector site.

Actin Cytoskeleton

Ras transformed cells have long been known to have undergone profound changes to their actin cytoskeleton, such as the loss of stress fibres.[58] When microinjected with activated Ras protein, fibroblasts show rapid membrane ruffling due to the polymerization of cortical actin.[59] Very similar effects have been seen with activated Rac,[60] and the effects of Ras have been shown to be inhibited by dominant negative forms of Rac, thus placing Ras upstream of Rac in the control of the cytoskeleton. Rho family protein signaling to the actin cytoskeleton is discussed in the chapter 14. The mechanism connecting Ras to Rac has been somewhat unclear until recently. Raf proteins do not have rapid effects on the cytoskeleton in fibroblasts, although longer term effects which are dependent on transcriptional regulation do occur. However, a number of other effectors could be involved. For example, Ral-GDS interacts with Ras and in turn activates Ral, which binds to Ral-BP1, a protein which contains a Rac GAP domain.[35,36,61] Ral-GDS might therefore be able to alter the activation state of Rac. In addition, expression of noncatalytic portions of $p120^{GAP}$ has effects on the cytoskeleton of fibroblasts:[38] a possible pathway by which $p120^{GAP}$ could influence Rho family proteins is through its associated protein p190,[62] which has GAP activity towards Rac and Rho.[40] Furthermore, in fission yeast, Ras has been shown to directly regulate Scd1, a GDS for CDC42, which is involved in regulation of cell morphology.[56] It is a possibility that a mammalian homolog of Scd1 may exist which is a Rac GDS that is directly regulated by interaction with Ras. Finally, a reasonable alternative is that Ras controls Rac through PI-3 kinase: PI-3 kinase has been shown to activate Rac, as determined both by morphological effects on cells[63] and by direct measurement of GTP levels on Rac in permeabilized cells.[64]

In order to distinguish between these various mechanisms, a number of approaches have been used. A potent technique has derived from the use of partial loss of function mutations in Ras.[65] Many mutations in the effector region of Ras, residues 32 to 40, are known to inhibit the biological function of Ras and to block the interaction of Ras with target proteins. Recently it has become clear that relatively subtle mutations in this region might lead to partial loss of function mutants in which interaction with some effectors is maintained but with others is lost. For example, White et al have shown that T35S Ras will still interact with Raf, but not the *S. pombe* MAP kinase kinase kinase Byr2, while D37G interacts with Byr2 but not Raf.[65] Both mutants are unable to transform mammalian cells by themselves but will cooperate together to give transformation; the normal mammalian targets for D37G Ras include Ral-GDS[66] and Rin.[52] Similarly, Joneson et al have reported that T35S Ras is able to induce MAP kinase activation but not membrane ruffling when microinjected into fibroblasts, while Y40C Ras induces membrane ruffling but not MAP kinase activation.[67] The two mutants acting together will induce DNA synthesis, but either one alone is ineffective. Y40C Ras has been shown to interact with PI-3 kinase,[68] but not Raf or Ral-GDS. It therefore seems very likely that PI-3 kinase, which interacts with Y40C Ras and is a known regulator of the actin cytoskeleton, is the link between Ras and actin rearrangement. However, a powerful argument against this mechanism was the fact that PI-3 kinase inhibitory drugs such as wortmannin or LY294002 failed to prevent Ras induced membrane ruffling.[63] Recently it has been shown that in fact these drugs do not effectively inhibit the ability of Ras to activate PI-3 kinase activity in some cells.[68] Other means of inhibiting PI-3 kinase using dominant negative mutants based on the regulatory p85 subunit show that Ras, but not Rac, induced ruffling is indeed dependent on PI-3 kinase activation. At least one isoform of PI-3 kinase, variously known as p170, Cpk and PI-3 kinase

C2α, has been identified that is very insensitive to wortmannin and LY294002;[69,70] this enzyme, or other drug-insensitive PI-3 kinases, may be responsible for the wortmannin resistance of Ras induced ruffling in some cells.

Gene Transcription

A fairly rapid effect of Ras activation is the stimulation of transcription from a number of mitogen responsive immediate early genes. One promoter which has been studied in detail is that of c-*fos*: this contains a serum responsive element (SRE) which complexes with the transcription factors serum response factor (SRF) and ternary complex factor (TCF), a family of ETS-related proteins including Elk-1. Elk-1 is phosphorylated by the MAP kinase ERK, acting downstream of the Ras effector Raf; this phosphorylation results in increased ternary complex formation and increased activity of the transcriptional activation domain.[71] In addition, the ERK pathway also leads to $p90^{Rsk2}$ (a kinase immediately downstream of ERK) phosphorylation and activation of CREB, the binding protein for the cAMP responsive element in the *fos* promoter.[72]

The Raf effector pathway is thus one means by which Ras controls transcription. However, several other signaling pathways downstream of Ras are also involved. For example, JNK is also able to phosphorylate and activate Elk-1. Rac is able to activate JNK,[73,74] so Ras may also regulate the SRE through a PI-3 kinase, Rac, JNK pathway. However, although activation of PI-3 kinase invariably causes actin cytoskeleton rearrangement, its ability to activate JNK is variable, with some reports of JNK activation by PI-3 kinase[75,76] and some reports of lack of activation.[77,78] Ras is not a particularly strong activator of JNK compared with cellular stresses: it is possible that various other pathways are involved in its activation, including autocrine production of growth factors,[79] direct interaction of Ras with MEKK1[44] or direct interaction of Ras with JNK.[53] In addition to the ability of Rac to regulate Elk-1 through the JNK pathway, there is also an as yet uncharacterized pathway connecting Rac, and hence Ras, to activation of SRF through a MAP kinase independent mechanism.[80] In addition, Ral-GDS has also been implicated in regulation of *fos* gene transcription, although the mechanism for this has not yet been determined.

Proliferation and Transformation

The ability of Ras to regulate both transcriptional and cytoskeletal events may be important for its control of cell cycle progression and transformation. Use of partial loss of function effector mutants of Ras has clearly shown that multiple pathways are required downstream of Ras for efficient transformation,[65,67,68,81] not just the Raf/MAP kinase pathway, although this pathway alone does have some transforming ability.[19] Dominant negative forms of MEK, which block the MAP kinase pathway, inhibit Ras transformation,[19,68] as do dominant negative forms of PI-3 kinase.[68] In addition, dominant negative Rac and Rho have also been found to inhibit Ras transformation,[82,83] suggesting that these pathways are also important. The picture emerging suggests that Ras transformation requires the function of several signaling pathways: however, a degree of caution should be used when interpreting data from dominant negative experiments of this type. It is possible that the dominant negative proteins are not as specific as claimed and may affect other related pathways, and also that they may affect pathways required for cell transformation or proliferation that are not normally controlled by Ras, but which are permissive for its function.

Effector mutants of Ras suggest that both the Raf/MAP kinase and PI-3 kinase pathways are needed for transformation. Rac appears to be able to provide a large part of the oncogenic signal downstream of PI-3 kinase in fibroblasts: partial loss of function effector mutants in Rac suggest that the ability to activate JNK is dispensable for transformation, while the ability to control the actin cytoskeleton is required.[84,85] Indeed, membrane ruffling

is closely connected with cell motility which may be an important component of metastatic potential in vivo.[86] The Rac GDS, Tiam-1, has been found as an oncoprotein in human T cell lymphomas, and increases cell invasiveness in vitro and metastasis in mice.[87] Another effector system activated by Rac that may be important in transformation is the generation of reactive oxygen species (ROS),[88,89] although the precise mechanisms involved in this process are currently unclear.

An area of considerable interest at present is how the various signaling pathways activated by Ras promote passage through the cell cycle; in other words, how the early signaling events described above translate into longer term regulation of cell cycle check points many hours later. Recent work has shown that Ras function is required for inactivation of the retinoblastoma protein (Rb) in late G1.[90,91] A considerable part of this signaling appears to be due to the ability of the Ras to induce Cyclin D1 expression through the Raf/MAP kinase pathway.[92] However, there are several indications that other pathways acting downstream of Ras may be important in cell cycle regulation. It is particularly interesting that the activation state of Ras, as measured by the ratio of bound GTP to GDP, varies throughout the cell cycle, and is high late in G1 at a time when the Raf/MAP kinase pathway is not activated.[93] It has been known for a considerable time that Ras function is required late in G1,[94,95] suggesting that Ras may be influencing the cell cycle through pathways other than Raf. In addition to the role of cellular Ras protein in promoting cell cycle progression, it has recently been shown that continuous strong activation of Ras by oncogenic mutation leads to increased expression of the cyclin-dependent kinase inhibitors $p16^{INK4A}$ and $p21^{WAF1}$ through a Raf pathway.[96,97] This leads to growth arrest and cell senescence, and presumably represents a safety mechanism to protect cells from Ras transformation.

In many cell types, particularly epithelial cells, which are of primary importance in human malignant disease, Ras controls another set of potentially oncogenic responses through PI-3 kinase which do not involve Rac. Oncogenic Ras mutants protect epithelial cells from apoptosis, in particular the apoptosis induced by loss of attachment to the extracellular matrix, a response sometimes referred to as "anoikis".[98,99] Detachment induced apoptosis is likely to be very important in ensuring that epithelial cells do not survive in locations in the body distant from their site of origin; this mechanism probably has to be overcome before a cell can metastasize. Study of the signaling pathways involved in the Ras suppression of detachment induced apoptosis reveals that this is achieved through activation of PI-3 kinase. However, Rac does not protect cells from apoptosis, rather the effect is mediated through the serine/threonine kinase known as Akt or PKB.[100] PKB/Akt is activated by a combination of PI-3 kinase produced lipids, such as phosphatidylinositol (3,4) bisphosphate and phosphatidylinositol (3,4,5) trisphosphate, binding to its amino terminal pleckstrin homology domain, and phosphorylation of PKB/Akt by upstream kinases that are directly activated by these lipids (PDK1 and PDK2).[101] The ability of Ras to promote cell survival in the absence of attachment through this pathway is likely to be a very important part of the mechanism whereby Ras transforms cells. However, while activated forms of PKB/Akt can cause cells to survive in suspension, they do not promote their growth: presumably other pathways downstream of Ras are required for this, probably predominantly the Raf/MAP kinase pathway.

Future Directions

The realization that Ras proteins signal through multiple effector pathways has greatly complicated our view of growth regulation. It is not yet clear whether all the significant signaling pathways acting downstream of Ras have been identified, and it is entirely possible that new effectors may still be discovered. Much work remains to be done to determine the physiological significance of many of the known effectors. Our understanding of these path-

ways is considerably complicated by the fact that in mammals many proteins exist that are closely related to Ras, such as Rap1, Rap2, R-Ras and TC21 which interact with most of the same effectors, and also most of the effectors exist as large families of related proteins. There is therefore scope for much subtlety and complexity to these signaling pathways. It is very likely that many of the methods used to study Ras effector pathways are not adequate to analyze this type of system; overexpression of components in cultured cells may well lead to loss of distinction between the function of related proteins and the specificity of dominant negative mutants has rarely been established satisfactorily. While biochemical and molecular biological studies have been very successful in establishing the strong interactions between signaling molecules in these pathways, new methods may be required to adequately address the complexities of Ras signaling pathways in mammals; certainly more use of mouse genetics and more cell biological study of culture systems expressing only endogenous levels of proteins will be very important in the future. Due to the importance of Ras to human malignant disease, it is very probable that the broad range of signaling mechanisms controlled by Ras will remain the subject of intense study and will continue to produce interesting insights for some time to come.

References

1. Bos JL. Ras oncogenes in human cancer: A review. Cancer Res 1989; 49:4682-4689.
2. Lowy DR, Willumsen BM. Function and Regulation of RAS. Annu Rev Biochem 1993; 62:851-891.
3. McCormick F. Signal Transduction: How receptors turn Ras on. Nature 1993; 363:15-16.
4. Buday L, Downward J. EGF regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein and Sos nucleotide exchange factor. Cell 1993; 73:611-620.
5. Downward J, Graves JD, Warne PH et al. Stimulation of $p21^{ras}$ upon T-cell activation. Nature 1990; 346:719-723.
6. Willumsen BM, Papageorge AG, Kung HF et al. Mutational analysis of a ras catalytic domain. Mol Cell Biol 1986; 6:2646-54.
7. Zhang X-F, Settleman J, Kyriakis JM et al. Normal and Oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 1993; 364:308-313.
8. Warne PH, Vicinia PR, Downward J. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature 1993; 364:352-355.
9. Vojtek AB, Hollenberg SM, Cooper JA. Mammalian Ras interacts directly with the Serine/Threonine kinase Raf. Cell 1993; 74:205-214.
10. Van Aelst L, Barr M, Marcus S et al. Complex formation between Ras and Raf and other protein kinases. Proc Natl Acad Sci USA 1993; 90:6213-6217.
11. Finney RE, Robbins SM, Bishop JM. Association of pRas and pRaf-1 in a complex correlates with activation of a signal transduction pathway. Current Biology 1993; 3:805-812.
12. Hallberg B, Rayter SI, Downward J. Interaction of Ras and Raf in intact mammalian cells upon extracellular stimulation. J Biol Chem 1994; 270:3913-3916.
13. Leevers SJ, Paterson HF, C.J. M. Requirement for ras in raf activation is overcome by targeting raf to the plasma-membrane. Nature 1994; 369:411-414.
14. Stokoe D, MacDonald SG, Cadwallader K et al. Activation of raf as a result of recruitment to the plasma-membrane. Science 1994; 264:1463-1467.
15. Fabian JR, Daar IO, Morrison DK. Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. Mol Cell Biol 1993; 13:7170-7179.
16. Yamamori B, Kuroda S, Shimizu K et al. Purification of a Ras-dependent mitogen-activated protein kinase kinase kinase from bovine brain cytosol and its identification as a complex of B-Raf and 14-3-3 proteins. J Biol Chem 1995; 270:11723-11726.
17. Muslin AJ, Tanner JW, Allen PM et al. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell 1996; 84:889-897.
18. Marshall CJ. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. Curr Opin Genet Develop 1994; 4:82-89.

19. Cowley S, Paterson H, Kemp P et al. Activation of map kinase kinase is necessary and sufficient for pc12 differentiation and for transformation of NIH 3T3 cells. Cell 1994; 77:841-852.
20. Downward J. KSR: A novel player in the RAS pathway. Cell 1995; 83:831-834.
21. Stephens LR, Jackson TR, Hawkins PT. Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-trisphosphate: A new intracellular signalling system. Biochim Biophys Acta 1993; 1179:27-75.
22. Rodriguez-Viciana P, Warne PH, Dhand R et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 1994; 370:527-532.
23. Rodriguez-Viciana P, Warne PH, Vanhaesebroeck B et al. Activation of phosphoinositide-3 kinase by interaction with Ras and by point mutation. EMBO J 1996; 15:2442-2451.
24. Kodaki T, Woscholski R, Hallberg B et al. The activation of phosphatidylinositol-3 kinase by Ras. Current Biology 1994; 4:798-806.
25. Klinghofer RA, Duckworth B, Valius M et al. PDGF-dependent activation of PI3 kinase is regulated by receptor binding of SH2 domain-containing proteins which influence Ras activity. Mol Cell Biol 1996; 16:5905-5914.
26. Rubio I, Rodriguez-Viciana P, Downward J et al. Ras interaction with phosphoinositide-3 kinase γ. Biochem J 1997; 326:891-895.
27. Vanhaesebroeck B, Welham MJ, Kotani K et al. p110δ, a novel phosphoinositide-3 kinase in leukocytes. Proc Natl Acad Sci USA 1997; 94:4330-4335.
28. Albright CF, Giddings BW, Liu J et al. Characteri-zation of a guanine nucleotide dissociation stimulator for a ras-related GTPase. EMBO-J 1993; 12:339-47.
29. Hofer F, Fields S, Schneider C et al. Activated ras interacts with the ral guanine-nucleotide dissociation stimulator. Proc Natl Acad Sci USA 1994; 91:11089-11093.
30. Kikuchi A, Demo SD, Ye ZH et al. Ral-GDS family members interact with the effector loop of ras p21. Mol Cell Biol 1994; 14:7483-7491.
31. Spaargaren M, Bischoff JR. Identification of the guanine-nucleotide dissociation stimulator for ral as a putative effector molecule of r-ras, h-ras, k-ras, and rap. Proc Natl Acad Sci USA 1994; 91:12609-12613.
32. Wolthuis RM, Bauer B, van't Veer LJ et al. RalGDS-like factor (Rlf) is a novel Ras and Rap 1A-associating protein. Oncogene 1996; 13:353-362.
33. Urano T, Emkey R, Feig LA. Ral-GTPases mediate a distinct downstream signaling pathway from ras that facilitates cellular-transformation. EMBO J 1996; 15:810-816.
34. Jiang H, Luo JQ, Urano T et al. Involvement of Ral GTPase in v-src induced phospholipase-D activation. Nature 1995; 378:409-412.
35. Cantor SB, Urano T, Feig LA. Identification and characterization of Ral-binding protein-1, a potential downstream target of Ral GTPases. Mol Cell Biol 1995; 15:4578-4584.
36. Jullien-Flores V, Dorseuil O, Romero F et al. Bridging ral GTPase to rho pathways: rlip76, a ral effector with CDC42/rac GTPase-activating protein activity. J Biol Chem 1995; 270:22473-22477.
37. Schweighoffer F, Barlat I, Chevalier-Multon M-Cet al. Implication of GAP in Ras-dependent transactivation of a polyoma enhancer sequence. Science 1992; 256:825-827.
38. McGlade J, Brunkhorst B, Anderson DM G et al. The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. EMBO J 1993; 12:3073-3081.
39. Medema RH, de Laat WL, Martin GA et al. GTPase activating protein SH2-SH3 domains induce gene expression in a ras-dependent fashion. Mol Cell Biol 1992; 12:3425-3430.
40. Settleman J, Narasimhan V, Foster LC et al. Molecular cloning of cDNAs encoding the GAP-associated protein p190: Implications for a signalling pathway from ras to the nucleus. Cell 1992; 69:539-549.
41. van der Geer P, Henkemeyer M, Jacks T et al. Aber-rant Ras regulation and reduced p190 tyrosine phosphorylation in cells lacking p120-Gap. Mol Cell Biol 1997; 17:1840-1847.
42. Guo HF, The I, Hannan F et al. Requirement of Drosophila NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. Science 1997; 276:795-798.
43. The I, Hannigan GE, Cowley GS et al. Rescue of a Drosophila NF1 mutant phenotype by protein kinase A. Science 1997; 276:791-794.

44. Russell M, Lange-Carter C, Johnson GL. Direct interaction between ras and the kinase domain of mitogen activated protein kinase kinase kinase (mekk1). J Biol Chem 1995; 270:11757-11760.
45. Minden A, Lin A, McMahon M et al. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science 1994; 266:1719-1723.
46. Diaz-Meco MT, Lozano J, Municio MM et al. Evidence for the in vitro and in vivo interaction of ras with protein kinase C zeta. J Biol Chem 1994; 269:31706-31710.
47. Berra E, Diaz-Meco MT, Dominguez I et al. Protein kinase C ζ isoform is critical for mitogenic siganl transduction. Cell 1993; 74:555-563.
48. Diaz-Meco MT, Municio MM, Frutos S et al. The product of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. Cell 1996; 86:777-786.
49. Van Aelst L, White MA, Wigler MH. Ras partners. Cold Spring Harb Symp Quant Biol 1994; 59:181-186.
50. Kuriyama M, Harada N, Kuroda S et al. Identification of AF-6 and canoe as putative targets for Ras. J Biol Chem 1996; 271:607-610.
51. Han L, Colicelli J. A human protein selected for interference with Ras function interacts directly with Ras and competes with Raf1. Mol Cell Biol 1995; 15:1318-1323.
52. Han L, Wong D, Dhaka A et al. Protein binding and signaling properties of RIN1 suggest a unique effector function. Proc Natl Acad Sci U S A 1997; 94:4954-4959.
53. Adler V, Pincus MR, Brandtrauf PW et al. Complexes of p21(ras) with jun n-terminal kinase and jun proteins. Proc Natl Acad Sci USA 1995; 92:10585-10589.
54. Adler V, Pincus MR, Polotskaya A et al. Activation of c-Jun-NH2-kinase by UV irradiation is dependent on p21ras. J Biol Chem 1996; 271:23304-23309.
55. Toda T, Uno I, Ishikawa T et al. In yeast Ras proteins are controlling elements of adenylate cyclase. Cell 1985; 40:27-36.
56. Chang EC, Barr M, Wang Y et al. Cooperative interaction of S. pombe proteins required for mating and morphogenesis. Cell 1994; 79:131-141.
57. Zheng Y, Bender A, Cerione RA. Interactions among proteins involved in bud-site selection and bud-site assembly in saccharomyces cerevisiae. J Biol Chem 1995; 270:626-630.
58. Weber K, Lazarides E, Goldman RD et al. Localization and distribution of actin fibers in normal, transformed and revertant cells. Cold Spring Harb Symp Quant Biol 1974; 39:363-369.
59. Bar Sagi D, Feramisco JR. Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. Science 1986; 233:1061-1068.
60. Ridley A, Paterson HF, Johnston CL et al. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 1992; 70:401-410.
61. Park SH, Weinberg RA. A putative effector of ral has homology to rho/rac GTPase-activating proteins. Oncogene 1995; 11:2349-2355.
62. Ellis C, Moran M, McCormick F et al. Phosphorylation of GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature 1990; 343:377-381.
63. Nobes CD, Hawkins P, Stephens L et al. Activation of the small GTP-binding proteins rho and rac by growth factor receptors. J Cell Sci 1995; 108:225-233.
64. Hawkins PT, Eguinoa A, Qiu RG et al. PDGF stimulates an increase in GTP-rac via activation of phosphoinositide-3 kinase. Current Biology 1995; 5:393-403.
65. White MA, Nicolette C, Minden A et al. Multiple ras functions can contribute to mammalian-cell transformation. Cell 1995; 80:533-541.
66. White MA, Vale T, Camonis JH et al. A role for the Ral guanine nucleotide dissociation stimulator in mediating Ras-induced transformation. J Biol Chem 1996; 271:16439-16442.
67. Joneson T, White MA, Wigler MH et al. Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of Ras. Science 1996; 271:810-812.
68. Rodriguez-Viciana P, Warne PH, Khwaja A et al. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell 1997; 89:457-467.
69. Virbasius JV, Guilherme A, Czech MP. Mouse p170 is a novel phosphatidylinositol-3 kinase containing a c2 domain. J Biol Chem 1996; 271:13304-13307.

70. Domin J, Pages F, Volinia S et al. Cloning of a human phosphoinositide-3 kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin. Biochem J 1997; 326:139-147.
71. Treisman R. Regulation of transcription by MAP kinase cascades. Curr Opin Cell Biol 1996; 8:205-215.
72. Xing J, Ginty D, Greenberg M. Coupling of the Ras-MAPK pathway to gene activation by RSK2, a growth factor regulated CREB kinase. Science 1996; 273:959-963.
73. Coso OA, Chiariello M, Yu JC et al. The small GTP-binding proteins Rac1 and CDC42 regulate the activity of the JNK/SAPK signaling pathway. Cell 1995; 81:1137-1146.
74. Minden A, Lin A, Claret FX et al. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell 1995; 81:1147-1157.
75. Klippel A, Reinhard C, Kavanaugh WM et al. Membrane localization of phosphatidylinositol-3 kinase is sufficient to activate multiple signal transducing kinase pathways. Mol Cell Biol 1996; 16:4117-4127.
76. Logan SK, Falasca M, Hu P et al. Phosphatidylinositol-3 kinase mediates epidermal growth factor-induced activation of the c-Jun N-terminal kinase signaling pathway. Mol Cell Biol 1997; 17:5784-5790.
77. Marte BM, Rodriguez-Viciana P, Wennström S et al. PI-3 kinase and PKB/Akt act as an effector pathway for R-Ras. Current Biology 1997; 7:63-70.
78. Reif K, Nobes CD, Thomas G et al. Phosphatidylinositol-3 kinase signals activate a selective subset of Rac/Rho-dependent effector pathways. Curr Biol 1996; 6:1445-1455.
79. McCarthy SA, Samuels ML, Pritchard CA et al. Rapid induction of heparin-binding epidermal growth factor/diphtheria toxin receptor expression by Raf and Ras oncogenes. Genes Dev 1995; 9:1953-1964.
80. Hill CS, Wynne J, Treisman R. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell 1995; 81:1159-1170.
81. Khosravi-Far R, White MA, Westwick JK et al. Oncogenic Ras activation of Raf/MAP kinase independent pathways is sufficient to cause tumorigenic transformation. Mol Cell Biol 1996; 16:3923-3933.
82. Qiu RG, Chen J, Kirn D et al. An essential role for rac in ras transformation. Nature 1995a; 374:457-459.
83. Qiu RG, Chen J, McCormick F et al. A role for rho in ras transformation. Proc Natl Acad Sci USA 1995b; 92:11781-11785.
84. Joneson T, McDonough M, Bar-Sagi D et al. RAC regulation of actin polymerization and proliferation by a distinct pathway from Jun kinase. Science 1996; 274:1374-1376.
85. Lamarche N, Tapon N, Stowers L et al. Rac and CDC42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. Cell 1996; 87:519-529.
86. Jiang W. Membrane ruffling of cancer cells: A parameter of tumor cell motility and invasion. Eur. J Surg Oncol 1995; 21:307-309.
87. Michiels F, Habets GGM, Stam JC et al. A role for rac in tiam1-induced membrane ruffling and invasion. Nature 1995; 375:338-340.
88. Irani K, Xia Y, J.L. Z et al. Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. Science 1997; 275:1649-1652.
89. Sulciner DJ, Irani K, Yu ZX et al. rac1 regulates a cytokine-stimulated, redox-dependent pathway necessary for NF-kappaB activation. Mol Cell Biol 1996; 16:7115-7121.
90. Peeper DS, Upton TM, Ladha MH et al. Ras signalling linked to the cell cycle machinery by the retinoblastoma protein. Nature 1997; 386:177-181.
91. Mittnacht S, Paterson H, Olson MF et al. Ras signalling is required for inactivation of the tumour suppressor pRb cell cycle control protein. Current Biology 1997; 7:219-221.
92. Lavoie JN, L'Allemain G, Brunet A et al. Cyclin D1 expression is regulated positively by the p42/p44 MAPK and negatively by the p38/HOG MAPK pathway. J Biol Chem 1996; 271:20608-20616.

93. Taylor SJ, Shalloway D. Cell cycle-dependent activation of Ras. Current Biology 1996; 6:1621-1627.
94. Durkin JP, Whitfield JF. Characterization of G1 transit induced by the mitogenic-oncogenic Ki-ras gene product. Mol Cell Biol 1986; 6:1386-1392.
95. Dobrowolski S, Harter M, Stacey DW. Cellular ras activity is required for passage through multiple points of the G0/G1 phase in BALB/c 3T3 cells. Mol Cell Biol 1994; 14:5441-5449.
96. Lloyd AC, Obermuller F, Staddon S et al. Cooperating oncogenes target cyclin/cdk activity. Genes & Develop 1997; 11:663-677.
97. Serrano M, Lin AW, McCurrach ME et al. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997; 88:593-602.
98. Meredith JE, Fazeli B, Schwartz MA. The extracellular matrix as a cell survival factor. Mol Biol Cell 1993; 4:953-961.
99. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994; 124:619-626.
100. Khwaja A, Rodriguez-Viciana P, Wennstrom S et al. Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. EMBO J 1997; 16:2783-2793.
101. Marte BM, Downward J. PKB/Akt: Connecting PI-3 kinase to cell survival and beyond. Trends Biochem Sci 1997; 22:355-358.

CHAPTER 13

Structures of Ras and Its Complexes with Raf and GAP

Klaus Scheffzek and Alfred Wittinghofer

Introduction

Ras is the 21 kDa GTP-binding protein essential for the control of eukaryotic cell growth and becomes an oncoprotein when it is mutated as found in a large number of cancerous tumors.[1,2] Ras functions as a molecular switch cycling between GTP-, bound 'ON' and GDP-bound 'OFF' states with the GDP-bound state being prevalent in the resting cell.[3,4] Post-translational modification of the C-terminus involving farnesylation and palmitoylation is required for linkage of Ras to the inner cell membrane.[5] Activation as triggered by extracellular signals is facilitated by guanine nucleotide exchange factors.[6,7] Only in the GTP-bound form does Ras interact with its effectors.[8,9] Signal termination is accomplished by GTP hydrolysis mediated by Ras itself. This process is intrinsically slow but can be accelerated by orders of magnitude upon interaction with Ras specific GTPase activating proteins (RasGAPs).[10] A vast amount of information about biochemistry, biology, and structure of Ras and related proteins has been accumulated in many laboratories. In a metaphorical overlay accounting for its properties to function as a cycling molecular switch, its heart-like shape, and its crucial role in the processing of extracellular signals arriving at the plasma membrane, Ras has also been called the 'beating heart of signal transduction' (H. Bos, personal communication). Structural information as derived from X-ray crystallographic analyses or nuclear magnetic resonance (NMR) techniques provides the most detailed knowledge about molecular functions. During the past 3 years application of these methods to the issues of Ras-effector and Ras-RasGAP interactions has revealed structural details of Ras signaling, of its downregulation by RasGAPs, and most importantly has provided simple explanations how oncogenic Ras mutants, as found in a large number of cancerous tumors, escape the regulation by RasGAPs. In this chapter we will give a brief overview of the current structural knowledge of Ras and of its interactions with effector molecules and RasGAPs.

Structure of Ras

Overview

From the structure of elongation factor Tu (EF-Tu)[11,12] along with a comparison of the primary sequences of Ras and EF-Tu,[13] models of the three-dimensional architecture of Ras had been derived.[14] Structures of Harvey-(H)-Ras have been determined by X-ray crystallography and NMR spectroscopy. C-terminal truncation by 23 or 18 residues, which are variable in sequence among Ras proteins from different species, has resulted in dramatically

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

improved crystallization properties of Ras and made structure determination feasible.[15,16] This led to initial structural models of H-Ras(1-166)[17] complexed with GppNHp, a nonhydrolyzable GTP analog and of GDP-bound H-Ras(1-171).[18]

Detailed structural analyses have been carried out for catalytic domains of H-Ras complexed with GTP analog[19-21] and with modified guanine nucleotides to probe nucleotide binding or GTP hydrolysis.[22] In addition, the structures of several Ras mutants have been determined (see below). The solution structure of catalytic domains bound to GDP[23] or to $GppCH_2p$[24] have been determined by NMR spectroscopy and were basically consistent with the X-ray structures. They also confirmed the mobility of regions that have been implicated to be involved in interactions with regulatory or target molecules.[25,26]

The G-Domain and Nucleotide Binding

The so called G-domain is the principal folding unit of Ras which in its basic architecture has been found in GTP-binding proteins, the structures of which are known so far.[27] It comprises a mixed 5-stranded β-sheet (Fig. 13.1A) that is surrounded by 5 α-helices forming a globular protein of approximately 40 Å in diameter.[28]

According to crystallographic analyses of the full length H-Ras(1-188) bound to GDP the C-terminal region extending the G-domain is mobile in the crystals.[29] The guanine nucleotide (GTP/GDP) is bound in a pocket that is formed by residues belonging to the sequence motifs (G1-G5, Fig. 13.1B) characteristic for GTP binding proteins. Their contributions can be subdivided into parts involved in binding of the phosphate chain (P-loop, loop L2, loop L4) and of the guanine base (Phe28, NKXD, SAK/L). Numerous polar interactions derived from the crystal structure account for the high affinity for GTP/GDP.[19]

The guanine base is bound in a hydrophobic cavity that is lined by residues derived from the G4 (NKXD) and G5 (SAK/L) motifs.[19] Guanine is sandwiched by the side chain of Lys117 (from NKXD) on one face; on the other face Phe28, that is conserved in Ras related GTP binding proteins,[2] undergoes an aromatic-aromatic interaction with the base and is itself stabilized by the side chain of Lys147 from the SAK/L motif. Its mutation to leucine leads to a dramatic increase in the dissociation rates of GDP/GTP accompanied by destabilization of the protein.[30]

Binding specificity for guanine nucleotides has been ascribed mainly to the interaction between the main chain amide group of Ala146 and the exocyclic O6-atom of the purine ring.[31] Another contribution comes from Asp119 that contacts the exocyclic NH_2- and the endocyclic NH at position 1 of the ring. Its mutation to asparagine changes the specificity to xanthine nucleotides making the protein an XTPase which is nevertheless sensitive to GTPase activating proteins.[32]

The G1-motif forms the so called phosphate binding loop or P-loop that is found in many other nucleotide binding proteins including myosin and nucleoside monophosphate kinases.[33-35] Together with the helix-dipole of α1 it forms a positively polarized environment that is responsible mainly for stabilization of the β-phosphate of the nucleotide. This is demonstrated by numerous interactions of P-loop residues with this part of the nucleotide[19] and by the observation that guanine nucleotides lacking the β-phosphate (GMP- and guanosine derivatives) bind with 10^5-fold reduced affinity.[36]

In the structures of the GTP-bound form the loops L2 and L4 of Ras are tightly involved in binding of the γ-phosphate. Threonine35 and Gly60 form main chain contacts with the phosphate oxygens, and most importantly the hydroxyl group of Thr35 coordinates Mg^{2+}, the octahedral coordination shell of which is completed by oxygens of Pβ and Pγ, Ser17 and by two water molecules, with one being stabilized by the invariant Asp57 from the G3-motif. Residues 61-63 in loop L4 are highly flexible in crystals of both active and inactive conformations of Ras.[19,29] The NMR structure of H-Ras•GDP suggests considerable flexibility of both the L2 and L4 regions.[23]

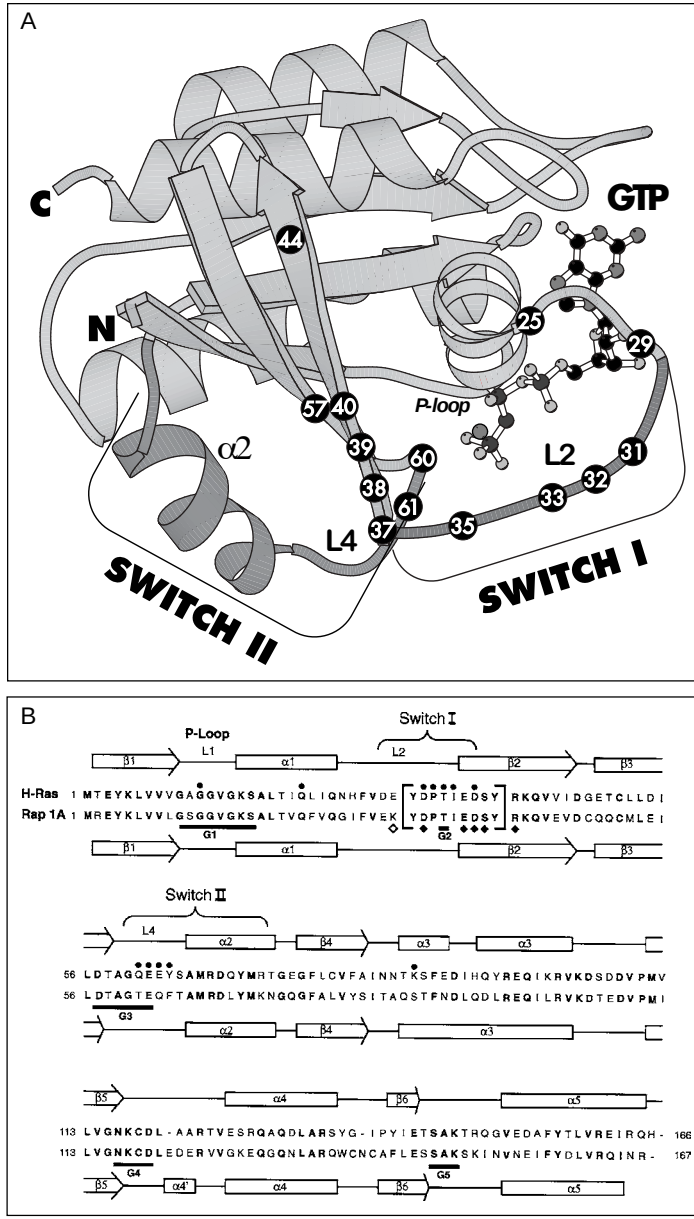


Fig. 13.1. Structure of Ras. (A) Ribbon representation of the GTP conformation of H-Ras (modified from ref. 9). Selected residues are indicated by their numbers in the sequence. (B) Sequence alignment of human H-Ras and Rap1A with assignment of secondary structure elements included. Polar residues important for the interaction with Raf-RBD as seen in the complex with Rap1A[81,83] are labeled by '◆'. The '◊' indicates importance as determinant of effector specificity. Selected residues important for the interaction with GAP as seen in the Ras-RasGAP complex[128] are marked by '•', (modified from ref. 81).

The role of Thr35 in coordination of the metal ion in the GTP-bound form has been demonstrated by the structures of GDP-bound Gα proteins[37,38] and of Ras•GDP where its interaction with the metal ion is lost,[29,39] and most impressively in the EF-Tu system. In EF-Tu crystals the homologous residue coordinates Mg^{2+} in the triphosphate form[40,41] but is 18 Å away from Mg^{2+} when GDP is bound to the protein,[42,43] similar to the situation observed in the structures of GDP-bound Arf1 and Ran.[44,45] Mg^{2+} coordination and its involvement in the conformational changes occurring upon transition between GTP- and GDP-bound forms[29,39] most likely provide an explanation why the Thr35 homologue is strictly conserved among all known GTP binding proteins.[4]

GTP Hydrolysis and Associated Conformational Changes

GTP hydrolysis as performed by Ras means the transfer of the γ-phosphoryl group of GTP to a water molecule. This involves inversion of configuration on the γ-phosphate, which is consistent with nucleophilic attack of a water molecule on the γ-phosphate occurring as a direct in line displacement.[46] The mechanism how Ras hydrolyzes GTP has been a matter of controversial debate. In the crystal structure of the GTP-bound form a water molecule has been identified that was proposed to represent the attacking nucleophile on which the γ-phosphoryl group is transferred.[19] Corresponding water molecules have also been found in the structures of EF-Tu[40] and of the α-subunits of transducin ($G_{t\alpha}$) or $G_{\alpha i1}$, when bound to GTPγS[47,48] or GDP•AlF_x.[48,49]

Glutamine 61, that is in the vicinity of the γ-phosphate, has been proposed as a candidate residue that could play the role of the general base activating the attacking water molecule,[19,28,50,51] and has stimulated a controversial discussion.[52,53] Several lines of evidence have been presented by Schweins et al[54,55] that GTP itself acts as the general base activating the water molecule. A possible role of Gln61 would be the stabilization of the transition state of the reaction[55] as proposed by Privé et al.[56]

Using Mn^{2+} as divalent cation in analyses of GTP hydrolysis it was found that this transition metal increases the intrinsic and GAP accelerated GTPase rate. In addition, atomic absorption spectroscopy measurements and crystallographic studies with Mn^{2+} as a 'heavy' Mg^{2+} analog suggested one divalent metal ion to be sufficient for catalysis.[57]

The regions involved in binding of the γ-phosphate moiety in the active conformation of Ras have been shown to change their conformations upon transition between GDP- and GTP-bound states.[29,39] The resulting structural changes have been derived by comparing the structures of the di- and triphosphate conformations of Ras as found in crystals of the Ras complexed with the nonhydrolyzable GTP analog $GppCH_2p$ and GDP[29] or by a highly elegant approach using time resolved crystallographic analysis where GTP hydrolysis occurs in crystals of Ras complexed with 'caged' GTP[58] after flash photolysis of the photolabile 'caged' group.[39,59] Most of the structural changes have been proposed to occur as a result of the loss of interactions of L2 and L4 with the γ-phosphate, with Thr35 and Gly60 playing an important role to promote the conformational changes. Threonine 35 is no longer coordinated to Mg^{2+} in the GDP-form but is rather exposed to the solvent; the side chain of Tyr32 swings out of its position in proximity of the γ-phosphate into a more solvent exposed orientation, accompanied by a shift of neighboring residues.[29,39] The role of Thr35 in triggering the conformational transitions between GTP- and GDP-bound Ras has been disputed in spectroscopic studies of liquid and frozen solution using Mn^{2+} to probe the metal binding site;[60,61] the authors found the Cβ of Th35 more distant from the divalent cation than in the crystal structure of Ras•GppNHp.

In summary, most of the conformational changes were localized to residues 30 to 38 and 60 to 76 (which includes helix α2); these regions have been called the 'switch I' and 'switch II', respectively[29] (Fig. 13.1A). The details of the 'real' conformations corresponding

to active and inactive Ras are not clear yet. It has to be considered that both switch I and switch II are located near the surface of the protein and may in principle have conformations favored by crystal packing forces. In the NMR- structure they have been demonstrated to be flexible[23] or to show several distinct conformations.[24]

Oncogenic Ras Mutants

The structures of several oncogenic Ras mutants have been determined by X-ray crystallography; these include G12V,[18,39,62] G12R,[62] Q61H,[62] Q61L,[56,62] G12D,[63] and A59T.[56] All mutations investigated left the overall structure of Ras unchanged. From the local structures hypotheses have been put forward to explain the oncogenicity of these mutants. While mutations in position 12 or 61 were suggested to interfere in some ways with the catalytic competent conformation of GTP hydrolysis, introduction of threonine in position 59 was reported to cause a conformational shift in the whole loop L4 that would block the conformation necessary for GTP hydrolysis. No satisfactory explanation could be given by these studies why the nononcogenic mutant G12P, the structure of which is very similar to that of wild-type Ras has increased GTPase activity.[63]

Interactions with Effectors

In the GTP-bound form Ras is able to interact with effector proteins. These are operationally defined as molecules that interact more strongly with the GTP-conformation than with Ras in its GDP bound state. To date several candidate effectors of Ras are known: Among them are Raf-kinase, PI-3-kinase, Ral-GDS (Ral GDP-dissociation stimulator, also termed RalGEF or RGF), all with several isoforms, and Byr2 from yeast.[8,64] In the case of C-Raf1 (with isoforms A-Raf and B-Raf) it has been shown that Ras binds to its N-terminal domain, termed Ras-Binding-Domain (RBD) that comprises residues 51-131 of the 648 amino acid molecule.[65-69] Ras•GTP dependent interaction has also been demonstrated for PI-3-kinase[70,71] and Ral-GDS and its isoforms Rgl, Rlf and Rlf2.[72-76] In the case of Ral-GDS a domain comprising approximately 100 C-terminal residues (RGF-RBD) is responsible for the interaction.[77] The structures of the Ras binding domains (RBDs) of c-Raf1 and of Ral-GDS have been determined by NMR-spectroscopy[78,79] and X-ray crystallography.[80,81]

Structure of Rap1A•Raf-RBD Complex

As originally derived from NMR spectroscopy[78] c-Raf-RBD has a fold similar to that of ubiquitin.[82] The X-ray crystallographic analysis of a complex between c-Raf-RBD (Raf-RBD), and Rap1A (Rap), that shares > 50% identical amino acids with Ras (Fig. 13.1B), bound to GppNHp revealed the structural details of not only Raf-RBD and Rap but most importantly of the protein-protein interactions involved in downstream signaling of Ras.[81,83]

The structural fold of Raf-RBD shows a five stranded mixed β-pleated sheet containing an α-helix (A1; nomenclature according to Nassar et al[81]) followed by a loop and a 3_{10}-helix between β-strand B2 and B3 and another 3_{10}-helix located between β-strands B4 and B5 (Fig. 13.2A,B).[81] The structure of Rap resembles Ras[28] very closely with root mean square deviations of 0.88 Å for 163 corresponding Cα atoms.[81] Given the similarity of the molecules and especially of the effector regions it is considered a suitable model system to study Ras effector interactions.

The Rap1A•Raf-RBD Interface

In the complex with Rap an inter protein β-sheet is formed involving parts of switch I from Rap and mainly residues from B2 of Raf-RBD, but not residue of switch II of Rap. The N-terminal regions of the second β-strand of Rap and of Raf-RBD are running antiparallel making the hydrogen bonding pattern typical for β-sheets. (Fig. 13.2A). This mode of

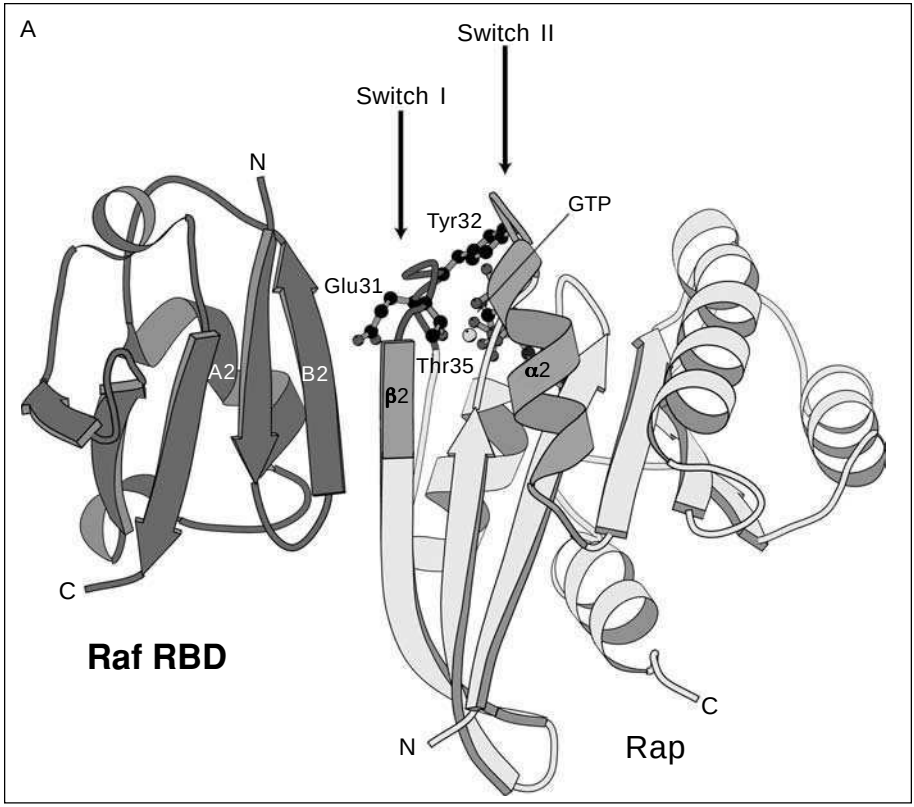


Fig.13.2 Structure of Rap1A•Raf-RBD complex.

A. Ribbon drawing of the complex in an orientation showing the inter protein β-sheet. Selected residues are indicated, see text (modified from ref. 96).

B. Sequence alignment of Raf isoforms and RBDs from Byr2 and RGF, based on the structures of Raf-RBD[81] and RGF-RBD.[79] Corresponding to Figure 1B residues involved in polar interactions with Rap1A are indicated by '◆' (modified from ref. 81.).

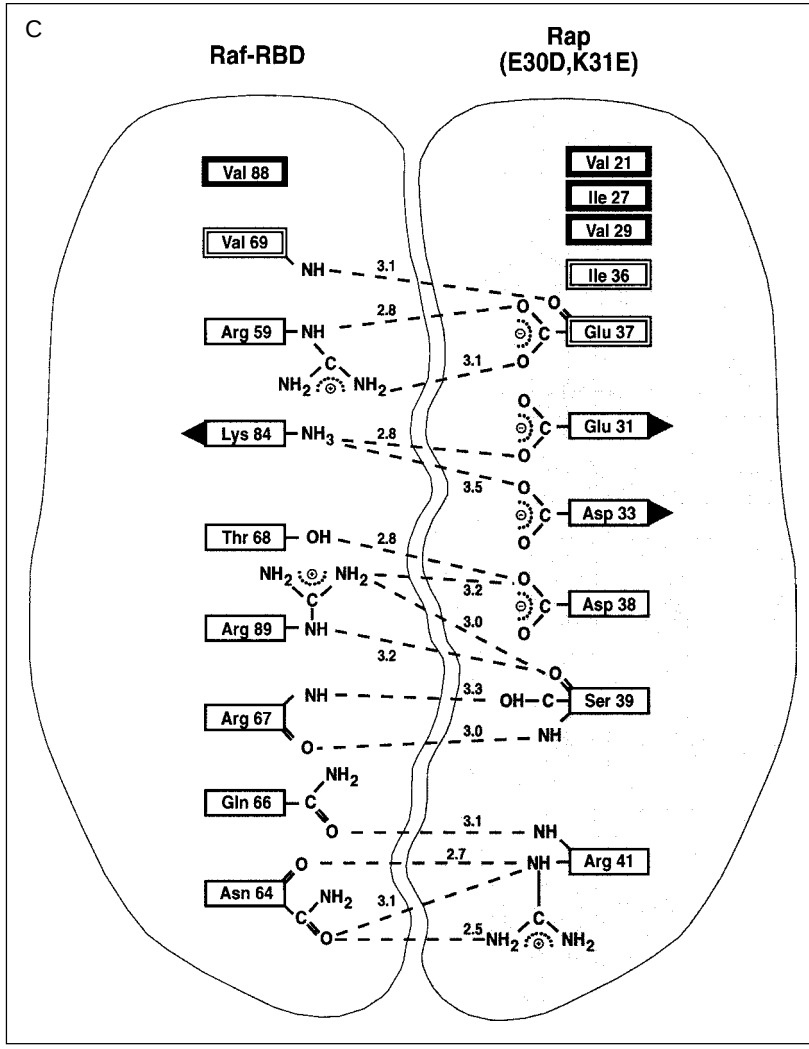


Fig. 13.2C. Schematic diagram of the Rap1A•Raf-RBD interface as derived from the complex between the double mutant (E31D,K31E) and Raf-RBD showing the predominantly polar character of Rap-Raf communication. Residues involved in interactions resulting from the mutations are labeled by black triangles. Residues belonging to hydrophobic pockets are indicated by bold rimmed boxes (modified from ref. 81).

interaction has been found in other protein complexes as well[84,85] where the individual components do not share sequence homologies with the protein system reviewed in this chapter. It is also reminiscent of the interprotein β-sheets found in the dimers observed in the asymmetric units of crystals from GDP bound Arf1[44] or RGF-RBD.[80] It thus appears that Ras and Raf are exploiting a common scheme of protein-protein communication rather than inventing one.

On the Rap side Glu37(CO) and Ser39(NH) are involved in β-strand formation, facing on the RBD side the complementary groups of Val69 and Arg67, respectively. Apart from B2 RBD contributes only the C-terminal part of the following helix (A2) to the interface. The interaction pattern is governed almost exclusively by polar contacts between residues stabilizing the complex (Fig. 13.2C), with residues from the effector region of Rap (exactly the same as in Ras) being tightly involved in the interaction with RBD. Hydrophobic contacts involving Val21, Ile27, Val29, Ile36 of Rap1A and Val69 and Val88 of RBD appear to be of minor importance for the interaction between the two molecules.[81]

Critical Residues and Effector Specificity

The effector region of Ras has been extensively characterized with respect to the importance of its amino acids for downstream signaling.[9,25,26,63] The critical amino acids in this and the neighboring regions are Asn26, His27, Asp38, Glu31, Pro34, Thr35, Ile36, Glu37, Asp38, Tyr40, Val45 and Gly48. In the complex structure most of the residues that have been shown by many laboratories to be important for Ras signaling were found in the interface region,[81,83] confirming the functional importance of these residues.

The effect of several mutations on the structure has been reviewed by Herrmann and Nassar.[86] It is obvious from the architecture of the interface which is stabilized mainly by electrostatic interaction, that disruption of polar contacts is likely to influence binding to effectors. The mutation of Asp38 to alanine leads to decreased binding of Ras to Raf-RBD[87] and disrupts signaling.[88] Glutamate, in principle, could be accommodated in this region but nevertheless decreases signaling effects.[88-90]

Mutation of Tyr40 to lysine may lead to unfavorable interaction with Arg89 of Raf-RBD[86] which might explain its negative effect on signaling.[88,90] Glu37 interacts with Arg67 and Arg59 in Raf-RBD. Disruption of this interaction by mutation of GLu37 to alanine destroys the biological activity, while its conservative replacement by Asp has transforming properties similar to wild type.[89]

From genetic studies on *Drosophila melanogaster* an arginine (Arg89) has been identified to be important for the interaction with Ras.[91] A systematic mutational study based on the interactions observed in the complex has demonstrated Gln66, Lys84, and Arg89 to be the major contributors to the binding affinity between Ras and Raf which correspond to only one third of the amino acids involved in direct intermolecular interactions.[92] The observation that only a few amino acids account for the binding affinity is in agreement with corresponding results obtained for other protein protein complexes,[93,94] where only a few amino acids account for binding affinity.

The mutation Arg89→Leu disrupts numerous polar interactions with Asp38, Ser39, and Thr35 and leads to an increased dissociation constant in the micromolar range, suggesting that this residue is most important for the interaction with Ras. Lysine84 is not involved in Rap binding in the complex; however its mutation to alanine reduces the affinity for Ras by more than 100-fold and leads to disruption of Ras/Raf signaling in vivo.[92] Together with the proximity of Lys84 to residues Glu30 and Lys31 in the complex with Rap, (which are the only residues in the extended effector region ranging from Phe28 to Val44 that are different in Ras) these results suggested that Lys84 accounts for the effector specificity by recognition of the side chains present in position 30 and 31 of the respective Ras

protein.[92] Mutational studies in which Rap was made Ras-like with respect to switch I by introducing the double mutation (E30D, K31E) demonstrated an increase in affinity to Raf to a value close to that observed for the interaction between Ras and Raf. The main contribution to this effect has been ascribed to the charge reversal of Lys31 (in Rap) as judged from the binding data for the single mutant K31E. Consistent with this interpretation, X-ray crystallographic analysis of the complex with Raf-RBD showed Lys84 (in Raf) forming a salt bridge with the mutant Glu31, in addition to a weak salt bridge with Asp33 and a water mediated contact with the main chain carbonyl group of Glu31[83] (Fig. 13.2C).

GDI Effect and Importance of Cysteine Rich Domain

The guanine nucleotide binding site is not located at the interface of the complex, which was unexpected since Raf binding has a guanine nucleotide dissociation inhibitory (GDI) effect on Ras.[77,87] Protein nucleotide interactions appear to be tighter in the complex as compared with the GTP-bound form of Ras.[19] Most importantly Tyr32, conserved in Ras, Rho, and Ran subfamily members[2] forms a water mediated contact with the γ-phosphate. Tyrosine 32 in solution is mobile and flips between two conformations which are in rapid equilibrium, one of them being stabilized upon interaction with Raf-RBD.[95] Together with the tighter network of the remaining protein nucleotide interactions this might be responsible for the GDI effect of Raf-RBD on Rap/Ras, that has been used to determine binding affinities between Ras and Raf.[77,87] Release of the γ-phosphate upon intrinsic GTP hydrolysis (which in fact terminates the Ras-Raf interaction) might be the trigger for conformational changes involving the release of Tyr32 and residues Thr35 and Gly60 that are both contributing to γ-phosphate binding.[28]

The conformation of the effector region as found in the complex is not compatible with that observed in crystals of isolated H-Ras in the GDP- or GTP-bound form.[96] This is not surprising, since it appears to be biased by crystal packing forces. For example, Tyr32 is forming a hydrogen bond with a γ-phosphate oxygen from a neighboring molecule in crystals of the GppNHp bound form of H-Ras.[19] The various conformations of the switch I (and switch II) region in Ras crystals and in the NMR structure rather suggest that the physiologically significant one is picked up by the respective effector molecule. This is in agreement with 'regional polysterism' of these regions, detected by NMR methods.[24]

The complex between Rap and Raf-RBD can be considered a model system for studying Ras effector interactions,[96] given the intriguing structural similarities between Ras and Rap.[81] Preliminary crystallographic analyses of crystals of the complex between H-Ras and Raf-RBD are indeed consistent with this assumption (Nassar, unpublished data). There is clear evidence that Ras binds also to regions outside the RBD.[9,97-99] Mutations at residues 31, 37 and 59 of H-Ras inhibit binding to full length Raf and mutations at residues 26, 29, 39, 40, 41, 44, 45 have been implicated as necessary for Ras-dependent Raf activation.[9,100] In addition the cysteine rich domain (CRD) has been shown to be involved in Ras-Raf interactions with switch II on Ras being a candidate region involved in the interaction.[97-100] The structure of Raf-CRD has been determined by NMR[101] and shown to resemble the phospholipid binding domain of protein kinase C.

Structure of RalGEF-RBD

The structure of human RGF-RBD has been solved by NMR-spectroscopy.[79] Huang et al[80] have determined the structure of a corresponding domain (residues 767-864) from rat by X-ray crystallography.

Although not related in sequence, RGF-RBD displays the same topology as Raf-RBD[78,81] forming a mixed 5 stranded β-sheet with a short helix followed by a loop and a 3_{10} helix inserted between β-strands B2 and B3 and another 3_{10}-helix between strands B4 and B5.[79,80]

The structure of an isoform has been solved recently by NMR methods and shown to be very similar to that of RGF-RBD.[102]

Based on a structural comparison with Raf-RBD and considering the mutational studies performed on Raf-RBD,[92] corresponding residues (Arg20/Arg59, Lys32/Gln66, Lys52/Arg89) were analyzed by site directed mutagenesis using a two hybrid system to monitor the effect or the mutation on Ras-binding.[80] The authors report similar results as Block et al.[92] However, it has to be considered that their results have a rather qualitative character as compared with the c-Raf study, where the mutants were quantitatively characterized in vitro and in vivo. The structure together with mutational studies and the structure of the Rap•Raf-RBD complex suggest a similar interaction mode using an interprotein β-sheet as basic element. Huang et al[80] could not identify a residue structurally homologous to Lys84 of c-Raf which has been demonstrated to be responsible for mediating effector specificity by recognition of the amino acids in position 30 and 31 in Ras/Rap.[83] They speculate that the absence of a Lys84 counterpart in RGF-RBD may account for the lower affinity of Ras for RGF-RBD. Geyer et al[79] have compared the surface charge distribution of RGF-RBD and Raf-RBD and identify residues Arg16, Lys28, Lys48, and His49 to form a positively charged cluster comparable to that formed by Arg67, Arg89, Lys84, and Lys87 in Raf-RBD.

Ras binding studies done with NMR using ^{15}N labeled RGF-RBD indicated conformational changes of the whole molecule and especially of the N-terminal β-strands, that are assumed to be involved in complex formation similarly as with the Rap•Raf-RBD complex.[79] As with Raf-RBD, binding of RGF-RBD to Ras fixes one of two conformations of the effector region, as monitored by ^{31}P-NMR.[79,95]

A detailed analysis of the network of interprotein interactions will have to await the structure determination of complexes of RGF-RBD with Ras or Ras-related proteins.

Interaction with GTPase Activating Protein

The interaction of Ras→GTP with Ras specific GTPase activating proteins (RasGAP) leads to an increase in the rate of Ras mediated GTP hydrolysis by up to five orders of magnitude.[103] p120GAP was the first GAP to be discovered.[104-106] GTPase activation was in fact the first direct regulatory effect of a protein on Ras to be observed.[104] p120GAP contains a number of signaling modules such as SH2, SH3, PH, CalB domains and is believed to be also a signaling molecule.[107] It was only two years after the discovery of p120GAP when neurofibromin, the protein product of the type I neurofibromatosis gene,[108] was reported to share sequence homology with p120GAP and to interact with Ras•GTP to stimulate Ras-mediated GTP hydrolysis.[109-112]

A number of other RasGAPs have been described to date.[104-106] They are molecules of various sizes and domain composition but they all share a region of sequence homology that is responsible for their GAP activity. The region with high homology between p120GAP, neurofibromin, and IRA1/IRA2 from yeast[113] was originally defined as the GAP related domain (GRD). Smaller fragments have consistently been used in biochemical studies and shown to have high enzymatic activity.[103,114,115] A minimum catalytic fragment of neurofibromin that retains full GAP activity comprises only 230 amino acids.[116] Neurofibromin has been considered the mammalian homolog of IRA1/IRA2 from yeast because the region of sequence homology is more extensive between these proteins. Gap1m,[117] a mammalian homolog of the Drosophila GAP1 gene,[118] and a close homolog GAPIII[119] have been described, both of which contain C2 domains and a PH domain in addition to the GRD. An inositol-4-phosphate (IP4) binding protein, GAP1(IP4BP), has been characterized to contain a RasGAP catalytic domain and stimulates the GTPase of both Ras and Rap.[120]

Structure of a Catalytic Domain of p120GAP (GAP-334)

The first structure determination of a RasGAP has revealed the catalytic domain of human p120GAP (residues 714-1047; GAP-334) as a purely helical protein of elongated shape with approximate dimensions 70x30x30 Å.[121] It appears to be composed of 2 domains (Fig. 13.3A), a central domain (GAP_c; residues 765-98) belonging to the inner portion of the protein of the sequence, comprising two thirds of the molecule and containing all residues conserved among RasGAPs and an extra domain (GAP_{ex}; residues 718-764; 982-1037) that is formed by an approximately equal number of residues derived from the N- and C-terminus each (Fig. 13.3A).[121] It is interesting to note that GAP_c corresponds essentially to what on the basis of proteolysis studies has been found to be a minimum catalytic domain of neurofibromin that retains full GAP activity.[116] Other regions of neurofibromin comprising residues 1441 to 1531 or 1518 were reported to have tumor suppressing activity with only ca. 20-fold reduced GAP activity.[122,123] In the structure of GAP-334 these regions correspond to the C-terminal part of the molecule including helices $\alpha 8_c$ and $\alpha 4_{ex}$ to $\alpha 6_{ex}$.[121] Given the requirements of amino acids outside this region for GTPase activation,[124-127] it appears to be unlikely that GTPase activation in this case occurs by a mechanism similar to that mediated by the central GAP related domain, a conclusion which is indeed supported by the structure of the Ras-RasGAP complex (see below).[128]

A sequence comparison between the GAP_c regions of several RasGAPs shows three characteristic blocks of sequence homology, termed block 1, 2, 3A/3B (Fig. 13.3B). Block 3 that contains most of the invariant residues, including the FLR...PA....P -finger print motif[129] appeared to be the region most critical for Ras-RasGAP interaction.[124-126] In the structure, amino acids of block 3 form two helices ($\alpha 6_c$ and $\alpha 7_c$) in antiparallel orientation that are connected by a large loop ($L5_c$) (Fig. 13.3A). These two helices form the bottom of a shallow groove in the surface of GAP_c which is bordered by helices and loops.[121] Of special importance are $L6_c$ and $L1_c$ together with $\alpha 2_c$. $L6_c$, that belongs to the sequence stretch immediately following block 3, is located at the C-terminal end of helix $\alpha 7_c$, and borders the groove on one side; $L1_c$ that comprises the middle part of block 1 borders the groove on the opposite side (Fig. 13.3A).

Most of the conserved residues are clustered around the surface groove in GAP_c. The FLR motif is located in an apparently four residue turn which appears to interrupt $\alpha 6_c$, with Phe901 contributing to a hydrophobic core stabilizing the helical arrangement, Leu902 exposed to solvent, and Arg903 interacting with the main chain of $L1_c$ in the vicinity of the second invariant arginine Arg789. The invariant residues Lys935, Gln938, and Asn942 are located in the C-terminal portion of $\alpha 7_c$ being accessible from the groove.[121]

In a vast number of studies, regions in Ras and RasGAP have been identified to be important for successful Ras-RasGAP interaction. In Ras they have been mapped extensively and found to involve mainly the switch I/II and P-loop regions[25] and the N-terminal part of helix $\alpha 3$.[130,131] Mutations of Gly12,[132] Gln61,[133] Gly13, and Ala59 as found in a large number of human tumors lead to oncogenic activation of Ras, that in this state is unable to hydrolyze GTP at a rate sufficient to switch off the transmitted signal and is insensitive to RasGAPs.[104,105,134]

On the basis of numerous mutagenesis studies on neurofibromin[129,135-139] and p120GAP[103,124-126,140] and peptide binding studies,[141] residues critical for successful Ras-RasGAP interaction have been mapped to the block 1 and block 3 regions. Using these studies a docking model was proposed to show how RasGAPs might bind to Ras[121] that was basically confirmed by the structure determination of the Ras-RasGAP complex.[128]

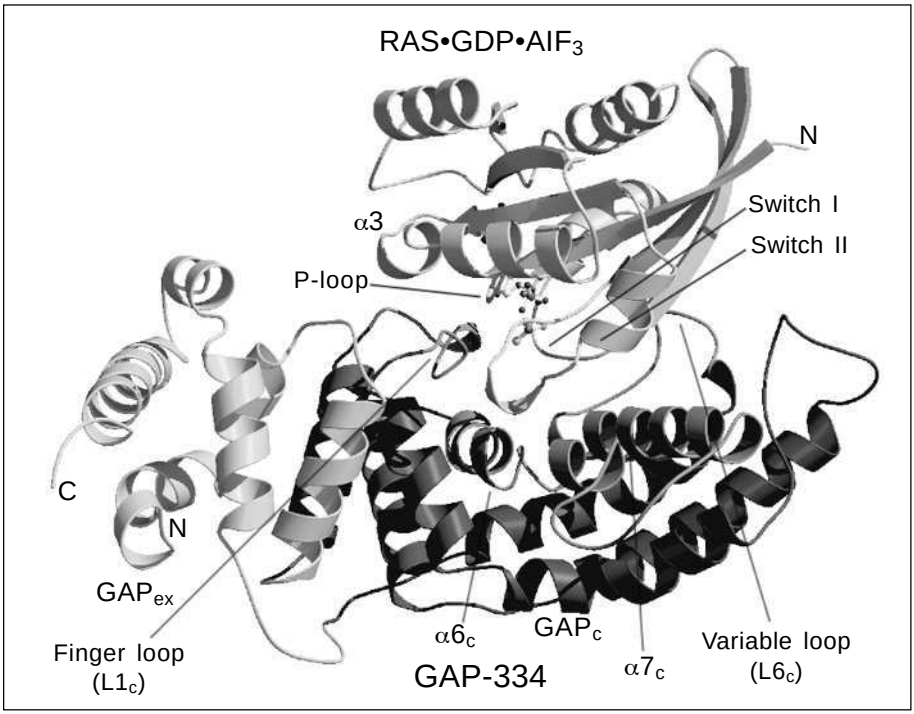


Fig. 13.3. GAP-334 and its interactions with Ras (modified from ref. 128).
Fig. 13.3A. Ribbon representation of the complex showing the elongated shape of GAP-334 and its binding to Ras, with important elements indicated (see text), GDP·AlF_3 is in ball and stick.

The Ras•GAP-334 Complex

The complex was crystallized from solutions containing H-Ras(1-166)•GDP and GAP-334 in the presence of aluminum fluoride.[128] Aluminum fluoride is believed to be a transition state analog of phosphoryl transfer reactions mimicking the transferred phosphoryl group.[142] It activates GDP-bound α-subunits of heterotrimeric G-proteins and has been found in corresponding crystal structure in the position usually occupied by the γ-phosphate.[48,49] The former enigma that Ras•GDP does not bind aluminum fluoride was resolved by the observation that addition of RasGAP (NF1-GRD or GAP-334) to a solution containing Ras•GDP and aluminum fluoride results in the formation of a ternary complex between Ras•GDP, RasGAP, and aluminum fluoride. Stoichiometric amounts of RasGAP are needed for this effect.[143]

The Ras•GAP-334 Interface

Ras binds to the surface groove of GAP_c with the tip of its heart-like shape deeply penetrating into it (Fig. 13.3A). Consistent with many biochemical studies switch I/II and the P-loop and partly helix α3 are involved in the protein-protein contact which on the GAP side is mediated mainly by residues belonging to loop $L1_c$, helices $\alpha6_c$ and $\alpha7_c$ together with $L6_c$ (Fig. 13.3A,C). The interface is stabilized by hydrophobic and hydrophilic contacts involving several water molecules, in agreement with the sensitivity of the Ras-Ras GAP interactions towards salts and lipids.[135,144,145] The situation is depicted in Figure 13.3D. Residues

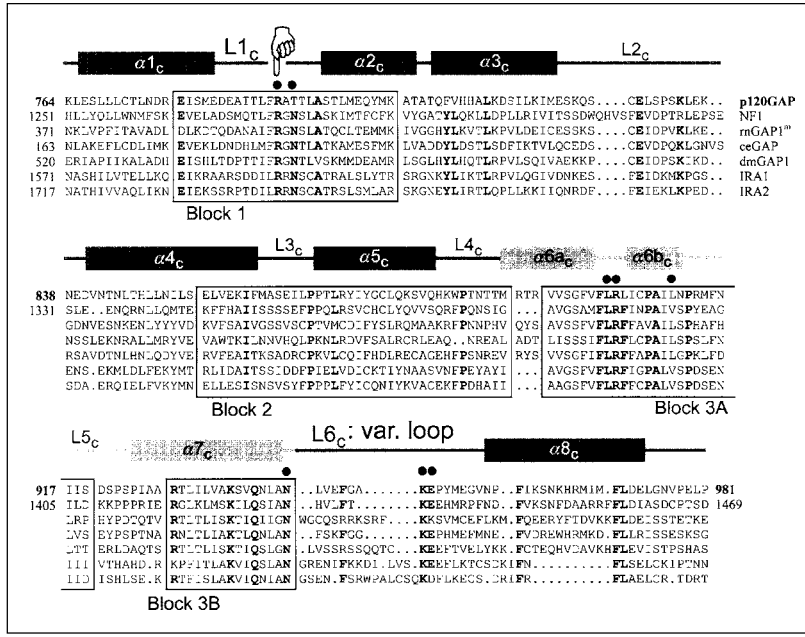


Fig. 13.3B. Sequence alignment of selected RasGAPs according to the central catalytic domain of GAP-334, that is the presumed minimum Ras GAP module containing all conserved residues. α-helical secondary structure elements are included, selected residues involved in the interaction with Ras are indicated by '•', corresponding to Figure 13.1B.

from Ras involve Tyr32, Pro34, Ile36 from the effector region, and Tyr64 from switch II that interact hydrophobically with residues belonging to the groove region, including Leu902, and Leu910 that make closer contacts. Consistent with NMR experiments RasGAP appears to stabilize a conformation of Tyr32 distant to the nucleotide.[95]

Residues from the effector region of Ras have been identified to be important for Ras-Ras GAP interaction.[25,88,105,134,146] The P34R mutant fails to interact with RasGAP,[147] and an arginine side chain could indeed not be accommodated in position 34 without disturbing the structural arrangement. Mutation of Ile36 to alanine abrogates Ras-RasGAP interaction while mutation to Leu or Met only moderately reduces GAP sensitivity. Of more critical importance is Leu902: Its conservative replacement by Ile disrupts GAP catalysis.[124] The requirement of Tyrosine in position 64 is demonstrated by the observation that it can be replaced by Phe but not by Glu without disrupting Ras-RasGAP interaction.[148] A corresponding Trp mutant binds to but is not stimulated by RasGAP;[149] probably because of its size Trp64 would interfere with the catalytically competent conformation required to accelerated GTP hydrolysis.

The effector region also participates in a number of polar interactions involving, among several water molecules, particularly the $L6_c$ region of GAP. Lys949 and Glu950, belonging to loop $L6_c$ are tightly involved in these interactions (Fig. 13.3C,D). Taking this observation as a starting point to revise the current sequence comparison with respect to a presumed 'KE'-motif C-terminal of block 3 results in an alignment that proposes $L6_c$ to be of variable length among RasGAPs (Fig. 13.3B), assuming the structures of RasGAPs to be similar;

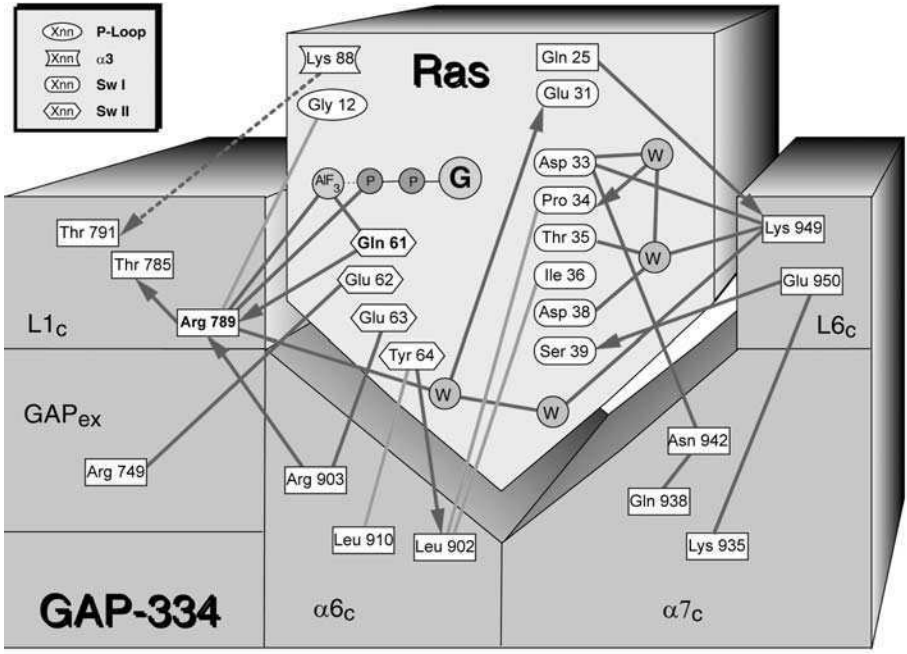


Fig. 13.3C. Cartoon drawing of the complex illustrating the complex pattern of interactions[128] They are shown as lines in light gray for hydrophobic and in dark gray for polar interactions. Arrows indicate side chain-main chain contacts with the arrow head marking the main chain partner of the contact. Residues directly involved in stabilization of the presumed transition state of the GTPase reaction are labeled in bold.

hence $L6_c$ has been called the 'variable loop'.[128] The resulting alignment also revealed additional invariant residues the importance of which is currently being investigated by site directed mutagenesis (R. Ahmadian, personal communication).

Lysine 949 forms a salt bridge with Asp33 along with indirect contacts to the side chains of Thr35 and Asp38; Glu950 forms a tight intramolecular salt bridge with the invariant Lys935 in helix $\alpha 6_c$, that has been reported to be mutated to Glu in solid tumors and neurofibromas.[150] Mutational analysis has led to controversial results suggesting Lys935 is important in Ras-binding, GAP catalysis, or stabilization of the protein.[136,138,150] The intramolecular salt bridge observed in the complex indeed suggests structural destabilization as a major consequence of mutations in this position. Glu950 also interacts with the main chain amide group of Ser39, with no side chain interaction of Ser39 observed, consistent with the observation that Ser39 can be mutated to Ala without affecting GAP sensitivity.[134] In contrast, substitutions of the preceding Asp38 by alanine or glutamate both abrogate successful Ras-RasGAP interaction.[88,134,135] Whereas in the first casethe negative charge is removed from this region with deleterious consequences on binding and catalysis, sterical clashes due to the longer side chain of Glu might explain the effect in the latter case, although binding of RasGAP is not severely impaired by the Glu mutant.[62,126]

Asp33 contributes to the negative charge created by five acidic residues within the stretch comprising residues 30-38 (switch II). Besides several water-mediated contacts, it forms a polar interaction with the amide group of Asn942 located at the C-terminal end of helix $\alpha 7_c$.

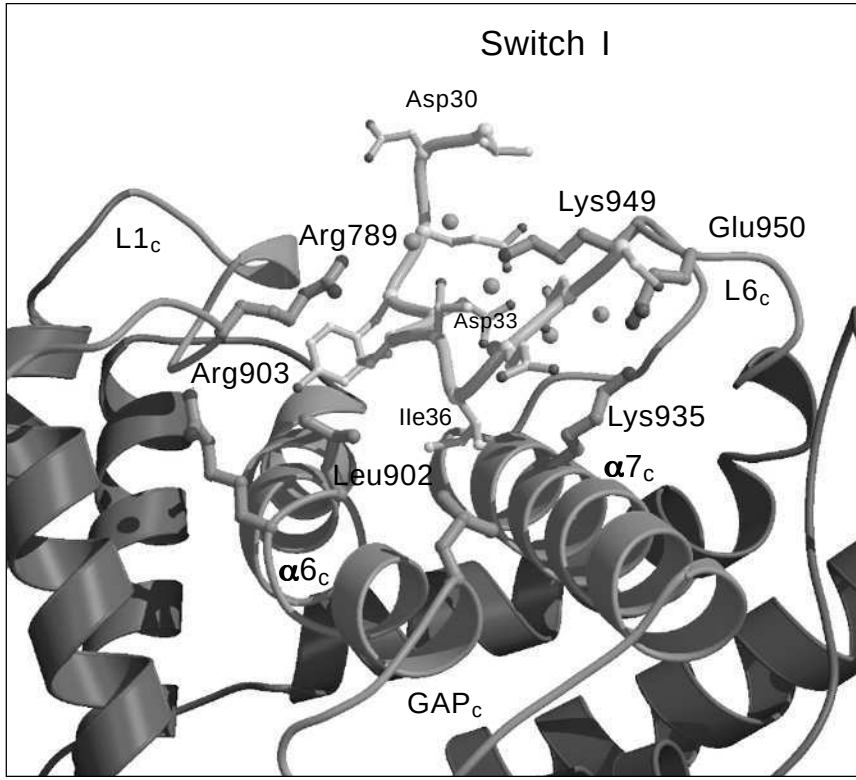


Fig. 13.3D. Structural details of interactions involving switch I (in light gray) and GAP_c (in dark gray), shown as a 'zoom in' view into the surface groove region of GAP_c in an orientation similar as in A. Selected residues are shown in 'ball and stick', water molecules as gray spheres. The view also demonstrates the positions of the invariant arginines Arg789 and Arg903, see text for details.

In addition, it forms a saltbridge with Lys950 from the variable loop and several water mediated contacts. Mutation of Asp33 to asparagine as well as the double mutation (D33H/H34S) lead to reduced RasGAP binding and abolishes RasGAP sensitivity[146,151,152] suggesting a critical role of this residue for Ras-RasGAP interaction. The structural arrangement in the effector binding region suggests that Lys949 could be replaced by arginine that might undergo additional polar interactions. Interestingly, a corresponding arginine mutant in neurofibromin binds Ras more tightly than wild type neurofibromin.[137,153]

Threonine35 is one of the most strictly conserved residues among GTP binding proteins. Several studies suggest the coordination of Mg^{2+} in the GTP-bound form as the reason for that. From mutational analysis, conservative substitution of Thr35 by serine in Ras does not affect intrinsic GTPase or RasGAP binding, but renders Ras insensitive to NF1-GRD suggesting that the aliphatic part of the threonine-side chain plays an important role in interface stabilization during catalysis. Similarly, conservative replacement of Leu902 in GAP l-334 by Ile leads to loss of GAP catalysis.[124,125]

From the structure Asp30 and Glu31, residues that have been found to be responsible for effector specificity do not appear to be directly involved in Ras-RasGAP interaction. However, introducing the double mutation (D30E, E31K) in Ras leads to impaired binding to NF1-GRD and to abrogation or GTPase stimulation.[146] Interestingly a chimera composed

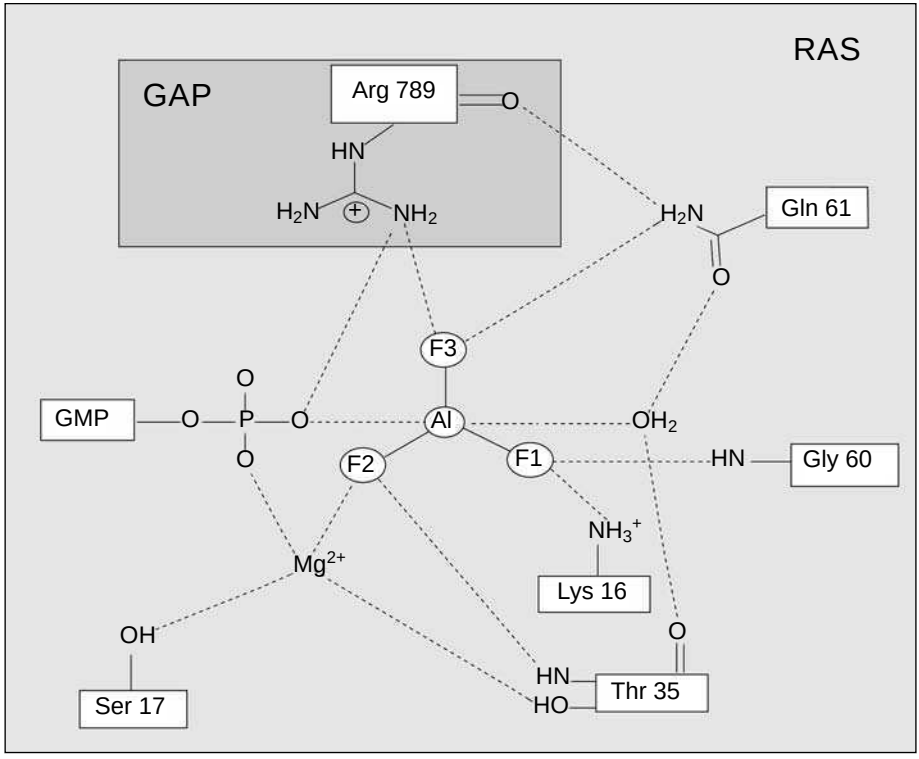


Fig. 13.3E. Schematic diagram of the active site, with polar interactions symbolized as dashed lines. The active site is constituted by Ras and GAP-334, two completely different proteins forming a heterodimer to accelerate the rate of GTP hydrolysis.

of the N-terminal part (1-60) from Rap (with Glu30 and Ly31) and the C-terminal portion (61-189) from Ras is not sensitive to neurofibromin but can still be stimulated by p120GAP.[154,155] Most mutations in the C-terminal part of β2 do not or only moderately affect Ras-RasGAP interaction[146] consistent with the observation that this region does not contact RasGAP in the crystalline complex.

Another contact region has been found in the N-terminal part of helix α3 (residues 87-102) of Ras, with Asp92 being a residue crucial for the interaction with RasGAP.[130] Neurofibromin mutants rescuing this mutation have been mapped to block 1 or block 3.[139] In the structure helix α3 is in proximity of $L1_c$ with Lys88 as a candidate residue to interact with the loop. Helix α3 has been identified to be in part responsible for the specificity of IRA2p for yeast Ras2p.[131]

The Active Site

In the Ras-RasGAP complex[128] the nucleotide GDP is bound in a similar mode as observed in isolated Ras,[18,19] with the characteristic interactions between the various parts of Ras and GDP, and Mg^{2+} present in the expected position (s. above) and coordinated by Ser17 from the P-loop and Thr35 from the switch I region. Electron density in the position usually occupied by the γ-phosphate was interpreted as aluminum trifluoride[128] that is bound in a way basically comparable with the situation in the corresponding complexes of the

α-subunits of heterotrimeric G-proteins (Fig. 13.3E).[48,49] However, in these structures and in the structure of the catalytic domain of p50rhoGAP complexed with RhoA•GDP and aluminum fluoride[156] four fluoride ligands of aluminum have been identified. Although aluminum trifluoride has been found in corresponding complexes of nucleoside mono- and diphosphate kinases,[157,158] the difference in the number of fluorines coordinating aluminum is presently not explained.

As a most important observation the nucleotide binding pocket is bordered by $L1_c$ of GAP-334.[128] This leads to placement of the guanidinium group of Arg789 in proximity of the phosphate moiety suggesting interactions with a fluoride ligand of the pentagonally coordinated aluminum fluoride and with a β-phosphate oxygen. In addition, the presence of $L1_c$ results in stabilization of L4, the N-terminal portion of the switch II region which is mobile in the structures of isolated Ras (Fig. 13.3E).[19,23,29] The involvement of both switch regions in communication with RasGAP is not surprising since these regions change their conformation upon transition between the GTP- and GDP-bound states.[29,39]

Mechanism of GTPase Activation

The mechanism by which GAPs accelerate the GTPase reaction of Ras has been a matter of controversial discussion.[6] In one model Ras itself is an efficient GTPase, and GAP induces the attainment of a catalytic competent conformation. Conflicting evidence for such a mechanism has been presented.[55,159-161] In another model the actual chemical cleavage step is modified by GAP directly, most likely by GAP supplying residues for GTP hydrolysis on Ras thereby stabilizing the transition state of the phosphoryl transfer reaction. Arginines have been discussed as candidate residues to play such a role.[157,162] Arginine can bridge intermolecular gaps and has a positively charged side chain that might be involved in transition state stabilization. In support of the second model, also called the 'arginine finger' hypothesis, RasGAPs have been found to stabilize binding of aluminum fluoride to Ras•GDP (that by itself does not bind aluminum fluoride) forming a ternary complex[143] that is believed to be the transition state mimic of the GTPase reaction.[48,49] Stoichiometric amounts of RasGAP are needed for this effect.[143]

The structure of the Ras-RasGAP complex did not provide evidence for a residue that would be a likely candidate as a general base for proton abstraction from the attacking water molecule. With the cautionary note that water chains may act as potential nucleophiles, which at the given resolution of 2.5 Å are not very likely to be unambiguously detected, it seems likely that the 'GTP as base' mechanism[55] is still valid in the case of GAP accelerated GTP hydrolysis.[95] This is also in agreement with the interpretation of results of linear free energy relationship analyses, according to which the mechanism of GTP hydrolysis need not be changed in the case of the GAP accelerated reaction.[163]

In the structure of the Ras-RasGAP complex, GAP-334 inserts Arg789 into the active site to contact the nucleotide and, most importantly AlF_3 that is believed to represent the transferred phosphoryl group in the transition state of the GTPase reaction. The structural situation was interpreted to mean that RasGAP stabilizes the transition state of Ras mediated GTP hydrolysis by neutralizing charges developing on the γ-phosphate during phosphoryl transfer.[128] In addition, RasGAP stabilizes the conformation of the switch II region and especially of Gln61 by a hydrogen bond between the carbonyl oxygen of Arg789 and the side chain amide group of Gln61 (Fig. 13.3E). This situation found in the complex structure is consistent with the explanation that RasGAP stabilizes the conformation of a catalytically important residue which in isolated Ras has been described to be flexible.[18,19,23]

A hydrogen bond between the main chain carbonyl group of Arg789 and the side chain amide group of Gln61 that in turn interacts with a fluoride ligand of AlF_3 stabilizes Gln61 to assume the presumed catalytically competent conformation in the transition state of the

phosphoryl transfer reaction.[128] As in unligated GAP-334[121] the orientation of $L1_c$ is stabilized by Arg903 with its guanidinium group contacting the main chain carbonyl oxygens of Phe 788, Arg 789, and Ala790 (Fig. 13.3D). Mutational analyses of the invariant Arg903 and Arg789 have demonstrated that conservative replacement by lysine abrogates transition state formation in the case of Arg789 but not with Arg903. The GAP catalyzed reaction is reduced 2000-fold and 200-fold, respectively, suggesting that Arg789 is much more critical for RasGAP mediated GTPase acceleration and that Arg903 plays an indirect role in the reaction.[127] The structure of the Ras-RasGAP complex and the biochemical analyses of the invariant arginines have led to the conclusion that Arg789 represents the 'arginine finger' by which RasGAPs actively participate in Ras mediated GTP hydrolysis. NMR studies suggest that in the ground state Arg789 does not contact the nucleotide, since no change in the ^{31}P-spectrum was observed when RasGAP was added to Ras•GppNHp.[95] In the Rho-RhoGAP system the ground state as represented in the RhoGAP•CDC42•GppNHp complex does not show an arginine in the vicinity of but not in interacting distance to the nucleotide.[164] In the transition state complex this arginine rotates into the active site and contacts aluminum fluoride ligand.[156]

Taken together, GAP action on Ras appears to work by two mechanisms: firstly, insertion of a catalytic residue (an arginine) into the active site to stabilize developing charges during the transition state of the GTPase reaction, and secondly, stabilization of the switch regions (especially switch II) thereby orienting residues (Gln61) that are critical for catalysis. The second mechanism appears to be of general importance as demonstrated by the structure of the α-subunit of the heterotrimeric G-protein Gαi complexed with aluminum fluoride and its GTPase activating protein RGS4 (Regulator of G-protein signaling).[165] Gα-proteins contain a critical arginine, which is located in the position corresponding to Tyr32 in Ras. This arginine is important for the GTPase reaction and in the structure contacts aluminum fluoride. In the complex structure RGS4 interacts with the switch regions without contributing additional residues directly involved in catalysis.[165]

Why Do Oncogenic Ras Mutants Escape GAP Regulation?

As stated above the P-loop region contacts GAP-334 in the immediate vicinity of Arg789 mainly by van der Waals interactions between Gly12 and $L1_c$. Mutation of Gly12 to all natural amino acid except proline renders Ras oncogenic;[132] similarly most mutations of Gln61 lead to cell transformation as well.[133] Oncogenic Ras mutants in either of the two positions are usually not sensitive to RasGAP.[104,105,134,135,152] As downregulation of Ras•GTP by RasGAPs appears to be a crucial step to keep levels of Ras•GTP low, it has long been an unanswered question how oncogenic Ras mutants escape control by GAPs. When looking at the structural situation in the active site as complemented by GAP l-334,[128] it is immediately clear that even the smallest possible amino acid substitution (G12A) in position 12 leads to steric hindrance with the carbonyl oxygen of Arg789 and also with the amino group of Gln61. Judging from the geometry of the presumed transition state mimic and the role of Gln61 to stabilize the attacking nucleophile, it is clear that in position 61 other amino acids would not be tolerated, although some of them bind to RasGAP.[135,152,166]

Conclusions

Extension of our knowledge about Ras signaling and regulation by RasGAPs to three dimensions has supplied a considerable amount of information on how Ras communicates with effectors and its GAPs, and to a limited extent what they are saying. Apparently Ras uses distinct structural patterns of intermolecular communication with effectors and RasGAPs. In the complex with Raf-RBD it uses an intermolecular β-sheet, a motif found in other protein complexes of unrelated function as well. In contrast, a complicated scheme of

loops and helices interacting with each other is used in the complex with RasGAP. This appears to represent a novel mode of protein-protein interaction as such, but is found similarly in complexes of other GTP binding proteins with their specific GTPase activating proteins as well.[156,164,165] These GAPs are also purely helical, but are related neither in structure nor in sequence.[6,167] The different modes of protein-protein interaction observed in the Rap•Raf-RBD and Ras•GAP-334 complexes agree with NMR studies, that detected two distinct conformations of the effector region in GTP-bound Ras, which are selectively stabilized upon interactions with either effectors or RasGAPs.[79,95] The interfaces observed in the GAP- and effector complexes overlap and thus account very well for the mutual exclusive binding of these proteins to Ras.[87] The structural information provided by the interface of the Rap•Raf-RBD complex has led to programs aimed at the design of molecules that might block Ras effector interaction thus blocking signaling of permanently activated Ras mutants in human tumors. Similarly the structure of the Ras•RasGAP complex has stimulated the search for small molecule compounds that might mimic the GAP function to restore the GTPase of oncogenic Ras mutants. This suggests that in principle, and using the knowledge of the 3D structure of the Ras-RasGAP complex, it might be possible to induce GTP hydrolysis on normal and maybe on mutant Ras, by molecules designed to stabilize the transition state of the GTPase reaction.

Acknowledgments

We thank Frank Schmitz, Friedhelm Dräger, and Reza Ahmadian for help with preparation of figures, Reza Ahmadian, Matthias Geyer, Christian Herrmann, Ilme Schlichting, and Axel Scheidig for discussions, Matthias Geyer for carefully reading the manuscript and Rita Schebaum for excellent secretarial assistance. Klaus Scheffzek acknowledges support by the Peter and Traudl Engelhorn Stiftung (Penzberg, Germany) and by the National Neurofibromatosis Foundation (New York, USA).

References

1. Barbacid M. *ras* genes. Ann Rev Biochem 1987; 56:779-82.
2. Valencia A, Chardin P, Wittinghofer A et al. The ras Protein Family: Evolutionary tree and role of conserved amino acids. Biochemistry 1991;30:4637-4648.
3. Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: a conserved switch for diverse cell functions. Nature 1990; 348:125-132.
4. Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: Conserved structure and molecular mechanism. Nature 1991; 349:117-127.
5. Gibbs JB. Lipid modifications of proteins in the Ras superfamily. In: Dickey BF, Birnbaumer L, eds. GTPases in Biology I, Handbook Exp Pharmacology 108/I. Springer Verlag 1993:335-344.
6. Boguski MS, McCormick F. Proteins regulating Ras and its relatives. Nature 1993; 366:643-654.
7. Lowy DR, Willumsen BM. Function and regulation of Ras. Ann Rev Biochem 1993; 62:851-891.
8. Marshall CJ. Ras effectors. Current Opinion in Cell Biology 1996;8:197-204.
9. McCormick F, Wittinghofer A. Interactions between Ras proteins and their effectors. Current Opinion in Biotechnology 1996; 7:449-456.
10. Wittinghofer A, Scheffzek K, Ahmadian MR. The interaction of Ras with GTPase-activating proteins. FEBS Letters 1997; 410:63-67.
11. LaCour TFM, Nyborg J, Thirup S et al. Structural details of the binding of guanosine diphosphate to elongation factor Tu from *E. coli* as studied by X-ray crystallography. EMBO J 1985; 4:2385-2388.
12. Jurnak F. Structure of the GDP domain of EF-Tu and location of amino acids homologous to ras oncogene proteins. Science 1985; 230:32-36.

13. Halliday KR. Regional homology in GTP-binding proto-oncogene products and elongation factors. J Cyclic Nucleotide and Protein Phosphorylation Res 1983; 9:435-448.
14. McCormick F, Clark BFC, la Cour TFM et al. A model for the tertiary structure of p21, the product of the *ras* oncogene. Science 1985; 230:78-82.
15. Scherer A, John J, Linke R et al. Crystallization and preliminary X-ray analysis of the human c-H-ras oncogene product p21 complexed with GTP analogues. J Mol Biol 1989; 206:257-259.
16. Jancarik J, DeVos AM, Kim,S-H et al. Crystallisation of human c-H-ras oncogene products. J Mol Biol 1988; 200:205-207.
17. Pai EF, Kabsch W, Krengel U et al. Structure of the guanine nucleotide binding domain of the H-ras oncogene product p21 in the triphosphate conformation. Nature 1989; 341:209-214.
18. Tong L, deVos AM, Milburn MV et al. Crystal structures at 2.2 A resolution of the catalytic domains of normal ras protein and an oncogene mutant complexed with GDP. J Mol Biol 1991; 217:503-516.
19. Pai EF, Krengel U, Petsko GA et al. Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 Å resolution: Implications for the mechanism of GTP hydrolysis. EMBO J 1990; 9: 2351-2359.
20. Brünger AT, Milburn MV, Tong L et al. Crystal structure of an active form of Ras protein, a complex of a GTP analog and the HRAS p21 catalytic domain. Proc Natl Acad Sci U.S.A. 1990; 87:4849-4853.
21. Krengel U. Struktur und Guanosintriphosphat-Hydrolysemechanismus des C-terminal verkürzten menschlichen Krebsproteins p21H-ras. Dissertation, University of Heidelberg 1990.
22. Scheidig AJ, Franken SM, Corrie JET et al. X-ray crystal structure analysis of the catalytic domain of the oncogene product $p21^{H\text{-}ras}$ complexed with caged GTP and mant dGppNHp. J Mol Biol 1995; 253:132-150.
23. Kraulis PJ, Domaille PJ, Campbell-Burk SL et al. Solution structure and dynamics of ras p21.GDP determined by heteronuclear three- and four-dimensional NMR spectroscopy. Biochemistry 1994; 33(12):3515-3531.
24. Ito Y, Yamasaki K, Iwahara J et al. Regional Polysterism in the GTP-bound form of the human c-Ha-Ras protein. Biochemistry 1997; 36:9109-9119.
25. Polakis P, McCormick F. Structural requirements for the interaction of $p21^{ras}$ with GAP, exchange factors, and its biological effector target. J Biol Chem 1993; 268:9157-9160.
26. Marshall MS. The effector interactions of $p21^{ras}$. TIBS 1993; 19:250-254.
27. Geyer M, Wittinghofer A. GEFs, GAPs, GDIs and effectors: Getting a closer (3D) look at the regulation of Ras-related GTP-binding proteins. Curr Opin Struct Biol 1997; 7:786-792.
28. Wittinghofer A, Pai E. The structure of ras protein: A model for a universal molecular switch. TIBS 1991; 16:383-387.
29. Milburn MV, Tong L, DeVos AM et al. Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic *ras* proteins. Science1990; 247:939-945.
30. Reinstein J, Schlichting I, Frech M et al. p21 with a Phenylalanine 28 → Leucine mutation reacts normally with the GTPase activating protein GAP but nevertheless has transforming properties. J Biol Chem 1991; 266:17700-17706.
31. Rensland H, John J, Linke R et al. Substrate and product structural requirements for binding of nucleotides to H-ras p21: The mechanism of discrimination between guanosine and adenosine nucleotides. Biochemistry 1995; 34:593-599.
32. Schmidt G, Lenzen C, Simon I et al. Biochemical and biological consequences of changing the specificity of $p21^{ras}$ from guanosine to xanthosine nucleotides. Oncogene 1996; 12:87-96.
33. Walker JE, Sarastre M, Runswick MJ et al. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J 1982; 1:945-951.
34. Dreusicke D, Schulz GE. The glycine-rich loop of adenylate kinase forms a giant anion hole. FEBS Letters 1986; 208:301-304.

35. Saraste M, Sibbald PR, Wittinghofer A. The P-loop—a common motif in ATP- and GTP-binding proteins. TIBS 1990; 15:430-434.
36. John J, Sohmen R, Feuerstein J et al. Kinetics of interaction of nucleotides with nucleotide free H-ras p21. Biochemistry 1990; 29:6058-6065.
37. Lambright DG, Noel JP, Hamm HE et al. Structural determinants for activation of the α-subunit of a heterotrimeric G protein. Nature 1994; 369:621-628.
38. Mixon MB, Lee E, Coleman DE et al. Tertiary and quaternary structural changes in $G_{i\alpha 1}$ induced by GTP hydrolysis. Science 1995; 270:954-960.
39. Schlichting I, Almo S, Rapp G et al. Time resolved X-ray structural studies on H-ras p21: Identification of the conformational change induced by GTP hydrolysis. Nature 1990; 345:309-315.
40. Berchtold H, Reshetnikova L, Reiser CPA et al. Crystal structure of active elongation factor Tu reveals major domain rearrangements. Nature 1993; 365:126-132.
41. Kjeldgaard M, Nissen P, Thirup S et al. The crystal structure of elongation factor EF-Tu from Thermus aquaticus in the GTP conformation. Structure 1993; 1:35-50.
42. Polekhina G, Thirup S, Kjeldgaard M et al. Helix unwinding in the effector region of elongation factor EF-TU-GDP. Structure 1996; 4(10):1141-1151.
43. Abel K, Yoder MD, Hilgenfeld R et al. An alpha to beta conformational switch in EF-Tu. Structure 1996; 4(19):1153-1159.
44. Amor JC, Harrison DH, Kahn RA et al. Structure of the human ADP-ribosylation factor 1 complexed with GDP. Nature 1994; 372:704-708.
45. Scheffzek K, Klebe C, Fritz-Wolf K et al. Crystal structure of the nuclear Ras-related protein Ran in its GDP-bound form. Nature 1995; 374:378-381.
46. Feuerstein J, Goody RS, Webb MR. The mechanism of guanosine nucleotide hydrolysis by p21 c-Ha-*ras*. J Biol Chem 1989; 264:6188-6190.
47. Noel JP, Hamm HE, Sigler PB. The 2.2 Å crystal structure of transducin-α complexed with GTPγS. Nature 1993; 366:654-663.
48. Coleman DE, Berghuis AM, Lee E et al. Structures of active conformations of G_{ia1} and the mechanism of GTP hydrolysis. Science 1994; 265:1405-1412.
49. Sondek J, Lambright DG, Noel JP et al. GTPase mechanism of G proteins from the 1.7-Å crystal structure of transducin α·GDP·AlF_4^-. Nature 1994; 372:276-279.
50. Goody RS, Pai EF, Schlichting I et al. Studies on the structure and mechanism of H-ras p21. Phil Trans R Soc Lond 1992; B 336:3-11.
51. Frech M, Darden TA, Pedersen LG et al. Role of Glutamine-61 in the hydrolysis of GTP by $p21^{H\text{-}ras}$: An experimental and theoretical study. Biochemistry 1994; 33:3237-3244.
52. Chung H-H, Benson DR, Schultz PG. Probing the structure and mechanism of ras protein with an expanded genetic code. Science 1993; 259:806-809.
53. Langen R, Schweins T, Warshel A. On the mechanism of guanosine triphosphate hydrolysis in ras p21 proteins. Biochemistry 1992; 31:8691-8696.
54. Schweins T, Langen R, Warshel A. Why have mutagenesis studies not located the general base in ras p21. Nature Struct Biol 1994; 1(7):476-484.
55. Schweins T, Geyer M, Scheffzek K et al. Substrate-assisted catalysis as a mechanism for GTP hydrolysis of $p21^{ras}$ and other GTP-binding proteins. Nature Struct Biol 1995; 2:36-44.
56. Privé GG, Milburn MV, Tong L et al. X-ray crystal structures of transforming p21 ras mutants suggest a transition-state stabilization mechanism for GTP hydrolysis. Proc Natl Acad Sci USA 1992; 80:3649-3653.
57. Schweins T, Scheffzek K, Aβheuer R et al. The role of the metal ion in the $p21^{ras}$ catalysed GTP-hydrolysis: Mn^{2+} versus Mg^{2+}. J Mol Biol 1997; 266:847-856.
58. Schlichting I, Rapp G, John J et al. Biochemical and crystallographic characterization of a complex of c-Ha-*ras* P21 and caged GTP with flash photolysis. Proc Natl Acad Sci USA 1989; 86:7687-7690.
59. Scheidig A, Sanchez-Llorente A, Lautwein A et al. Crystallographic studies on p21H-ras using the synchrotron Laue method: improvement of crystal quality and monitoring of the GTPase reaction at different time points. Acta Cryst 1994; D50:512-520.

60. Halkides CJ, Farrar CT, Larsen RG et al. Characterization of the active site of p21 *ras* by electron spin-echo envelope modulation spectroscopy with selective labeling: Comparisons between GDP and GTP forms. Biochemistry 1994; 33:4019-4035.
61. Farrar CT, Halkides CJ, Singel DJ. The frozen solution structure of p21 ras determined by ESEEM spectroscopy reveals weak coordination of Thr35 to the active site metal ion. Structure 1997; 5:1055-1066.
62. Krengel U, Schlichting, I, Scherer A et al. Three-dimensional structures of H-ras p21 mutants: molecular basis for their inability to function as signal switch molecules. Cell 1990; 62:539-548.
63. Franken SM, Scheidig AJ, Krengel U et al. Three-dimensional structures and properties of a transforming and a nontransforming glycine-12 mutant of $p21^{H\text{-}ras}$. Biochemistry 1993; 32:8411-8420.
64. Wittinghofer A, Herrmann C. Ras-effector interactions, the problem of specificity. FEBS Lett 1995; 369:52-56.
65. Vojtek AB, Hollenberg SM, Cooper JA. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell 1993; 74:205-214.
66. Aelst van L, Barr M, Marcus S et al. Complex formation between RAS and RAF and other protein kinases. Proc Natl Acad Sci USA 1993; 90:6213-6219.
67. Zhang X-F, Settleman J, Kyriakis JM et al. Normal and oncogenic $p21^{ras}$ proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 1993; 364:308-313.
68. Warne PH, Rodriguez Viciana P, Downward J. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature 1993; 364:352-355.
69. Koide H, Satoh T, Nakafuku M et al. GTP-dependent association of Raf-1 with Ha-Ras: Identification of Raf as a target downstream of Ras in mammalian cells. Proc Natl Acad Sci USA 1993; 90:8683-8686.
70. Kodaki T, Woscholski R, Hallberg B et al. The activation of phosphatidylinositol 3-kinase by Ras. Current Biology 1994; 4:798-806.
71. Rodriguez-Viciana P, Warne PH, Dhand R et al. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 1994; 370:527-532.
72. Albright CF, Giddings BW, Liu JL et al. Characterization of a guanine nucleotide dissociation stimulator for a ras-related GTPase. EMBO J 1992; 12:339-347.
73. Kikuchi A, Demo SD, Ye Zhi-Hai et al. ralDGS family members interact with the effector loop of *ras* p21. Mol Cell Biol 1994; 14:7483-7491.
74. Hofer F, Fields S, Schneider C et al. Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. Proc Natl Acad Sci USA 1994; 91:11089-11093.
75. Spaargaren, M. and Bischoff JR. Identification of the guanine nucleotide dissocition stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras and Rap. Proc Natl Acad Sci USA 1994; 91:12609-12613.
76. Wolthuis RMF, Bauer B, van 't Veer LJ et al. RalGDS-like factor (Rlf) is a novel Ras and Rap 1A-associating protein. Oncogene 1996; 13:353-362.
77. Herrmann C, Horn G, Spaargaren M et al. Differential interaction of the Ras family GTP-binding proteins H-Ras, Rap1A and R-Ras with the putative effector molecules Raf-kinase and Ral-guanine nucleotide exchange factor. J Biol Chem 1996; 271:6794-6800.
78. Emerson S., Madison VS, Palermo RE et al. Solution structure of the Ras-binding domain of c-Raf-1 and identification of its Ras interaction surface. Biochemistry 1995; 34:6911-6918.
79. Geyer M, Herrmann C, Wohlgemuth S et al. Structure of the Ras-binding domain of RalGEF and implications for Ras binding and signalling. Nature Structural Biology 1997; 4:694-699.
80. Huang L, Weng XW, Hofer F et al. Three-dimensional structure of the Ras-interacting domain of RALGDS. Nat Struct Biol 1997; 4(8):609-615.
81. Nassar N, Horn G, Herrmann C et al. The 2.2 Å crystal structure of the Ras-binding domain of the serine/threonine kinase c-Raf1 in complex with Rap1A and a GTP analogue. Nature 1995; 375:554-560.
82. Vijay-Kumar S, Bugg CE, Cook WJ. Structure of ubiquitin refined at 1•8 Å resolution. J Mol Biol 1987; 194:531-544.

83. Nassar N, Horn G, Herrmann C et al. Ras/Rap effector specificity determined by charge reversal. Nature Structural Biology 1996; 3:723-729.
84. Derrick JP, Wigley DB. Crystal structure of a streptococcal protein G domain bound to an Fab fragment. Nature 1992; 359:752-754.
85. Derrick JP, Wigley DB. The third IgG-binding domain from streptococcal protein G. J Mol Biol 1994; 243:906-918.
86. Herrmann C, Nassar N. Ras and its effectors. Prog Biophys molec Biol 1996; 66(1):1-41.
87. Herrmann C, Martin GA, Wittinghofer A. Quantitative analysis of the complex between $p21^{ras}$ and the Ras-binding domain of the human Raf-1 protein kinase. J Biol Chem 1995; 270:2901-2905.
88. Calés C, Hancock JF, Marshall CJ et al. The cytoplasmic protein GAP is implicated as the target for regulation by the ras gene product. Nature 1988; 332:548-551.
89. Stone JC, Blanchard RA. Genetic definition of ras effector elements. Mol Cell Biol 1991; 11:6158-6165.
90. Sigal IS, Gibbs JB, D'Alonzo JS et al. Identification of effector residues and a neutralizing epitope of Ha-ras-encoded protein. Proc Natl Acad Sci USA 1986; 83:4725-4729.
91. Fabian JR, Vojtek AB, Cooper A et al. A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. Proc Natl Acad Sci USA 1994; 91:5982-5986.
92. Block C, Janknecht R, Herrmann C et al. Quantitative structure-activity analysis correlating Ras/Raf interaction in vitro to Raf activation in vivo. Nature Structural Biology 1996; 3:244-251.
93. Cunningham BC, Wells JA. Comparison of a structural and a functional epitope. J Mol Biol 1993; 234:554-563.
94. Clackson T, Wells JA. A hot spot of binding energy in a hormone-receptor interface. Science 1995; 267:383-386.
95. Geyer M, Schweins T, Herrmann C et al. Conformational transitions in free $p21^{H\text{-}ras}$ and in $p21^{H\text{-}ras}$ complexed with the effector protein Raf-RBD and the GTPase activating protein GAP. Biochemistry 1996; 35:10308-10320.
96. Wittinghofer A, Nassar N. How Ras-related proteins talk to their effectors. TIBS 1996; 21:488-491.
97. Brtva TR, Drugan JK, Ghosh S et al. Two distinct Raf domains mediate interaction with Ras. J Biol Chem 1995; 270:9809-9812.
98. Hu CD, Kariya KI, Tamada M et al. Cysteine-rich region of Raf-1 interacts with activator domain of post-translationally modified Ha-Ras. J Biol Chem 1995; 270(30):274-277.
99. Moodie SA, Paris M, Villafranca E et al. Different structural requirements within the switch II region of the Ras protein for interactions with specific downstream targets. Oncogene 1995; 11:447-454.
100. Shirouzu M, Koide H, Fujita-Yoshigaki J et al. Mutations that abolish the ability of Ha-Ras to associate with Raf-1. Oncogene 1994; 9:2153-2157.
101. Mott HR, Carpenter JW, Zhong S et al. The solution structure of the Raf-1 cysteine-rich domain: A novel Ras and phospholipid binding site. Proc Natl Acad Sci USA 1996; 93:8312-8317.
102. Esser D, Bauer B, Wittinghofer A et al. Structure determination of the Ras-binding domain of the Ral-specific guanine nucleotide exchange factor Rlf. Biochemistry 1997; (In press).
103. Gideon P, John J, Frech M et al. Mutational and kinetic analysis of the GTPase-activating Protein (GAP)-p21 interaction: The C-terminal domain of GAP is not sufficient for full activity. Mol Cell Biol 1992; 12:2050-2056.
104. Trahey M, McCormick F. A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants. Science 1987; 238:542-545.
105. Gibbs JB, Schaber MD, Allard WJ et al. Purification of ras GTPase activating protein from bovine brain. Proc Natl Acad Sci USA 1988; 85:5026-5030.
106. Trahey M, Wong G, Halenbeck R et al. Molecular cloning of GAP Complementary DNA from Human Placenta. Science 1988; 242:1697-1700.

107. Tocque B, Delumeau I, Parker F et al. Ras-GTPase activating protein (GAP): A putative effector for Ras. Cell Signal 1997; 9(2):152-159.
108. Gutmann DH, Collins FS. The neurofibromatosis type 1 gene and its protein product, neurofibromin. Neuron 1993; 10:335-343.
109. Xu G, O'Connel P, Viskochil D et al. The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell 1990; 62:599-608.
110. Ballester R, Marchuk D, Boguski M et al. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. Cell 1990; 63:851-859.
111. Martin GA, Viskochil D, Bollag G et al. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. Cell 1990; 63:843-849.
112. Xu G, Lin B, Tanaka K et al. The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of S.cerevisiae. Cell 1991; 63:835-841.
113. Tanaka K, Matsumoto K. Toh-e A. *IRA1*, an inhibitory regulator of the RAS-cyclic AMP pathway in *Saccharomyces cerevisiae*. Mol Cell Biol 1989; 9:757-768.
114. Eccleston JF, Moore KJM, Morgan L et al. Kinetics of interaction between normal and proline 12 Ras and the GTPase-activating proteins, p120-GAP and neurofibromin. J Biol Chem 1993; 268:27012-27019.
115. Marshall MS, Hill WS, Ng AS et al. A C-terminal domain of GAP is sufficient to stimulate ras p21 GTPase activity. EMBO J 1989; 8:1105-1110.
116. Ahmadian MR, Wiesmüller L, Lautwein A et al. Structural Differences in the Minimal Catalytic Domains of the GTPase-Activating Proteins $p120^{GAP}$ and Neurofibromin. J Biol Chem 1996; 271:16409-16415.
117. Maekawa M, Li S, Iwamatsu A et al. A novel mammalian Ras GTPase-activating protein which has phospholipid-binding and Btk homology regions. Mol Cell Biol 1994; 14:6879-6885.
118. Gaul U, Mardon G, Rubin GM. A putative ras GTPase activating protein acts as a negative regulator of signalling by the sevenless receptor tyrosine kinase. Cell 1992; 68:1007-1019.
119. Baba H, Fuss B, Urano J et al. GapIII, a new brain-enriched member of the GTPase-activating protein family. J Neurosci Res 1995; 41:846-858.
120. Cullen PJ, Hsuan JJ, Truong O et al. Identification of a specific Ins(1,3,4,5)P_4-binding protein as a member of the GAP1 family. Nature 1995; 376:527-530.
121. Scheffzek K, Lautwein A, Kabsch W et al. Crystal structure of the GTPase-activating domain of human p120GAP and implications for the interaction with Ras. Nature 1996; 384:591-596.
122. Nur-E-Kamal MSA, Varga M, Maruta H. The GTPase-activating NF1 fragment of 91 amino acids reverses v-Ha-Ras-induced malignant phenotype. J Biol Chem 1993; 268:22331-22337.
123. Fridman M, Tikoo A, Varga M et al. The minimal fragments of c-Raf-1 and NF1 that can suppress v-Ha-Ras-induced malignant phenotype. J Biol Chem 1994; 269:30105-30108.
124. Skinner RH, Bradley S, Brown AL et al. Use of the Glu-Glu-Phe C-terminal epitope for rapid purification of the catalytic doamin of normal and mutant ras GTPase-activating proteins. J Biol Chem 1991; 266:14163-14166.
125. Brownbridge GG, Lowe PN, Moore KJM et al. Interaction of GTPase activating proteins (GAPs) with $p21^{ras}$ measured by a novel fluorescence anisotropy method. J Biol Chem 1993; 268:10914-10919.
126. Hettich L, Marshall M. Structural analysis of the Ras GTPase activating protein catalytic domain by semirandom mutagenesis: Implications for a mechanism of interaction with Ras-GTP. Cancer Research 1994; 54:5438-5444.
127. Ahmadian MR, Stege P, Scheffzek K et al. Confirmation of the arginine-finger hypothesis for the GAP-stimulated GTP-hydrolysis reaction of Ras. Nat Struct Biol 1997; 4(9):686-689.
128. Scheffzek K, Ahmadian MR, Kabsch W et al. The Ras-RasGAP complex: Structural basis for GTPase activation and its loss in oncogenic Ras mutants. Science 1997; 277:333-338.
129. Gutmann DH, Boguski M, Marchuk D et al. Analysis of the neurofibromatosis type 1 (NF1) GAP-related domain by site-directed mutagenesis. Oncogene 1993; 8:761-769.

130. Wood DR, Poullet P, Wilson BA et al. Biochemical characterization of yeast *RAS2* mutants reveals a new region of ras protein involved in the interaction with GTPase-activating proteins. J Biol Chem 1994; 269:5322-5327.
131. Parrini MC, Bernardi A, Parmeggiani A. Determinants of Ras proteins specifying the sensitivity to yeast Ira2p and human p120-GAP. EMBO J 1996; 15:1107-1111.
132. Seeburg PH, Colby WW, Capon DJ et al. Biological properties of human c-Ha-ras 1 genes mutated at codon 12. Nature 1984; 312:71-75.
133. Der CJ, Finkel T, Cooper GM. Biological and biochemical properties of human ras^{H} genes mutated at codon 61. Cell 1986; 44:167-176.
134. Adari H, Lowy DR, Willumsen BM et al. Guanosine triphosphatase activating protein (GAP) interacts with the p21 *ras* effector binding domain. Science 1988; 240:518-521.
135. Bollag G, McCormick F. Differential Regulation of rasGAP and neurofibromatosis gene product activities. Nature 1991; 351:576-579.
136. Wiesmüller L, Wittinghofer A. Expression of the GTPase activating domain of the neurofibromatosis type 1 (NF1) gene in *E. coli* and role of the conserved lysine residue. J Biol Chem 1992; 267:10207-10210.
137. Nakafuku M, Nagamine M, Ohtoshi A et al. Suppression of oncogenic Ras by mutant neurofibromatosis type 1 genes with single amino acid substitutions. Proc Natl Acad Sci USA 1993; 90:6706-6710.
138. Poullet P, Lin B, Esson K et al. Functional significance of lysine 1423 of neurofibromin and characterization of a second site suppressor which rescues mutations at this residue and suppresses $RAS2^{Val\text{-}19}$-activated phenotypes. Mol Cell Biol 1994; 14:815-821.
139. Morcos P, Thapar N, Tusneem N et al. Identification of neurofibromin mutants that exhibit allele specificity or increased Ras affinity resulting in suppression of activated *ras* alleles. Mol Cell Biol 1996; 16:2496-2503.
140. Miao W, Eichelberger L, Baker L et al. p120 Ras GTPase-activating protein interacts with Ras-GTP through specific conserved residues. J Biol Chem 271; 26:15322-15329.
141. Rubinfeld B, Munemitsu S, Clark R et al. Molecular cloning of a GTPase activating protein specific for the Krev-1 protein $p21^{rap1}$. Cell 1991; 65:1033-1042.
142. Chabre M. Aluminofluoride and beryllofluoride complexes: New phosphate analogs in enzymology. TIBS 1990; 15:6-10.
143. Mittal R, Ahmadian MR, Goody RS et al. Formation of a transition state analog of the Ras GTPase reaction by Ras•GDP, tetrafluoroaluminate and GTPase-activating proteins. Science 1996; 273:115-117.
144. Serth J, Lautwein A, Frech M et al. The inhibition of the GTPase activating protein-Ha-ras interaction by acidic lipids is due to physical association of the C-terminal domain of the GTPase activating protein with micellar structures. EMBO J 1991; 10:1325-1330.
145. Tsai M-H, Hall A, Stacey DW. Inhibition by phospholipids of the interaction between R-ras, Rho, and their GTPase-activating proteins. Mol Cell Biol 1989; 9:5260-5264.
146. Marshall MS, Hettich LA. Characterization of Ras effector mutant ineractions with the NF1-GAP related domain. Oncogene 1993; 8:425-431.
147. Stone JC, Colleton M, Bottorff D. Effector domain mutations dissociate $p21^{ras}$ effector function and GTPase-activating protein interaction. Mol Cell Biol 1993; 13:7311-7320.
148. Nur-E-Kamal MSA, Sizeland A, D'Abaco G et al. Asparagine 26, Glutamic Acid 31, Valine 45, and Tyrosine 64 of Ras Proteins are required for their Oncogenicity. J Bio Chem 1992; 267:1415-1418.
149. Antonny B, Chardin P, Roux M et al. GTP hydrolysis mechanisms in ras p21 and in the ras-GAP complex studied by fluorescence measurements on tryptophan mutants. Biochemistry 1991; 30:8287-8295.
150. Li Y, Bollag G, Clark R et al. Somatic mutations in the neurofibromatosis 1 gene in human tumors. Cell 1992; 69:275-281.
151. Farnsworth CL, Feig LA. Dominant inhibitory mutations in the Mg^{2+}-binding site of ras^{H} prevent its activation by GTP. Mol Cell Biol 1991; 11:4822-4829.
152. Schaber MD, Garsky VM, Boyla D et al. Ras Interaction with the GTPase-activating protein (GAP). Proteins 1989; 6:306-315.

153. Mori S, Satoh T, Koide H et al. Inhibition of Ras/Raf interaction by anti-oncogenic mutants of neurofibromin, the neurofibromatosis type 1 (NF1) Gene product, in cell-free systems. J Biol Chem 1995; 270:28834-28838.
154. Zhang K, Papageorge AG, Martin P et al. Heterogeneous amino acids in ras and rap1A specifying sensitivity to GAP proteins. Science 1991; 254:1630-1634.
155. Maruta H, Holden J, Sizeland A et al. The residues of ras and rap proteins that determine their GAP specificities. J Biol Chem 1991; 266:11661-11668.
156. Rittinger K, Walker PA, Eccleston JF et al. Structure at 1.65Å of RhoA and its GTPase-activating protein in complex with a transition-state analogue. Nature 1997; 389:758-762.
157. Schlichting I, Reinstein J. Structures of active conformations of UMP kinase from *Dictyostelium discoideum* suggest phosphoryl transfer is associative. Biochemistry 1997; 36(31):9290-9296.
158. Xu YW, Moréra S, Janin J et al. AlF3 mimics the transition state of protein phosphorylation in the crystal structure of nucleoside diphosphate kinas and MgADP. Proc Natl Acad Sci USA 1997; 94:3579-3583.
159. Neal SE, Eccleston J F, Webb MR. Hydrolysis of GTP by $p21^{NRAS}$, the *NRAS* protooncogene product, is accompanied by a conformational change in the wild type protein: Use of a single fluorescent probe at the catalytic site. Proc Natl Acad Sci USA 1990; 87:3652-3565.
160. Rensland H, Lautwein A, Wittinghofer A et al. Is there a rate limiting step before GTP cleavage by H-ras p21? Biochemistry 1991; 30:11181-11185.
161. Moore KJR, Webb MR, Eccleston JF. Mechanism of GTP hydrolysis by $p21^{N\text{-}ras}$ catalyzed by GAP. Studies with a fluorescent GTP analogue. Biochemistry 1993; 32:7451-7459.
162. Müller CW, Schulz GE. Structure of the complex between adenylate kinase from *Escherichia coli* and the inhibitor Ap_5A refined at 1.9 Å resolution. J Mol Biol 1992; 224:159-177.
163. Schweins T, Geyer M, Kalbitzer HR et al. Linear free energy relationships in the intrinsic and GTPase activating protein-stimulated guanosine 5'-triphosphate hydrolysis of p21ras. Biochemistry 1996; 35(45):14225-14231.
164. Rittinger K, Walker PA, Eccleston JF et al. Crystal structure of a small G protein in complex with the GTPase-activating protein RHOGAP. Nature 1997; 388:693-697.
165. Tesmer JJG, Berman DM, Gilman AG, Sprang SR. Structure of RGS4 bound to AlF_4^--activated G_{ia1}: Stabilization of the transition state for GTP hydrolysis. Cell 1997, 89:251-261.
166. Vogel U, Dixon RAF, Schaber MD et al. Cloning of bovine GAP and its interaction with oncogenic ras p21. Nature 1988; 335:90-93.
167. Dohlmann HG, Thorner J. RGS proteins and signaling by heterotrimeric G proteins. J Biol Chem 1997; 272:3871-3874.

Chapter 14

Rho Family GTPases and Actin Cytoskeleton

Anne J. Ridley

Introduction

Cancer cells exhibit changes in cell morphology, cell motility, and in cell-cell and cell-matrix adhesion when compared to their nontransformed counterparts. These changes reflect alterations in the structure and composition of the cytoskeleton and associated adhesion sites, and thus understanding the signaling pathways regulating cytoskeletal organization is important in defining how cancer cells escape normal controls on proliferation and migration. This chapter focuses on the central role of Rho family proteins in regulating actin organization.

Rho family proteins are small monomeric G proteins, sharing 30% sequence identity with Ras family GTPases, and have been identified in many eukaryotic organisms, from yeast to plants and mammals. In mammalian cells, they include Rho(A,B,C), Rac(1,2), CDC42 (two alternatively spliced variants), TC10, RhoD, RhoE, RhoG and TTF.[1,2] Rho, Rac and/or CDC42 have been implicated in regulating actin organization in a variety of different model systems, including *Saccharomyces, Drosophila, Dictyostelium,* and cultured mammalian cells. In addition, they have been ascribed many other functions, including synthesis of cell wall components in *S. cerevisiae*,[3] and regulation of transcription, secretion, endocytosis and mitogenesis in mammalian cells.[1,4]

Rho, Rac and CDC42 bind and hydrolyze GTP and interact with a number of proteins which regulate their GTPase cycle. These include GDP dissociation stimulators (GDSs), GTPase activating proteins (GAPs) and GDP dissociation inhibitors (GDIs) (see chapter 11). It is presumed that other Rho family members are similarly regulated, with the exception of RhoE which does not detectably hydrolyze GTP[5] and may be a member of a subgroup of Rho family proteins which are regulated distinctly.

The study of Rho family proteins has been greatly facilitated by the availability of a number of bacterial toxins and exoenzymes that specifically modify members of the family (see chapter 23).[6] The most well-known is C3 transferase, an exoenzyme produced by *Clostridium botulinum* that ADP-ribosylates and inactivates Rho. It will also modify Rac and CDC42, although with much lower efficiency.[7] Toxins that activate Rho have also been described,[6] including a toxin that deamidates a glutamine to glutamate at amino acid 63 of Rho (and probably also acts on Rac and CDC42) producing a Rho GTPase which is no longer susceptible to the action of Rho GAPs.[8,9] In addition, constitutively active and dominant negative forms of the proteins have been useful for assessing protein function. The majority of constitutively active mutants have decreased GTPase activity and are insensitive

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

to GAPs, allowing them to remain predominantly in the active, GTP-bound form in cells, and the remaining mutants have enhanced GDP/GTP exchange rate.[10] Dominant negative forms of the proteins can be created by mutating amino acid 17 (Ras numbering) from serine/threonine to asparagine. These proteins are locked in an inactive state and inhibit the activation of their respective endogenous G proteins by sequestering the GDSs.[11]

Rho

There are three highly homologous isoforms of Rho in mammalian cells: RhoA, RhoB and RhoC. The proteins are 92% identical (RhoA and RhoC) or 85% identical (RhoA/RhoC and RhoB) at the amino acid level, with the majority of differences lying within the last 15 amino acids of the C-terminus. Like Ras family GTPases, Rho proteins are modified by prenylation and carboxymethylation of a conserved cysteine four amino acids from the C-terminus, followed by removal of the last three amino acids.[12] All three proteins can be prenylated by a 20-carbon chain geranylgeranyl group, but RhoB can also be prenylated by a 15-carbon chain farnesyl group.[12] This difference in the modification of RhoB is important for its localization: RhoB localizes predominantly to an endosomal/lysosomal compartment, whereas RhoA/C are principally cytoplasmic (presumably because they are complexed with GDIs in the cytoplasm) although they also show some plasma membrane localization.[13] RhoB may therefore have a distinct function in cells to RhoA or RhoC.

In a variety of cultured adherent cells, microinjection or expression of activated Rho proteins stimulates the accumulation of stress fibers.[14] Rho is also required for the formation of stress fibers induced by growth factors, cytokines or by activation of integrins.[1,15,16] These stress fibers consist of bundles of actin filaments associated with myosin II filaments and other proteins, and are contractile. The contractile nature of Rho-induced stress fibers in fibroblasts is clearly demonstrated when the cells are plated on deformable substrata,[17] and consistent with this Rho is required for contraction of smooth muscle cells.[18] Stress fibers are linked at the plasma membrane to multi-protein complexes known as focal adhesions, where transmembrane integrins are associated with a large number of structural and signaling proteins.[19] The formation of focal adhesions is regulated by Rho and is intimately linked with stress fiber formation. The recruitment of signaling proteins to focal adhesions may underlie the ability of Rho to stimulate transcription and DNA synthesis.[1]

A number of target proteins interacting with Rho have been implicated in mediating actin reorganization (Fig. 14.1A). There is good evidence that Rho-stimulated contractility is mediated via one of these Rho targets, Rho-kinase (also known as ROCK or ROKα) (see chapter 15), a serine/threonine kinase which can induce the phosphorylation of myosin II light chain both by phosphorylating and inhibiting the activity of myosin II light chain phosphatase, and by directly phosphorylating myosin II light chain[20,21] (for reviews see refs. 1,19). This phosphorylation of myosin II light chain promotes the formation of myosin II filaments and interaction of the myosin with actin filaments and is hypothesized to lead to the bundling of actomyosin filaments into contractile stress fibers.[1,19]

Many other observations on Rho function are consistent with its ability to stimulate actomyosin-based contractility. For example, Rho is required in vivo for the healing of small wounds in chick embryos, which are closed through the contraction of an actin-based purse-string.[22] In addition, in macrophages microinjection of activated Rho proteins rapidly stimulates cell contraction, and although macrophages do not have stress fibers they possess fine Rho-regulated actin cables in the cytoplasm which are presumably contractile.[23] In neuronal cell lines, Rho also stimulates cell contraction and mediates neurite retraction in response to a variety of extracellular agents.[24,25] Finally, RhoA is localized to the cleavage furrow at cytokinesis and is required for actin filament assembly and constriction of the

actomyosin-based contractile ring.[26,27] Rho-kinase can also phosphorylate the intermediate filament protein glial fibrillary acidic protein (GFAP) at sites specifically phosphorylated during cytokinesis.[28] As GFAP is localized to the cleavage furrow, these results suggest that Rho could act via Rho-kinase to coordinate changes in the actin cytoskeleton and intermediate filament network required for cytokinesis.

As well as stimulating actomyosin-based contractility, Rho can stimulate an overall increase in F-actin in cells,[29] presumably by stimulating actin polymerization. Consistent with this, Rho is required for membrane 'folding' during *Shigella* entry into HeLa cells, a process involving new actin polymerization.[30] These membrane folds may be functionally similar to the Rho-dependent membrane folding induced by hepatocyte growth factor in KB cells.[31] One mechanism whereby Rho could stimulate actin polymerization is by enhancing synthesis of phosphatidylinositol (4,5) bisphosphosphate (PIP_2), which binds to a number of actin-binding proteins, including profilin, vinculin and gelsolin (see chapters 2 and 7). PIP_2 is synthesized from PI by the sequential action of two enzymes, PI 4-kinase and PI(4)P 5-kinase. The latter enzyme has been shown to interact with recombinant Rho, although the interaction may not be direct[32] (for a review see ref. 14). The downstream pathways activated in response to Rho-stimulated PIP_2 production are far from clear. However, as PIP_2 has the potential to interact with many proteins, its postulated roles in signaling are diverse, possibly involving several populations of PIP_2 in different cellular compartments.[33] For example, PIP_2 can remove actin-capping proteins such as gelsolin from the plus-ends of actin filaments, creating nucleation sites for new actin polymerization (see chapter 2). PIP_2 is also required for the activity of phospholipase D enzymes, which in vitro bind to and can be activated by Rho, as well as Rac and CDC42.[34] In cells, however, phospholipase D has been implicated as acting upstream of Rho,[35] suggesting that the ability of the GTPases to bind phospholipase D may reflect a requirement to colocalize the proteins, perhaps linked to their ability to stimulate PIP_2 production.

Another target for Rho that may be involved in stimulating actin polymerization is p140mDia, which binds to profilin and can induce actin filament formation when overexpressed in COS cells.[36] p140mDia is part of a family of formin-related proteins, and two members of this family in *S. cerevisiae* (Bni1p and Bnr1p) are targets for Rho proteins, bind profilin and are required for correct actin distribution and cytokinesis.[37] By recruiting profilin, p140mDia and related proteins could potentially direct actin polymerization to selected sites at the plasma membrane (Fig. 14.1A). Another Rho/Rac target, the serine/threonine protein kinase PRK2, is also a potential effector for Rho regulating actin organization.[38] It seems likely that Rho interacts with several different effector proteins to induce actin reorganization, allowing the coordination of actin polymerization with the formation of stress fibers.

Our understanding of Rho function has been considerably enhanced by studies in *Drosophila* and yeast. In *Drosophila*, overexpression of a Rho homolog, *Rho1,* leads to disorganization of the actin cytoskeleton and causes abnormalities in development coincident with the time when cells undergo major morphological changes.[39] In *S. cerevisiae*, four *RHO* genes have been isolated, *RHO1, RHO2, RHO3* and *RHO4*. Of these, *RHO1* shows the closest homology to mammalian RhoA and is essential for cell viability. It is required for bud growth and is localized to the growing bud tip and to the mother-daughter neck at cytokinesis.[3] Rho1p appears to coordinate bud construction by regulating glucan synthase, a major enzyme involved in cell wall synthesis and by concomitantly organizing the actin cytoskeleton during budding, probably via its interaction with the profilin-binding protein Bni1p[37] (see above). As with Rho in mammalian cells, the localization of Rho1p suggests that it has a distinct function in regulating cytokinesis.

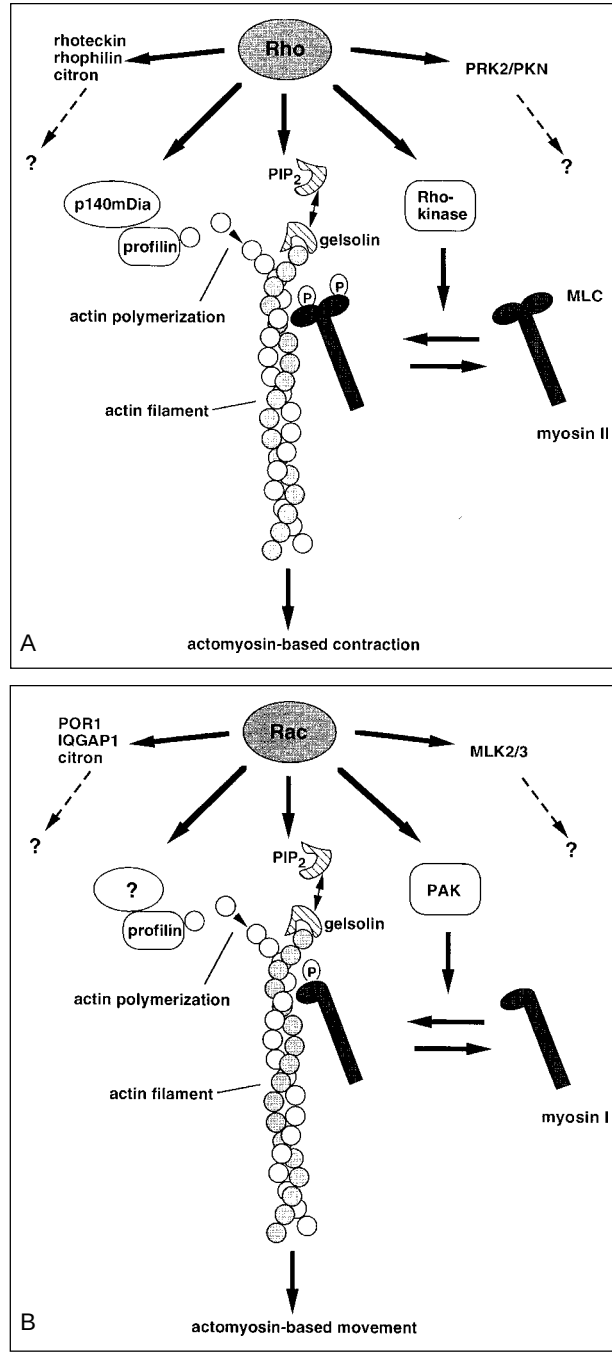
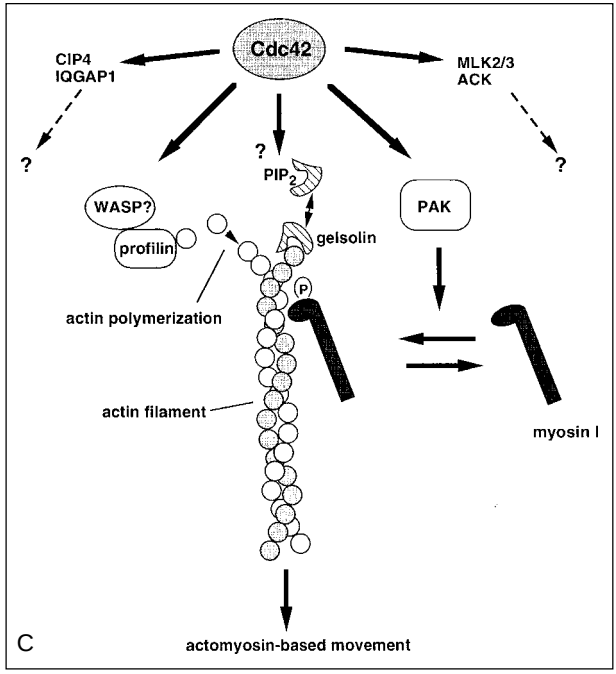


Fig. 14.1A. A model for signaling pathways regulated by Rho family proteins leading to actin reorganization. It is postulated that Rho, Rac and CDC42 initiate at least three independent signals required for actin reorganization. First, they activate a serine/threonine kinase which alters the phosphorylation status of myosins. Activated Rho (A) interacts with Rho-kinase to stimulate increased myosin light chain (MLC) phosphorylation, thereby inducing interaction of myosin II with actin to form contractile actomyosin filaments. Rac (B) and CDC42 (C) activate members of the PAK family, which phosphorylate the heavy chain of myosin I, stimulating its ability to move along actin filaments. Second, Rho, Rac and CDC42 stimulates PIP_2 production, thereby removing actin-capping proteins such as gelsolin from the ends of actin filaments. These then can act as nucleation sites for new actin polymerization. Third, they interact with proteins which recruit profilin (bound to actin monomers) to nucleation sites, where it then stimulates actin polymerization. Rho (A) interacts with p140mDia, which is known to bind profilin; CDC42 (C) interacts with WASP, which has the potential to bind profilin; a profilin-binding protein downstream of Rac (B) has not yet been identified. Rho, Rac and CDC42 also interact with a number of other proteins for which the precise function is unclear but which are likely to be involved in regulating actin polymerization and/or localization of proteins (see text for details).



Rac

The two mammalian *Rac* genes, *Rac*1 and *Rac*2, were initially cloned through their homology to *CDC42* (G25K).[40] Subsequently, the Rac1 and Rac2 proteins were purified as essential cytosolic components of the NADPH oxidase in phagocytic cells.[1] Rac1 and Rac2 proteins are 92% identical and differ primarily within the last 15 amino acids at the C-terminus, where Rac1 but not Rac2 contains a polybasic sequence.

Microinjection and transfection studies have revealed that, in contrast to Rho, Rac regulates the formation of lamellipodia and membrane ruffles in a variety of cell types.[14] Lamellipodia are plasma membrane protrusions containing a meshwork of actin filaments, and extend over the substratum to form new adhesive contacts known as focal complexes.[41] On adherent cells, membrane ruffles are similar in structure to lamellipodia, but protrude upwards from the dorsal surface of the cells. Both lamellipodia and membrane ruffles are formed by active actin polymerization occurring at the plasma membrane (see chapter 1). Interestingly, microinjection of activated Rac proteins is unable to stimulate lamellipodium formation in epithelial cells where intercellular junctions are present: instead, it actually appears to enhance actin filament accumulation at cell-cell junctions.[42] This suggests that the signaling pathways regulating lamellipodium formation are more complex in epithelial cells than in fibroblasts or macrophages.

As with Rho, a number of downstream targets for Rac have been identified, at least some of which may play a role in regulating actin reorganization (Fig. 14.1B). For example, members of the PAK family threonine/serine kinases are activated by Rac and CDC42 (see chapter 16), and experiments where either PAK or activated or inhibitory forms of PAK have been expressed in cells suggest that it regulates actin reorganization in a manner consistent with it acting downstream of Rac and CDC42.[43,44] PAK1 (α-PAK) has also been shown to localize to focal complexes in HeLa cells.[43] Other studies with Rac mutants, however, have

suggested that PAK interaction is not normally required for Rac-induced membrane ruffling.[45] A direct link between PAK and the actin cytoskeleton is suggested by the observation that myosin I heavy chain kinases in *Dictyostelium* and *Acanthameba* are related to PAKs, and that PAKs can phosphorylated myosin I heavy chains in *Dictyostelium*.[46-49] This phosphorylation event activates myosin I, stimulating actin-dependent ATP hydrolysis. In mammalian cells, myosin I is localized in lamellipodia and therefore may be a direct target of PAK involved in regulating the movement of actin filaments within lamellipodia.

Other possible targets for Rac include Rho-kinase (see above), which has the ability to interact with Rac in vitro,[45] and POR1, which has also been implicated in potentiating Ras- but not Rac-induced membrane ruffling.[50] In addition, Rac can interact with PI(4)P 5-kinase and in permeabilized platelets, Rac has been shown to stimulate PIP_2 formation.[51] As discussed above for Rho, Rac could therefore stimulate actin polymerization via production of PIP_2.

No Rac homolog exists in *S. cerevisiae*, but studies of Rac function in *Drosophila*, *C. elegans* and *Dictyostelium* have been very informative about Rac function in multicellular organisms, and suggest that it plays a key role during morphogenetic changes by regulating actin organization. In *Drosophila*, for example, DRac1 is involved in axonal outgrowth and expression of Rac1 mutants leads to abnormal actin organization,[52] consistent with studies in mammalian PC12 cells where Rac is required for NGF-induced neurite outgrowth.[53] A dominant negative mutant of Rac also disrupts cell shape changes and actin reorganization occurring during embryonic development, for example in dorsal closure.[54] In addition, in epithelial cells of the Drosophila wing disc, Rac is required for actin filament association with intercellular adherens junctions.[55] A direct role for Rac in cell migration during development has been implicated in studies of the *Drosophila* ovary.[56] Finally, PAK homolog in *Drosophila* and *C. elegans* and a potential activator (GDS) for Rho/Rac are highly expressed in cells undergoing morphogenetic changes and cytoskeletal organization,[57-59] consistent with a role in cell migration.

CDC42

CDC42 was first characterized in *S. cerevisiae*, where it was isolated as a cell cycle mutant defective in budding.[60] CDC42 is essential for polarization of the actin cytoskeleton in response to bud site selection, and is also a component of the pheromone-activated signaling pathway leading to polarization of cells towards a mating partner, again reflecting changes in the cytoskeleton.[61]

The two mammalian homolog of yeast CDC42 differ by 9 amino acids,[62] and are actually two alternatively spliced variants with different C-terminal sequences. Microinjection and transfection studies have demonstrated that CDC42 induces the formation of filopodia in several mammalian cell types, including fibroblasts and macrophages.[14,23] As with lamellipodia, filopodia in fibroblasts and macrophages are associated with focal complexes located approximately at the base of each filopodium.[23,63] Filopodia are fine plasma membrane protrusions containing bundles of actin filaments cross-linked by actin-binding proteins and extend from the leading edge of migrating cells, forming new contacts with the substratum.[64] Nerve growth cone extension is also dependent on filopodia, and in a neuroblastoma cell line, CDC42 has been shown to promote filopodium formation on growth cones.[25] These results suggest that, as in yeast, CDC42 may play a role in establishing cell polarity, a concept supported by studies in *Drosophila*. In the wing disc epithelium, CDC42 is required for epithelial cell elongation, which in turn is essential for the generation of apico-basal polarity.[55]

An interesting role for CDC42 has been defined from studies on *Salmonella typhimurium* uptake into cells. Binding of *S. typhimurium* to epithelial cells rapidly stimulates actin reor-

ganization and bacteria are internalized by subsequent macropinocytosis. These processes are dependent on CDC42 but not Rac or Rho,[65] but whether bacterial uptake involves filopodium extension is not clear.

A number of potential downstream targets for CDC42 have been identified that could mediate its effects on the actin cytoskeleton (Fig. 14.1C). As described above, PAKs are targets for both Rac and CDC42, and an increase in filopodia has been observed in cells injected with a PAK protein.[44] In *S. cerevisiae*, genetic studies have shown that two members of the PAK family, Ste20 and Cla4, are downstream targets of CDC42 involved in bud site selection. Ste20 also acts downstream of CDC42 in the signaling pathway activated by pheromones.[66] Possible targets for Ste20 and Cla4 are the heavy chains of the myosin I homologs in *S. cerevisiae*, Myo3 and Myo5, which are likely to be involved together with actin in generating cell polarity.[67]

Another effector of CDC42 is the Wiskott-Aldrich syndrome protein, WASP, which induces the formation of CDC42-dependent actin clusters in several cell types,[68] while its close relative N-WASP induces plasma membrane protrusions in COS 7 cells.[69] As well as interacting with CDC42, WASP and N-WASP have proline-rich sequences that can bind SH3 domains,[70-72] a pleckstrin homology (PH) domain and a domain homologous to the F-actin severing protein cofilin, through which N-WASP mediates actin depolymerization in vitro.[69,70] Interestingly, the proline-rich sequences of WASP resemble sequences in other proteins such as VASP that interact with profilin, suggesting that the role of WASP, like p140mDia, could be to recruit profilin to sites of CDC42-induced actin polymerization (Fig. 14.1C). Together, these results suggest that WASP could mediate at least some of the effects of CDC42 on the actin cytoskeleton, although a direct role for WASP in CDC42-induced filopodium formation has not yet been demonstrated. In fact, studies with CDC42 mutants in fibroblasts have suggested that neither PAK nor WASP are important for filopodium formation,[45] although it is not known whether these fibroblasts actually express WASP or a related protein. Finally, CIP4, another CDC42-interacting protein that could act as a transducer to the actin cytoskeleton, induces actin reorganization when overexpressed in Swiss 3T3 cells, and shows sequence homology to a region of the ERM (ezrin/radixin/moesin) family of actin-binding proteins.[73]

In addition to regulating filopodium formation, CDC42 has been shown to be involved in cytokinesis in *Xenopus* embryos,[27] and mammalian cells expressing activated CDC42 accumulate multiple nuclei, suggesting that cytokinesis is inhibited.[74] Studies in *Dictyostelium* have shown that correct cytokinesis requires RacE and two distinct members of the IQGAP family (Dd RAS GAP1 and GAPA), which in mammalian systems are targets for Rac and CDC42.[75-78] Interestingly, the effects of disrupting each *IQGAP* gene on cytokinesis are phenotypically distinguishable, suggesting that cytokinesis involves several steps regulated by different GTPases.[78] As IQGAPs bind F-actin,[78a] they provide a direct link between CDC42/Rac and the actin cytoskeleton (see chapter 18).

Other Rho Family Proteins

Although the roles of Rho, Rac and CDC42 in regulating actin organization have been best characterized, there is increasing evidence that other Rho family members can influence cell morphology and cytoskeletal arrangements. Expression of activated RhoD leads to actin rearrangement in several different mammalian cell types, and concomitantly alters the motility and distribution of early endosomes, suggesting a link between vesicular transport and the actin cytoskeleton.[2] In *Drosophila*, a novel member of the Rho family, RhoL, has been identified.[56] Expression of activated RhoL induces breakdown of cortical actin filaments in nurse cells associated with oocytes. Whether these proteins influence actin organization directly or via CDC42, Rac and Rho remains to be established.

Links Between Different Rho Family Proteins

Studies in quiescent, serum-starved Swiss 3T3 fibroblasts and in growth-arrested macrophages have shown that CDC42, Rac and Rho can act in a cascade, where activation of CDC42 leads to Rac activation, and Rac activation leads to Rho activation.[23,63,79] The functional basis of this cascade is probably to mediate cell migration, where extension of filopodia and lamellipodia at the leading edge of cells precedes the active contraction of the cell body, pulling the bulk of the cell forward.[64] If the cell body contraction is mediated by Rho, it may well be activated periodically in response to a critical level of lamellipodial extension. Little is known about the molecular links between CDC42, Rac and Rho, except that Rac-induced leukotriene production has been proposed to lead to Rho activation.[80] Studies in other cell types show that this cascade is not always wired up, however, and that Rac and Rho can actually have opposing effects on the cytoskeleton. For example, in cells already containing many stress fibers, such as growing MDCK epithelial cells, microinjection of Rac leads to a decrease in stress fibers.[42] As the presence of many stress fibers and focal adhesions inhibits cell migration, this action of Rac may facilitate migration, although presumably some level of Rho activation is required to mediate cell body contraction. Rho also acts antagonistically to Rac/CDC42 in neuroblastoma cells by inducing neurite retraction, while Rac/CDC42 promote neurite outgrowth.[25]

Rho Family Proteins, the Cytoskeleton and Transformation

In contrast to Ras, none of the Rho family proteins have been isolated as oncogenes or tumor suppressors from human cancers. There is evidence, however, that they can contribute to the transformation of rodent fibroblast lines: activated Rho, Rac and CDC42 are weakly oncogenic and can synergize with other oncogenes to induce transformation, and several GDSs for Rho, Rac and/or CDC42 have been isolated as oncogenes in NIH-3T3 fibroblast transformation assays.[4,81] In addition, suppression of Ras-induced transformation by the farnesyltransferase inhibitor, L-739,749, appears to be primarily due to its effects on RhoB, rather than on Ras itself.[82] These results raise the question of whether the ability of Rho family GTPases to stimulate transformation is a consequence of their effects on the cytoskeleton or whether the responses are separable. To address this issue, Westwick et al[83] have constructed a panel of Rac mutants which interact selectively with some but not all of its target proteins and have shown that it possible to separate the ability of Rac to transform cells from induction of lamellipodia. These experiments have been limited so far to rodent fibroblasts, and the definition of transformation has been predominantly one of increased growth rate in the absence of adhesion to a substratum. In vivo, however, very few cancers are of fibroblast origin, and cancers are also characterized by their ability to invade and metastasize. These latter functions involve cell migration and are therefore likely to be linked with changes in actin organization and cell adhesion regulated by Rho family proteins.

Conclusions

Rho, Rac and CDC42 each regulate the formation of different actin-based structures and associated adhesion sites. To do this they have to direct new actin polymerization to specific locations and coordinate this with recruitment of appropriate actin-binding proteins to allow the correct organization of these actin filaments. A model for how this complex process is achieved is that it requires each GTPase to interact with several target proteins (Fig. 14.1). Some of these targets are kinases which phosphorylate specific actin-binding proteins and change their interaction with actin. An example of this is Rho-kinase which phosphorylates myosin II light chain. Other targets are adaptor proteins which recruit actin-modulating proteins. In particular, recruitment of profilin via, for example, p140mDia appears to be important and is probably required to allow new actin polymerization.

Although it is clear that Rho family proteins can contribute to the transformed phenotype of rodent fibroblasts, whether they play a regulatory role in the development of human cancers is not known. An important question for the future therefore is whether Rho family proteins contribute to the transformed phenotype of human cancers and whether this involves their ability to regulate actin organization and cell adhesion. Their functions suggest, however, that they will play an active role in cancer metastasis as well as possibly in cancer growth, and that they may therefore be potential targets for therapeutic treatment of cancer.

References

1. Ridley AJ. Rho: Theme and variations. Curr Biol 1996; 6:1256-1264.
2. Murphy C, Saffrich R, Grummt M, Gournier H, Rybin V, Rubino M, Auvinen P, Lütcke A, Parton RG, Zerial M. Endosomal dynamics regulated by a Rho protein. Nature 1996; 384:427-432.
3. Bussey H. Cell shape determination: a pivotal role for Rho. Science 1996; 272:224-226.
4. Symons M. Rho family GTPases: the cytoskeleton and beyond. Trends Biochem Sci 1996; 21:178-181.
5. Foster R, Hu K-Q, Lu Y, Nolan KM, Thissen J, Settleman J. Identification of a novel human Rho protein with unusual properties: GTPase deficiency and in vivo farnesylation. Mol Cell Biol 1996, 16:2689-2699.
6. Machesky LM, Hall A. Rho: A connection between membrane receptor signalling and the actin cytoskeleton. Trends Cell Biol 1996; 6:304-310.
7. Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992; 70:401-410.
8. Flatau G, Lemichez E, Gauthier M, Chardin P, Paris S, Fiorentinin C, Boquet P. Toxin-induced activation of the G protein p21 Rho by deamindation of a glutamine. Nature 1997; 387:729-733.
9. Schmidt G, Sehr P, Wilm M, Selzer J, Mann M, Aktories K. Gln 63 of Rho is deamindated by *Escherichia coli* cytotoxic necrotizing factor-1. Nature 1997; 387:725-729.
10. Bourne HA, Sanders DA, McCormick F. The GTPase superfamily: conserved structure and molecular mechanism. Nature 1991; 349:117-127.
11. Farnsworth CL, Feig LA. Dominant inhibitory mutations in the Mg^{2+}-binding site of Ras^{H} prevent its activation by GTP. Mol Cell Biol 1991; 11:4822-4829.
12. Adamson P, Marshall CJ, Hall A, Tilbrook PA. Post-translational modifications of $p21^{rho}$ proteins. J Biol Chem 1992; 267:20033-20038.
13. Adamson P, Paterson HF, Hall A. Intracellular localization of the $p21^{rho}$ proteins. J Cell Biol 1992; 119:617-627.
14. Tapon N, Hall A. Rho, Rac and CDC42 GTPases regulate the organization of the actin cytoskeleton. Curr Opin Cell Biol 1997; 9:86-92.
15. Hotchin NA, Hall A. The assembly of integrin adhesion complexes requires both extracellular matrix and intracellular rho/rac GTPases. J Cell Biol 1995; 131:1857-1865.
16. Barry ST, Flinn HM, Humphries M, Critchley DR, Ridley AJ. Requirement for Rho in integrin signalling. Cell Adh Commun 1997; 4:387-398.
17. Chrzanowska-Wodnicka M, Burridge K. Rho-stimulated contractility drives the formation of stress fibres and focal adhesions. J Cell Biol 1996; 133:1403-1415.
18. Hirata K, Kikuchi A, Sasaki T, Kuroda S, Kaibuchi K, Matsuura Y, Seki H, Saida K, Takai Y. Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. J Biol Chem 1992, 267:8719-8722.
19. Burridge K, Chrzanowska-Wodnicka M. Focal adhesions, contractility, and signaling. Annu Rev Cell Dev Biol 1996; 12:463-519.
20. Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 1996; 271:20246-20249.

21. Kureishi Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, Ito M. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. J Biol Chem 1997; 272:12257-12260.
22. Brock J, Midwinter K, Lewis J, Martin P. Healing of incisional wounds in the embryonic chick wing bud: characterization of the actin purse-string and demonstration of a requirement for Rho activation. J Cell Biol 1996; 135:1097-1107.
23. Allen WE, Jones GE, Pollard JW, Ridley AJ. Rho, Rac and CDC42 regulate actin organization and cell adhesion in macrophages. J Cell Sci 1997; 110:707-720.
24. Jalink K, van Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenaar WH. Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein rho. J Cell Biol 1994, 126:801-810.
25. Kozma R, Sarner S, Ahmed S, Lim L. Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. Mol Cell Biol 1997; 17:1201-1211.
26. Ridley AJ. Rho-related proteins: Actin cytoskeleton and cell cycle. Curr Opin Genet Dev 1995; 5:24-30.
27. Drechsel DN, Hyman AA, Hall A, Glotzer M. A requirement for Rho and CDC42 during cytokinesis in Xenopus embryos. Curr Biol 1996; 7:12-23.
28. Kosako H, Amano M, Yanagida M, Tanabe K, Nishi Y, Kaibuchi K, Inagaki M. Phosphorylation of glial fibrillary acidic protein at the same sites by cleavage furrow kinase and Rho-associated kinase. J Biol Chem 1997; 272:10333-10336.
29. Norman JC, Price LS, Ridley AJ, Hall A, Koffer A. Actin filament organization in activated mast cells is regulated by heterotrimeric and small GTP-binding proteins. J Cell Biol 1994; 126:1005-1015.
30. Adam T, Giry M, Boquet P, Sansonetti P. Rho-dependent membrane folding causes *Shigella* entry into epithelial cells. EMBO J 1996; 15:3315-3321.
31. Nishiyama T, Sasaki T, Takaishi K, Masaki K, Hideaki Y, Araki K, Matsuura Y, Takai Y. rac p21 is involved in insulin-induced membrane ruffling and rho p21 is involved in hepatocyte growth factor- and 12-*0*-tetradecanoylphorbol-13-acetate (TPA)-induced membrane ruffling in KB cells. Mol Cell Biol 1994; 14:2447-2456.
32. Ren X-D, Bokoch GM, Traynor-Kaplan A, Jenkin GH, Anderson RA, Schwartz MA. Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. Mol Biol Cell 1996, 7:435-442.
33. Hsuan JJ, Tan SK. Growth factor-dependent phosphoinositide signalling. Int J Biochem Cell Biol 1997; 29:415-435.
34. Frohman MA, Morris AJ. Rho is only ARF the story. Curr Biol 1996; 6:945-947.
35. Cross MJ, Roberts S, Ridley AJ, Hodgkin MN, Stewart A, Claesson-Welsh L, Wakelam MJO. Stimulation of actin stress fibre formation in porcine aortic endothelial cells is mediated by the activation of phospholipase D. Curr Biol 1996; 6:588-597.
36. Watanabe N, Madaule P, Reid T, Ishizaki T, Watanabe GG, Kakizuka A, Saito Y, Nakao K, Jockusch BM, Narumiya S. p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho smal GTPase and is a ligand for profilin. EMBO J 1997; 16:3044-3056.
37. Imamura H, Tanaka K, Hihara T, Umikawa M, Kamei T, Takahashi K, Sasaki T, Takai Y. Bni1p and Bnr1p: downstream targets of the Rho family small G-proteins which interact with profilin and regulate actin cytoskeleton in *Saccharomyces cerevisiae*. EMBO J 1997; 16:2745-2755.
38. Vincent S, Settleman J. The PRK2 kinases is a potential effector target of both Rho and Rac GTPases and regulates actin cytoskeletal organization. Mol Cell Biol 1997; 17:2247-2256.
39. Hariharan IK, Hu K-Q, Asha H, Quitanilla A, Ezzell RM, Settleman J. Characterization of rho GTPase family homologues in *Drosophila melanogaster*: overexpressing Rho1 in retinal cells causes a late developmental defect. EMBO J 1995; 14:292-302.

40. Didsbury J, Weber RF, Bokoch GM, Evans T, Snyderman R. Rac, a novel ras-related family of proteins that are botulinum toxin substrates. J Biol Chem 1989; 264:16378-16382.
41. Mitchison TJ, Cramer LP. Actin-based cell motility and cell locomotion. Cell 1996; 84:371-379.
42. Ridley AJ, Comoglio PM, Hall A. Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac and Rho proteins in MDCK cells. Mol Cell Biol 1995; 15:1110-1122.
43. Manser E, Huang H-Y, Loo T-H, Chen X-Q, Dong J-M, Leung T, Lim L. Expression of constitutively active α-PAK reveals effects of the kinase on actin and focal complexes. Mol Cell Biol 1997; 17:1129-1143.
44. Sells MA, Knaus UG, Bagrodia S, Ambrose DM, Bokoch GK, Chernoff J. Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. Curr Biol 1997; 7:202-210.
45. Lamarche N, Tapon N, Stowers L, Burbelo PD, Aspenström P, Bridges T, Chant J, Hall A. Rac and CDC42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and JNK/SAPK MAP kinase cascade. Cell 1996; 87:519-529.
46. Lee SF, Egelhoff TT, Mahasneh A, Cote GP. Cloning and characterization of a *Dictyostelium* myosin I heavy chain kinase activated by CDC42 and Rac. J Biol Chem 1996; 271: 27044-27048.
47. Brzeska H, Szczepanowska J, Hoey J, Korn ED. The catalytic domain of *Acanthamoeba* myosin I heave chain kinase. II. Expression of active catalytic domain and sequence homolgoty to p21-activated kinase (PAK). J Biol Chem 1996; 271:27056-27062.
48. Brzeska H, Knaus UG, Wang ZY, Bokoch GM, Korn ED. P21-activated kinase hase subtrate specificity similar to *Acanthamoeba* myosin I heavy chain kinase and activates *Acanthamoeba* myosin I. Proc Natl Acad Sci USA 1997; 94:1092-1095.
49. Wu C, Lee S-F, Furmaniak-Kazmierczak E, Côté, Thomas DY, Leberer E. Activation of myosin-1 by members of the Ste20p protein kinase family. J Biol Chem 1996; 271: 31787-31790.
50. van Aelst L, Joneson T, Bar-Sagi D. Identification of a novel Rac1-interacting protein involved in membrane ruffling. EMBO J 1996; 15:3778-3786.
51. Hartwig JH, Bokoch GM, Carpenter CL, Janmey PA, Taylor LA, Toker A, Stossel TP. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell 1995; 82:643-653.
52. Luo L, Liao YJ, Jan LY, Jan YN. Distinct morphogenetic functions of simular small GTPases: *Drosophila* Drac1 is involved in axon outgrowth and myoblast fusion. Genes Dev 1994; 8:1787-1802.
53. Lamoureux P, Altun-Gultekin SF, Lin C, Wagner JA, Heidemann SR. Rac is required for growth cone function but not neurite assembly. J Cell Sci 1997; 110:635-641.
54. Harden J, Loh HY, Chia W, Lim L. A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in Drosophila. Development 1995; 121:903-914.
55. Eaton S, Auvinen P, Luo L, Jan YN, Simons K. CDC42 and Rac1 control different actin-dependent processes in the Drosophila wing disc epithelium. J Cell Biol 1995; 131:151-164.
56. Murphy AM, Montell DJ. Cell tyep-specific roles for CDC42, Rac and RhoL in *Drosophila* oogenesis. J Cell Biol 1996; 133:617-630.
57. Chen W, Chen S, Yap SF, Lim L. The Caenorhabditis elegans p21-activated kinase (CePAK) colocalizes with CeRac1 and Cdc42Ce at hypodermal cell boundaries during embryo elongation. J Biol Chem 1996; 271:26362-26368.
58. Harden J, Lee J, Loh HY, Ong YM, Tan I, Leung T, Manser E, Lim L. A *Drosophila* homolog of the Rac- and CDC42-activated serine/threonine kinase PAK is a potential focal adhesion and rocal complex protein that colocalizes with dynamic actin structures. Mol Cell Biol 1996; 16:1896-1908.
59. Werner LA, Manseau LJ. A *Drosophila* gene with predicted RhoGEF, pleckstrin homology and SH3 domains is highly expressed in morphogenic tissues. Gene 1997; 187:107-114.
60. Johnson DI, Pringle JR. Molecular characterization of CDC42, a Saccharomyces cerevisiae gene involved in the development of cell polarity. J Cell Biol 1990; 111:143-152.

61. Chant J. Generation of cell polarity in yeast. Curr Opin Cell Biol 1996; 8:557-565.
62. Shinjo K, Koland JG, Hart MJ, Narasimhan V, Johnson DI, Evans T, Cerione RA. Molecular cloning of the gene for the human placental GTP-binding protein Gp (G25K): Identification of this GTP-binding protein as the human homolog of the yeast cell-division-cycle protein CDC42. Proc Natl Acad Sci USA 1990; 87:9853-9857.
63. Nobes CD, Hall A. Rho, Rac, and CDC42 GTPases regulate the assembly of multimolecular focal adhesion complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 1995; 81, 53-62.
64. Lauffenburger DA, Horwitz AF. Cell migration: A physically integrated molecular process. Cell 1996; 84:359-369.
65. Chen LM, Hobbie S, Galan JE. Requirement of CDC42 for Salmonella-induced cytoskeletal and nuclear responses. Science 1996; 274:2115-2118.
66. Kyriakis JM, Avruch J. Protein kinase cascades activated by stress and inflammatory cytokines. BioEssays 1996; 18:567-577.
67. Goodson HV, Anderson BL, Warrick HM, Pon LA, Spudich JA. Synthetic lethality screen identifies a novel yeast myosin I gene (MYO5): myosin I proteins are required for polarization of the actin cytoskeleton. J Cell Biol 1996; 133:1277-91.
68. Symons M, Derry JMJ, Karlak B, Jiang S, Lemahieu V, McCormick F, Francke U, Abo A. Wiskott-Aldrich Syndrome protein, a novel efector for the GTPase Cdc42Hs, is implicated in actin polymerization. Cell 1996; 84:723-734.
69. Miki H, Miura K, Takenawa T. N-WASP, a novel actin-depolymerizing protein regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. EMBO J 1996; 15:5326-5335.
70. Kirchhausen T, Rosen FS. Unravelling Wiskott-Aldrich syndrome. Curr Biol 1996; 6;676-678.
71. Banin S, Truong O, Katz DR, Waterfield MD, Brickell PM, Gout I. Wiskott-Aldrich syndrome protein (WASp) is a binding partner for c-Src family protein-tyrosine kinases. Curr Biol 1996; 6:981-988.
72. Finan PM, Soames CJ, Wilson L, Nelson DL, Stewart DM, Truong O, Hsuan JJ, Kellie S. Identification of regions of the Wiskott-Aldrich syndrome protein responsible for association with selected Src homology 3 domains. J Biol Chem 1996; 271:26291-26295.
73. Aspenström P. A CDC42 target protein with homology to the non-kinase domain of FER has a potential role in regulating the actin cytoskeleton. Curr Biol 1997; 7:479-487.
74. Dutartre H, Davoust J, Gorvel J-P, Chavrier P. Cytokinesis arrest and redistribution of actin-cytoskeleton regulatory components in cells expressing the Rho GTPase CDC42Hs. J Cell Sci 1996; 109:367-377.
75. Larochelle DA, Vithalani KK, De Lozanne A. A novel member of the rho family of small GTP-binding proteins is specifically required for cytokinesis. J Cell Biol 1996; 133:1321-1329.
76. Faix J, Dittrich W. DGAP1, a homologue of RasGTPawe activatin proteins that controls growth, cytokinesis, and development in *Dictyostelium discoideum*. FEBS Lett 1996; 394:251-257.
77. Lee S, Escalante R, Firtel RA. A RasGAP is essential for cytokinesis and spatil patterning in *Dictyostelium*. Development 1997; 124:983-996.
78. Adachi H, Takahashi Y, Hasebe T, Shirouzu M, Yokoyama S, Sutoh K. Dictyostelium IQGAP-related protein specifically involved in the completion of cytokinesis. J Cell Biol 1997; 137:891-898.
78a. Bashour AM, Fullerton AT, Hart MJ et al. IQGAP, a Rac-and CDC42-binding protein, directly binds and cross-links microfilaments. J Cell Biol 1997; 137: 1555-1566.
79. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 1992; 70:401-410.
80. Peppelenbosch MP, Qui R-G, de Vries-Smits AMM, Tertoolen LGJ, de Laat SW, McCormick F, Hall A, Symons MH, Bos JL. Rac mediates growth factor-induced arachidonic acid release. Cell 1995, 81:849-856.
81. Qui R-G, Abo A, McCormick F, Symons M. CDC42 regulates anchorage-independent growth and is necessary for Ras transformation. Mol Cell Biol 1997; 17:3449-3463.

82. Lebowitz P, Davide J, Prenderdast GC. Evidence tht farnesyltransferase inhibitors suppress Ras transformation by interfering with Rho activity. Mol Cell Biol 1995; 15: 6613-6622.
83. Westwick JK, Lambert QT, Clark GJ, Symons M, van Aelst L, Pestell RG, Der CJ. Rac regulation of transformation, gene expression, and actin organization by multipe, PAK-independent pathways. Mol Cell Biol 1997; 17:1324-1335.

CHAPTER 15

Rho Effectors: Structure and Function

Shuh Narumiya, Naoki Watanabe and Toshimasa Ishizaki

Introduction: Rho Actions in the Cell

The small GTPase Rho is involved in a variety of cellular processes in mammals. First, it is involved in several processes requiring the reorganization of the actin cytoskeleton, such as stimulus-evoked cell-substrate adhesion mediated by integrins[1-3] and cytokinesis during cell division.[4,5] The cell-substrate adhesion process involves several steps such as integrin activation to bind to its ligand, complex formation of the ligated integrin with cytoskeletal proteins, clustering of these complexes and the bundling of bound actin filaments (for a review, see refs. 6,7). A typical phenotype of the cell-substrate adhesion is focal adhesions and stress fibers seen in fibroblasts and epithelial cells.[1] This process is suppressed by the inactivation of endogenous Rho and reproduced by microinjection with or the expression of activated Rho. Cytokinesis requires site selection for the division, actin polymerization and the contraction of the formed actin ring (for a review see ref. #8). The inactivation of Rho inhibits this process, and completely dissociates nuclear division from cytoplasmic division to produce multinucleate cells. Site selection and localized actin formation are also believed to be some of the actions of the RHO gene products of the yeast *Saccharomyces cervisiae*, where Rho is involved in the budding process.[9] The involvement of Rho in the generation of contraction is more evident in smooth muscles, where Rho increases the sensitivity of myofibrils to free calcium ion and induces contraction.[10] In addition to these Rho actions on the actin cytoskeleton, Rho also mediates the activation of serum response factor and is involved in nuclear signaling: this action of Rho is presumed to be mediated by a kinase cascade mobilized by Rho, which is analogous to the Ras/Raf/MAP-kinase pathways.[11] Furthermore, Rho is also involved in cell growth and transformation.[12,13] These diverse actions suggest that there are multiple Rho target molecules, each mediating a separate action, and that Rho acts on different combinations of these target molecules in a different context to perform a particular function. Recently, several molecules showing selective binding to the GTP-bound form of Rho have been isolated as putative Rho target molecules or effectors. The examination of the biological functions of these molecules has begun to reveal the biochemical mechanisms underlying the actions of Rho. In this review, we discuss current progress in the structure and function of these target molecules.

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

Rho Effectors and Their Functions in Mammalian Cells

Several groups have used either a yeast two-hybrid system, affinity chromatography on immobilized GTP-Rho or a ligand overlay assay, and have looked for Rho targets by screening for molecules that showed selective binding to GTP-Rho. These studies identified several molecules as putative target molecules or effectors for Rho. They include the p160ROCK/ROK/Rho-kinase family of protein kinases, the PKN/PRK family of protein serine/threonine kinases, two proteins named rhophilin and rhotekin which have a similar Rho-binding motif to PKN but have unidentified activities, and p140mDia which can bind to both GTP-Rho and an actin-binding protein, profilin. In addition, several molecules have been shown to be activated by GTP-Rho in crude membrane or cell lysates, they include phosphatidylinositol (PI) -4-phosphate 5-kinase and phospholipase D. The structure and biological functions of these molecules are described below.

Structure and Function of the p160ROCK/ROK/Rho-Kinase Family of Protein Serine/Threonine Kinases

Ishizaki et al[14] used a ligand overlay assay and purified a GTP-Rho binding protein from platelet cytosol. cDNA cloning for this protein revealed that it was a serine/threonine protein kinase, with a molecular weight of 160 kDa. This kinase was named p160ROCK (Rho-associated coiled-coil containing kinase) and has multiple functional domains, such as a kinase domain in the N-terminus, followed by a long amphipathic α-helix capable of forming a coiled-coil structure, a pleckstrin homology region and a Cys-rich zinc finger in the C-terminus. The N-terminal region of this kinase containing the kinase domain and the coiled-coil forming region show a significant homology to myotonic dystrophy kinase, a product of the causative gene of myotonic dystrophy. An isozyme of p160 ROCK was also isolated as a putative Rho target, and was named either ROKα by Leung et al,[15] Rho kinase by Matsui et al[16] or ROCK-II by Nakagawa et al.[17] In addition, another coiled-coil containing molecule without a kinase domain was isolated by a two-hybrid system using RhoC as bait and was named citron.[18] This molecule has several splicing variants, and a recent study indicated that one of these variants contained a kinase domain with about 60% identity to the ROCK kinases (Madaule P, Narumiya S. unpublished observation). Thus, these molecules appear to constitute a family of kinases. Their structures are shown schematically in Figure 15.1. The Rho-binding domains of these three molecules appear to be localized in the carboxy terminal end of the coiled-coil structure,[15,18,19] suggesting the possibility that the binding of Rho to this region may expose the N-terminal coiled-coil region and the C-terminal lipid binding region for complex formation and membrane attachment, respectively. Indeed, ROKα was translocated to the membrane in COS cells coexpressing the Val^{14}–Rho.[15] In thrombin-activated platelets, part of the endogenous p160ROCK is translocated to the cytoskeleton in an integrin-dependent manner (Fujita A, Narumiya, S. unpublished observation).

The functions of this family of kinases have been examined both in vitro and in vivo in intact cells. Kimura et al[20] found that Rho-kinase was coeluted with the p130 myosin binding subunit of myosin phosphatase from a GTP-Rho affinity column, and that Rho-kinase can phosphorylate this subunit protein. This phosphorylation was then shown to suppress its phosphatase activity against phosphorylated myosin light chain. This finding was consistent with previous studies using a permeabilized smooth muscle preparation, where Rho induced calcium sensitization through the inhibition of myosin light chain phosphatase[21] and where the phosphorylation of the p130 myosin binding subunit of the phosphatase inhibited its activity.[22] Amano et al[23] further showed that Rho-kinase could directly phosphorylate the myosin light chain in vitro, and proposed both the direct phosphorylation of the myosin light chain and the inhibition of myosin phosphatase as the mechanism respon-

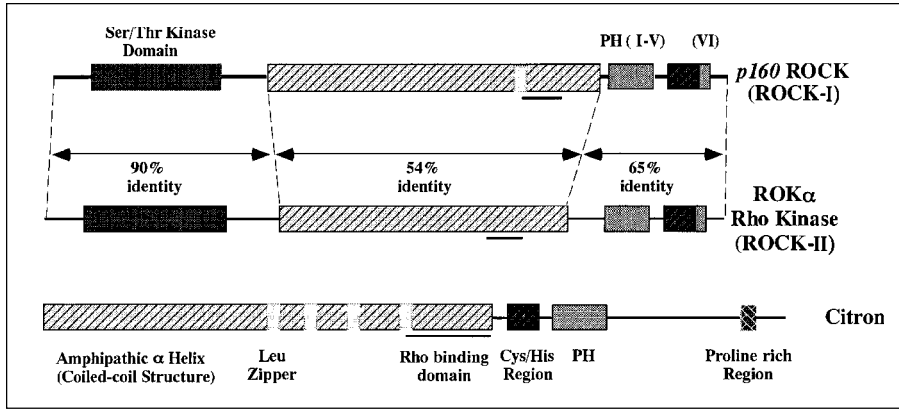


Fig. 15.1. Structures of p160ROCK, ROK/Rho-kinase/ROCK-II and citron.[14-19] Structures of p160ROCK, ROK/Rho-kinase/ROCK-II and a nonkinase variant of citron are shown. All of these proteins contain a long amphipathic α-helix, and the Rho-binding domain shown by an underline is localized at the carboxyl end of this helix. Amino Acid identity is shown in the respective regions of p160ROCK and ROKα/Rho-kinase/ROCK-II.

sible for the Rho-induced enhancement of myosin-based contractility (Fig. 15.2). The same group also showed that Rho-kinase added exogenously restored the calcium sensitization mechanism in a Triton X-100 -extracted smooth muscle preparation by both mechanisms.[24] Whether these two mechanisms work in vivo in intact muscles and how much Rho/ROCK-mediated calcium sensitization contributes to smooth muscle tone under physiological conditions, remain to be elucidated. On the other hand these findings further suggest that the ROCK/ROK/Rho-kinase mediates the contractile responses seen with other Rho actions. Cytokinesis is one of these candidate actions. Rho activity is required for both the induction and maintenance of the contractile ring in cytokinesis, and the actomyosin system mediates contraction of this ring. It is therefore very likely that the ROCK/ROK/Rho-kinase is involved in this process. However, direct proof for the involvement of ROCK/ROK/Rho-kinase in this process has not been provided. Instead, a recent paper suggested another in vitro role for Rho-kinase in cytokinesis by showing that it phosphorylates an intermediate filament protein, glial fibrillary acidic protein (GFAP), at the same sites as seen in the cleavage furrow during cytokinesis.[25] However, this paper did not demonstrate the phosphorylation of GFAP by endogenous Rho-kinase in vivo during cytokinesis. The physiological importance of this phosphorylation is also not known, because GFAP is not ubiquitously distributed in cells other than astroglia. Another candidate for the involvement of a ROCK/Rho-kinase-mediated contractile response is Rho-induced neurite retraction. This action has been extensively studied in cultured neuroblastoma cells in which external stimuli such as thrombin and lysophosphatidic acid applied to the tip of the neurites caused the collapse of the growth cone and retracted the neurites in a Rho-dependent manner.[26] Detailed analysis revealed that this was due to the generation of actomyosin contractility induced by the activated Rho. It is quite likely that ROCK/ROK/Rho-kinase also works as a Rho-effector in this system.

The role of the ROCK/ROK/Rho-kinase in the cell-substrate adhesion became apparent from studies on the expression of these kinases in cultured cells. Leung et al[27] microinjected ROKα cDNA into HeLa cells and then examined the cell morphology. They found that the expression of full-length ROKα and its C-terminal truncation mutants induced

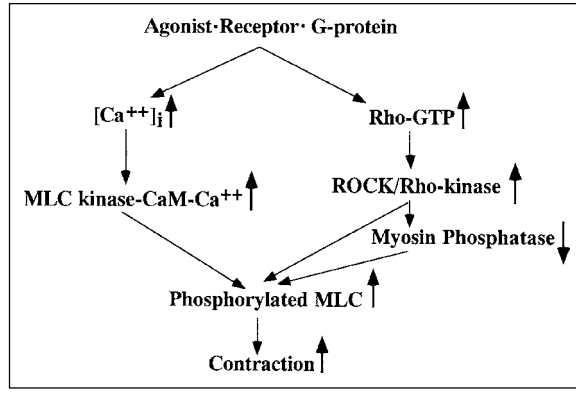


Fig. 15.2. A current model for Rho-induced calcium sensitization mechansm.[20-23] Agonist stimulation mobilizes two pathways to induce contraction in smooth muscle. One is the calcium dependent, Rho-independent pathway, where the elevated free calcium ion in the cell binds to calmodulin (CaM) and activates myosin light chain (MLC) kinase. This activation leads to enhanced phosphorylation of MLC. The other pathway activates Rho, and accumulated GTP-Rho then activates ROCK/Rho-kinase, which then phosphorylates either the myosin binding subunit of myosin phosphatase to inactivate this enzyme or directly phosphorylates MLC.

both focal adhesion-like structures and stress fiber-like actin bundles in these cells, and that this morphological phenotype required kinase activity. Ishizaki et al[28] reported similar morphological changes in HeLa cells transfected with the wild type and various mutants of p160ROCK. Focal adhesion- and stress fiber-like structures became condensed with the C-terminal truncated mutants and the cells became contracted, suggesting that myosin-based contractility was present, and become stronger with these truncations. They also used a kinase-negative, Rho-binding-defective mutant as a potential dominant negative form, and examined its role in the Rho-mediated response. This double mutant blocked the Rho-induced formation of focal adhesions and stress fibers, indicating that p160ROCK indeed functions downstream of Rho. On the other hand, this dominant negative mutant did not suppress Rac-induced membrane ruffling, excluding the conjecture by two groups[29,30] that p160ROCK and ROKα may work downstream of Rac. Amano et al[31] carried out a similar study using several truncation mutants of the Rho-kinase (ROKα) and found that a kinase-defective catalytic domain mutant as well a pleckstrin homology domain mutant could block lysophosphatidic acid-induced stress fiber formation, a finding consistent with other reports.[27,28] It is thus clear that ROCK/ROK/Rho-kinase works downstream of Rho to induce focal adhesions and stress fibers (Fig. 15.3). Since recent studies[7,32] have shown that myosin-based contractility was required for focal adhesion and stress fiber formation and, as described, Rho-kinase is involved in the enhancement of actomyosin contractility, it is likely that this family of kinases induces focal adhesions and stress fibers by the contraction-induced clustering of focal complexes and F-actin bundling. The question is whether ROCK/Rho-kinase is involved in other steps in cell-substrate adhesion. The regulation of the Na^+-H^+ exchanger is of interest in this respect. It has been reported that the activation of this antiporter is mediated by Rho and is essential for stress fiber formation.[33]

Structure and Function of p140mDia

Some of the Rho-mediated processes such as cytokinesis do not simply reorganize pre-existing filamentous actin but may require de novo actin polymerization. Recently, a candidate molecule for this effect has been cloned: p140mDia was isolated from a mouse cDNA library by yeast two hybrid screening.[34] p140mDia has a Rho-bindng domain in the N-terminus, a repeated polyproline stretch in the middle and the so-called FH-2 region in the C-terminus (Fig. 15.4). This molecule shows significant homology to the *Drosophila*

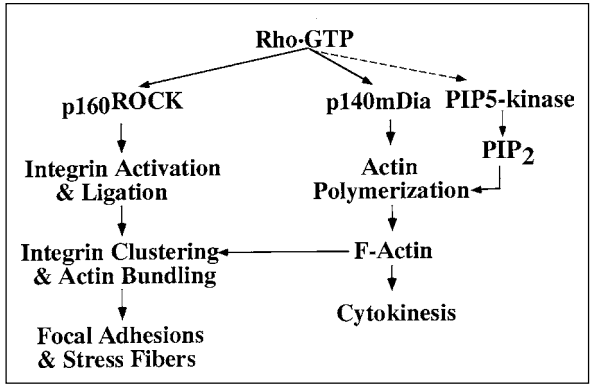


Fig. 15.3. Presumed roles of Rho effectors, ROCK/ROK/Rho-kinase, p140mDia and PIP-5-kinase in reorganization of actin cytoskeleton.

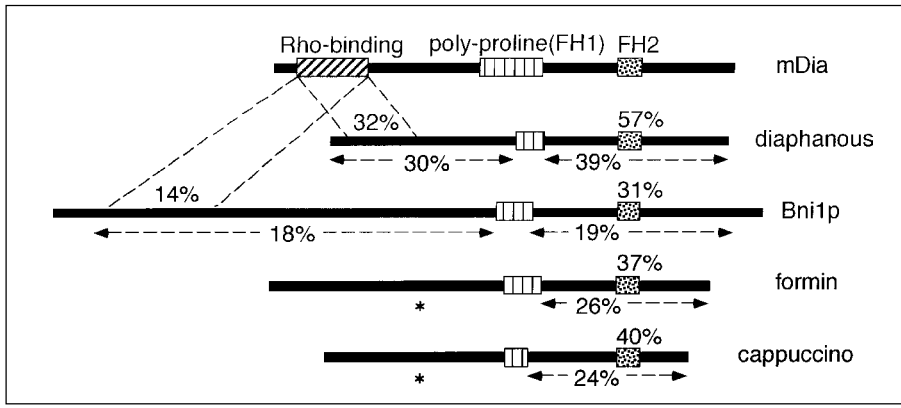


Fig. 15.4. Structures of p140mDia and related proteins.[34-36] Structures of p140mDia and some formin-related proteins are shown. Note that mDia, diaphanous and Bni1p show homology in the N-terminal part containing the Rho-binding domain, whereas this homology is absent in formin and cappucino.

protein diaphanous[35] throughout this entire sequence, and the name mDia (a mammalian homolog of diaphanous) was derived from this. It also shows lower but still significant homology to Bni1p of *Sacchromyces cervisiae*.[36] Interestingly, previous genetic studies[35,36] indicated that Bni1p and diaphanous were involved in yeast budding and cytokinesis, respectively. Bni1p has been identified as a target of yeast Rho1p, and this study will be discussed later below. Watanabe et al[34] found that p140mDia binds to both GTP-Rho and to an actin binding protein, profilin in vitro, and that these three proteins are colocalized in vivo in the membrane lamellae of spreading cells. Rho, profilin and p140mDia are also recruited together to phagocytic cups by fibronectin-coated beads in a Rho-dependent manner. Watanabe et al also found that the transient expression of p140mDia induced homogeneous actin filament formation in COS cells. They suggested that p140mDia caused the accumulation of profilin in the membrane, which in turn stimulates actin polymerization. Profilin is known as an actin monomer binding protein and is thought to function to sequester unpolymerized actin. However, recent studies have indicated that it promotes actin polymerization in vitro and in vivo.[37,38] Interestingly, some immunoreactive p140mDia was observed in the cleavage furrow of dividing cells, indicating that this protein also functions

during contractile ring formation. This is consistent with the findings that its yeast homologs, Bni1p and Bnr1p of *S. cerevisiae* and cdc12p of *S. pombe*, are involved in budding and cytokinesis, and thus are concentrated in the division sites (see below).

PKN/PRK and Molecules with a PKN/Rhophilin Rho-Binding Motif

Watanabe et al[39] used a yeast two-hybrid screening system combined with a ligand overlay assay with [^{35}S]GTPγS-Rho. They identified the serine/threonine protein kinase PKN, and rhophilin, a molecule containing a Rho-binding sequence homologous to PKN, as putative targets for Rho (Fig. 15.5). The identification of PKN as a putative Rho target has also been reported by Amano et al.[40] PKN[41] or PRK[42] is a serine/threonine kinase activated by the addition of lipids such as unsaturated fatty acids, and has a kinase domain highly homologous to that of protein kinase C. Although PKN activity is stimulated by the binding of Rho to its N-terminal regulatory domain and PKN is activated downstream of Rho in intact cells, the role of this kinase in Rho signaling remains obscure. Mukai et al[43] carried out a yeast two-hybrid screening for other proteins interacting with this kinase, and found that the subunits of neurofilament (L, M and H proteins) can bind PKN. PKN phosphorylates these subunits proteins, and this phosphorylation inhibited the in vitro polymerization of the neurofilaments. Using the same strategy, they also found that an actin-binding protein, α-actinin, binds to the N-terminal region of PKN, and that this binding was stimulated by the presence of phosphatidylinositol-4,5-bisphosphate.[44] Although these results indicate a role for PKN in the reorganization of the intermediate as well as the actin cytoskeleton, the relationship of these findings to the actions of Rho remains to be shown. These authors also reported that PKN was translocated from the cytosol to the nucleus following various stresses such as heat, sodium arsenite and serum starvation.[45] They suggested that this translocation was involved in mediating stress signals to the nucleus. However, the role of Rho in this process once again remains to be shown. PKN/PRK has a kinase domain homologous to protein kinase C, and Rho1p of the yeast *S. cerevisiae* acts on Pkc1p, a yeast homolog of protein kinase C, as a direct target to initiate a kinase cascade (see below). Therefore, PKN was expected to mobilize an analogous kinase cascade in mammalian cells for nuclear signaling. However, evidence for this action is still lacking; only a 2-fold increase in SRE activation was found with the expression of PRK2 in control as well as in activated Rho-expressing NIH3T3 cells.[46]

Both PKN and rhophilin have a homologous Rho-binding motif of about 70 amino acids in length in their N-terminal portion. This motif is also shared by another molecule called rhotekin[47] (Fig. 15.5). Notably, there appear to be no domains with catalytic activity in rhophilin or rhotekin. Instead, rhophilin has a domain homologous to the N-terminal portion of *C. elegans* YNK-1[39] and Bro1p of *S. cerevisiae*[48] in its middle (aa 150-450), and a PDZ domain in the C-terminal third (aa 496-607). Rhotekin has a putative SH3 binding motif near the C-terminus. These results indicate that these two proteins function as adaptor proteins linking GTP-Rho to other signaling molecules or structural proteins.

Phosphatidylinositol-4-Phosphate 5-Kinase and Phospholipase D

Chong et al[49] demonstrated that GTPγS-loaded Rho added to lysates of C3H fibroblasts increased the activity of phosphatidyl-4-phosphate (PIP) -5-kinase. This is particularly interesting, since the product formed by this reaction, PIP_2, binds to a variety of F-actin capping proteins that have capped the barbed end of actin filament and dissociates them from this end, leading to uncapping and induction of actin polymerization.[50] Indeed, the overexpression of a PIP-5-kinase produced a generalized actin polymerization in COS-7 cells.[51] Thus, the activation of profilin by PIP-5-kinase, together with its recruitment by p140mDia, is expected to induce focal actin polymerization at a site directed by activated

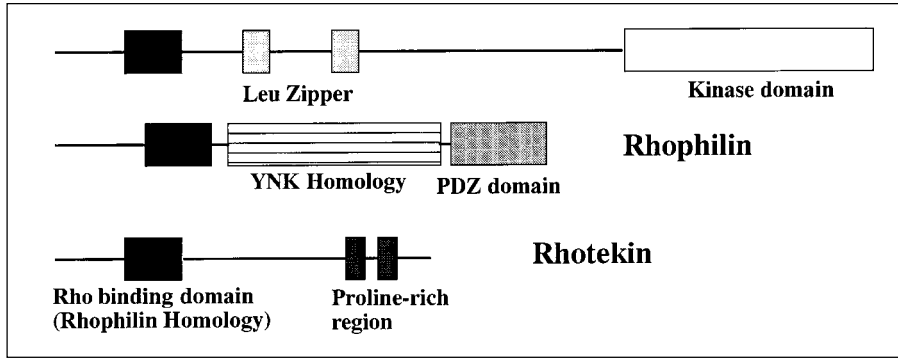


Fig. 15.5. Structures of PKN, rhophilin and rhotekin. The three proteins contain the conserved Rho-binding motif in their N-terminal end.

Rho in the cell. The physical association of PIP-5-kinase with Rho was examined by incubating cell lysates with immobilized Rho protein.[52] A 68-kDa protein with PIP-5-kinase activity was precipitated with immobilized Rho, and this protein was immunostained with an anti-type I PIP-5-kinase antibody. This antibody also coimmunoprecipitated PIP-5-kinase with Rho. However, this binding was independent of whether Rho was in its GDP- or GTP-bound form. Surprisingly, ADP-ribosylation of Rho enhanced this binding by about 8-fold. Thus, the binding of PIP-5-kinase to Rho may not necessarily lead to its activation, and it may require complex formation with another component for full activity This may be also true for Rac, because Rac was shown to bind to PIP-5-kinase both in vitro and in vivo without GTP-dependency.[53] Nevertheless, using permeabilized platelets, Hartwig et al[54] found that activated Rac mediated thrombin-induced phosphoinositide formation, which then facilitated the uncapping of actin barbed ends and triggered actin filament elongation.

Recent studies have demonstrated that Rho also activates phospholipase D (PLD) in several systems and this activation constitutes part of the signal transduction of some growth factors. It has been known for some time that PLD activity was enhanced by the addition of GTP and/or cytosolic factors. The examination of these effects revealed the involvement of two small GTPases in this activation: ARF and Rho. The involvement of Rho was verified by the inhibition of activation by Rho-GDI and/or C3 exoenzyme, and the subsequent restoration by adding GTP-Rho in these systems.[55,56] Until now, two types of PLD have been cloned: PLD-1 and PLD-2. PLD-1 has two isoforms, PLD-1a and b, generated by alternative splicing. Both isoforms of PLD-1 can be activated by GTPγS-ARF and to a lesser extent by RhoA,[57] whereas PLD-2 is constitutively active and was not activated in the presence of these GTPases.[58,59] PLD is a signal transducer that functions in the transmembrane signaling to generate second messenger molecules. A recent study showed that different members of the Rho family of GTPases were involved in PLD activation by different growth factors: RhoA in PLD activation by PDGF, lysophosphatidic acid, endothelin or phorbol esters and Rac1 in PLD activation by EGF.[60,61] In addition, Baldassare et al[62] reported that α-thrombin stimulation in IIC9 cells caused the translocation of a part of RhoA to the nuclear envelope, where it stimulated PLD activity.

Rho Effectors and Their Functions in Yeast

There are five *RHO* genes in the yeast *Saccharomyces cerevisiae: RHO1* to *4*[9,63] and *CDC42*.[64,65] Of these genes, *RHO1* is the yeast homolog of the mammalian *rho* genes. *RHO1*

is an essential gene, as its disruption causes death. The involvement of *RHO1* in the budding process was suggested by the finding that a diploid strain containing an activated allele of *RHO1* did not sporulate,[9] and that a strain with a temperature-sensitive mutant of *RHO1* (*rho-104*) showed growth arrest with a phenotype of a tiny bud.[66] Simultaneous nuclear staining and flow cytometry revealed a peak at 4n, suggesting that the nuclear cell cycle was not affected in the *rho-104* mutant. These results indicate that Rho1p regulates cell growth and bud formation in concert with the nuclear cycle. The expression of an epitope-tagged Rho1p and subsequent immunocytochemistry demonstrated that Rho1p was concentrated on the tip of the growing bud and at the site of cytokinesis and that it colocalized with actin microfilaments at these sites.[66] These results suggested that Rho1p exerts a growth controlling function on the cell. Recently, several putative target proteins for Rho1p have been identified, and their functional roles are being examined.

Qadota et al[67] substituted *RHO1* in *S. cerevisiae* with human *rhoA*. This strain then showed conditional lethality, being unable to grow at 37°C due to osmotic fragility at this temperature. Osmotic fragility is usually caused by some defect in the cell wall, indicating that Rho1p was involved in cell wall biosynthesis. Taking the same approach, Nonaka et al[68] screened for dominant mutations that suppressed this phenotype and isolated a mutant of *PKC1*, a yeast protein kinase C homolog as a suppressor. They further showed that Pkc1p interacted directly with GTP-Rho1p in a two-hybrid system. *PKC1* directs the activation of the yeast MAP kinase cascade *MKK1* and *MPK1*, and dominant active mutants of these kinases also suppress the *rhoA* phenotype, indicating that Rho1p was indeed located upstream of this kinase pathway. This is consistent with the osmotic fragility seen in the *rhoA* mutant because *pkc1* null mutants also showed osmotic fragility and are rescued by high osmolarity media.[69] In addition, a genetic interaction has been shown between the *PKC1* pathway and some cell wall synthesizing enzymes.[70] *RHO1* is not only indirectly involved in yeast cell wall synthesis through the PKC1 pathway; it has been recently reported that Rho1p directly regulated the activity of a cell wall synthesizing enzyme.[71,72] One of the main structural components of the yeast cell wall are 1,3-β-linked glucan polymers, which are synthesized by 1,3-glucan synthase encoded by Fks1 and 2 genes. Since it is known that this glucan synthase is activated by GTPγS, Drgonová et al[71] and Qadota et al[72] examined the role of Rho1p in this activation. Both groups found decreased glucan synthases activity in the *rho1* mutants which were insensitive to GTP. This defect was corrected by adding the wild type Rho1p back to the incubation. Qadota et al[70] further showed that Rho1p complexed with the Fsk1 subunit of the enzyme and colocalized at the bud tip where the cell remodels its wall. Thus, *RHO1* exerts two distinct actions in yeast cell wall synthesis: an activation of the PKC1 pathway to induce the glucan synthetases and an association with Fsk1p to activate the enzyme directly. It is quite likely that Rho1p also drives other pathway(s) in yeast, because *rho1* null mutants are not rescued in high osmolarity media such as 1M sorbitol.[66]

This apparent discrepancy appears to have been solved by the recent discovery of new Rho1p target proteins. Kohno et al[73] used a yeast two hybrid system with an active form of Rho1p [Rho1p(Q68L)] as bait and identified Bni1p as an interacting protein. The preferential binding of Bni1p to the GTP-bound form of Rho1p was then demonstrated by the coprecipitation of a MBP fusion protein of Bni1p with GTPγS-bound Rho1p. As described above, Bni1p is a yeast homolog of diaphanous and p140mDia and has been reported to be involved in the control of the budding pattern and cytokinesis.[36] This is consistent with the role of Rho1p in the yeast budding process, and the genetic linkage of *RHO1* and *BNI1* was confirmed by the synthetic lethality of *bni1* mutants with either *pkc1* or the *RhoA* mutation in the place of *RHO1* as described above. This group has also found a homolog of Bni1p in *Saccharomyces cervisiae*, which they termed Bnr1p.[74] An overlap of the functions of these two proteins is suggested by the temperature-sensitive growth phenotype of the *bni1 bnr1*

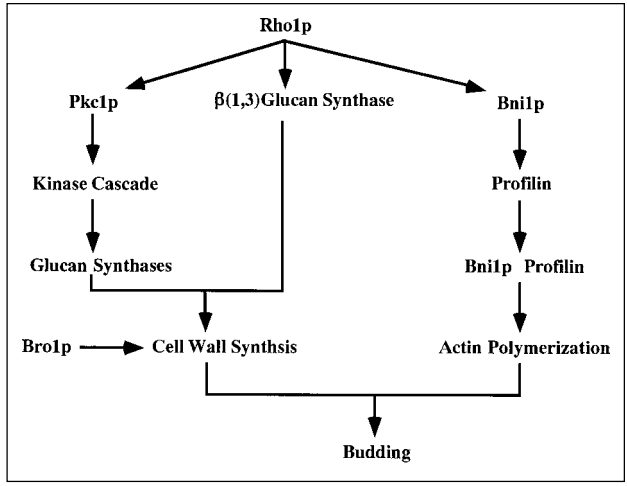


Fig. 15.6. Functions of RHO effectors in the yeast *Saccharomyces cerevisiae.*

mutant. These mutant cells are uniformly enlarged at the restrictive temperature due to a budding deficiency, and have a delocalized pattern of actin and chitin, which are normally localized to the polarized growth sites and bud scar, respectively. As described for p140mDia, both Bni1p and Bnr1p physically interact with profilin, and the bni1 bnr1 mutant showed a similar phenotype as the yeast profilin null pfy mutant, such as sensitivity to 1M sorbitol. Two-hybrid screening with different Rho proteins as baits indicated that Bnr1p interacted preferentially with Rho4p, and that Bni1p interacted with Rho1p. On the other hand, Evangelista et al[75] identified an interaction of Bni1p with CDC42 by a two hybrid system. They further demonstrated the interaction of Bni1p with not only profilin (Pfy1) but also with Bud6p, a protein which is involved in bipolar budding and is capable of interacting with actin. On the basis of these results, they suggested that Bni1p formed a complex with profilin, Bud6p and actin downstream of Cdc42p. Whether Bni1p preferentially complexes with Rho1p or Cdc42p or both in the yeast budding process remains to be determined. In contrast to budding yeast, cell division in the fission yeast *Schizosaccharomyces pombe* is performed by the formation of an actin ring that circumscribes the middle of the cell, followed by the formation of a septal cell wall. *Cdc12* has been identified as one of the genes required for the proper formation of this actin ring. Chang et al[76] recently cloned cdc12, and identified cdc12p as the Bni1p homologue of *S. pombe*. Cdc12p is located specifically in the cell division actin ring, and its mutant *cdc12* exhibits delocalized actin patches during mitosis. *Cdc12* has a synthetic lethal genetic interaction with the cdc3 (profilin) mutant, and a proline-rich region of cdc12p binds directly to profilin cdc3p. Thus, cdc12p shares many properties in common with Bni1p, as well as with *Drosophila* diaphanous and p140mdia. It is also quite likely that it binds selectively to the active form of Rho and is recruited to the site of its action by virtue of this binding.

Thus, in yeast, Rho appears to coordinate cell division and nuclear division in three ways: first by inducing glucan synthesizing enzymes, second by directly activating one of these enzymes and finally by inducing actin polymerization at the site of cell growth (Fig. 15.6).

Perspectives

Significant advances have been made in recent years in the identification of the structure and function of Rho effectors, and the biochemical mechanisms underlying Rho ac-

tions are now becoming clear. In the next few years, the interaction of several Rho effectors will be elucidated to explain the sequence of events in complex Rho actions such as cytokinesis, cell adhesion and cell transformation. Rho has also been reported to be involved in processes such as bacteria entry into cells[77,78] and the regulation of tight junction.[79] These actions will be described in molecular terms in the coming years. The physiological significance of Rho itself and Rho effectors in the body will also be clarified by the gene disruption or pharmacological manipulation of each molecule. Through these studies, unexpected Rho actions will likely be discovered. One remaining question is how Rho actions are regulated temporarily and spatially in the cell. We have been investigating whether the activated GTP-bound form of Rho is targeted from the cytoplasm to its action site at the plasma membrane. However, various screening experiments with GTP-Rho as the bait have not identified any candidate proteins for targeting and anchoring in the membrane. It may be that the activation of a Rho exchange protein occurs at the site of cell stimulation, and this then recruits Rho and converts it to the active GTP-form, followed by the accumulation of effector molecules at this site. This point will be another focus for future Rho research.

References

1. Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992; 70:389-399.
2. Morii N, Teru-uchi T, Tominaga T et al. A *rho* gene product in human blood platelets. II. Effects of the ADP-ribosylation by botulinum C3 ADP-ribosyltransferase on platelet aggregation. J Biol Chem 1992; 267:20921-20926.
3. Tominaga T, Sugie K, Hirata M et al. Inhibition of PMA-induced, LFA-1-dependent lymphocyte aggregation by ADP-ribosylation of the small molecular weight GTP-binding protien, *rho*. J Cell Biol 1993; 120:1529-1537.
4. Kishi K, Sasaki T, Kuroda S et al. Regulation of cytoplasmic division of Xenopus embryo by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI). J Cell Biol 1993; 120:1187-1195.
5. Mabuchi I, Hamaguchi Y, Fujimoto H et al. A rho-like protein is involved in the organisation of the contractile ring in dividing sand dollar eggs. Zygote 1993; 1:325-331.
6. Yamada KM, Miyamoto S. Integrin transmembrane signaling and cytoskeletal control. Cur Opin Cell Biol 1995; 7:681-689.
7. Burridge K, Chrzanowska-Wodnicka M. Focal adhesions, contractility, and signaling. Annu Rev Cell Dev Biol 1996; 12:463-519.
8. Fishkind DJ, Wang Y-L. New horizons for cytokinesis. Cur Opin Cell Biol 1995; 7:23-31.
9. Madaule P, Axel R, Meyers AM Characterization of two members of the rho gene family from the yeast *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 1987; 84:779-783.
10. Hirata K, Kikuchi A, Sasaki T et al. Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. J Biol Chem 1992; 267:8719-8722.
11. Hill CS, Wynne J, Treisman R. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell 1995; 81:1159-1170.
12. Khosravi-Far R, Solski PA, Clark GJ, Kinch MS, Der CJ. Activation of Rac1 and RhoA, and mitogen-activated protein kinases, is required for Ras transformation. Mol Cell Biol 1995; 15:6443-6453.
13. Qiu R-G, Chen J, McCormick F, SYmons M. A role for Rho in Ras transformation. Proc Natl Acad Sci USA 1995; 92:11781-11785.
14. Ishizaki T, Maekawa M, Fujisawa K et al. The small GTP-binding protein Rho binds to and activates a 160 lDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J 1996; 15:1885-1893.
15. Leung T, Manser E, Tan L et al. A novel serine/threonine kinase binding the Ras-relaated RhoA GTPase which translocates the kinase to peripheral membranes. J Biol Chem 1995; 270:29051-29054.

16. Matsui T, Amano M, Yamamoto T et al. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for the small GTP binding protein Rho. EMBO J 1996; 15:2208-2216.
17. Nakagawa O, Fujisawa K, Ishizaki T et al. ROCK-I and ROCK-II; two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. FEBS Lett 1996; 92:189-193.
18. Madaule P, Furuyashiki T, Reid T et al. A novel partner for the GTP-bound forms of rho and rac. FEBS Lett 1995; 377:243-248.
19. Fujisawa K, Fujita A, Ishizaki T et al. Identification of the Rho binding domain of p160ROCK, a Rho-associated coiled coil containing protein kinase. J Biol Chem 1996; 271:23022-23028.
20. Kimura K, Ito M, Amano M et al. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 1996; 273:245-248.
21. Noda M, Yasuda-Fukazawa C, Moriishi K et al. Involvement of rho in GTPgS-induced enhancement of phosphorylation of 20 kDa myosin light chain in vascular smooth muscle cells: inhibition of phosphatase activity. FEBS Lett 1995; 367:246-250.
22. Trinkle-Mulcahy L, Ichikawa K, Hartshorne DJ et al. Thiophosphorylation of the 130 kDa subunit is associated with a decreased activity of myosin light chain phosphatase in a-toxin-permeabilized smooth muscle. J Biol Chem 1995; 270:18191-18194.
23. Amano M, Ito M, Kimura K et al. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 1996; 271:20246-20249.
24. Kureishi Y, Kobayashi S, Amano M et al. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. J Biol Chem 1997; 272:12257-12260.
25. Kosako H, Amano M, Yanagida M et al. Phosphorylation of glial fibrillary acidic protein at the same sites by cleavege furrow kinase and Rho-associated kinase. J Biol Chem 1997; 272:10333-10336.
26. Jalink K, van Corven EJ, Hengeveld T et al. Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP-ribosylation of the small GTP-binding protein Rho. J Cell Biol 1994; 126:801-810.
27. Leung T, Chen X-Q, Manser E et al. The p160 RhoA-binding kinase ROKα is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol Cell Biol 1996; 16:5313-5327.
28. Ishizaki T, Naito M, Fujisawa K et al. p160ROCK, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. FEBS Lett 1997; 404:118-124.
29. Lamarche N, Tapon N, Stowers L et al. Rac and CDC42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JUN/SAPK MAP kinase cascade. Cell 1996; 87:519-529.
30. Joneson T, McDonough M, Bar-Sagi D et al. RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase. Science 1996; 274:1374-1376.
31. Amano M, Chihara K, Kimura K et al. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 1997; 275:1308-1311.
32. Chrzanowska-Wodnicka M, Burridge K. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J Cell Biol 1996; 133:1403-1415.
33. Vexler ZS, Symons M, Barber DL. Activation of Na^+-H^+ exchange is necessary for RhoA-induced stress fiber formation. J Biol Chem 1996; 271:22281-22284.
34. Watanabe N, Madaule P, Reid T et al. p140mDia, a mammalian homolog of Drosophila diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. EMBO J 1997; 16:3044-3056.
35. Castrillon DH, Wasserman SA. Diaphanous is required for cytokinesis in Drosophila and shares domains of similarity with the products of the limb deformity gene. Development, 1994;120:3367-3377.
36. Zahner JE, Harkins HA, Pringle JR. Genetic analysis of the bipolar pattern of bud site selection in the yeast Saccharomyces cerevisiae. Mol Cell Biol 1996; 16:1857-1870.

37. Pantaloni D, Carlier M-F. How profilin promotes actin filament assembly in the resence of thymosin b4. Cell 1993; 75:1007-1014.
38. Cao L-G, Babcock GG, Rubenstein PA et al. Effects of profilin and profilactin on actin structure and function of living cells. J Cell Biol 1992; 117:1023-1029.
39. Watanabe G, Saito Y, Madaule P et al. Protein kinase-N (PKN) and PKN-related protein rhophilin as targets of small GTPase Rho. Science 1996; 271:645-648.
40. Amano M., Mukai H., Ono Y et al. Identification of a putative target for Rho as the serine-threonine kinase protein kinase N. Science 1996; 271:648-650.
41. Mukai H, Ono Y. A novel protein kinase with leucine zipper-like sequences: its catalytic domain is highly homologous to that of protein kinase C. Biochem Biophys Res Commun 1994; 199:897-904.
42. Palmer RH, Ridden J, Parker PJ. Cloning and expression patterns of two members of a novel protein-kinase-C-related kinase family. Eur J Biochem 1995; 227:344-351.
43. Mukai H, Toshimori M, Shibata H et al. PKN associates and phosphorylates the head-rod domain of neurofilament protein. J Biol Chem 1996; 271:9816-9822.
44. Mukai H, Toshimori M, Shibata H et al. Interaction of PKN with α-actinin. J Biol Chem 1997; 272:4740-4746.
45. Mukai H, Miyahara M, Sunakawa H et al. Translocation of PKN from the cytosol to the nucleus induced by stresses. Proc Natl Acd Sci USA 1996; 93:10195-10199.
46. Quilliam LA, Lambert QT, Mickelson-Young LA et al. Isolation of a NCK-associated kinase, PRK2, an SH3-binding protein an protential effector of Rho protein signaling. J Biol Chem 1996; 271:28772-28776.
47. Reid T, Furuyashiki T, Ishizaki T et al. Rhotekin, a new putative target for Rho bearing homology to a serine/threonine kinase, PKN, and rhophilin in the Rho-binding domain. J Biol Chem 1996; 271:23022-23028.
48. Nickas ME, Yaffe MP. BRO1, a novel gene that interacts with components of the Pkc1p-mitogen-activated protein kinse pathway in Saccharomyces cerevisiae. Mol Cell Biol 1996; 16:2585-2593.
49. Chong LD, Traynor-Kaplan A, Bokoch GM et al. The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. Cell 1994; 79:507-513.
50. Janmey PA. Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. Ann Rev Physiol 1994; 56:169-191.
51. Shibasaki Y, Ishihara H, Kizuki N et al. Massive actin polymerization induced by phosphatydylinositol-4-phosphate 5-kinase in vivo. J Biol Chem 1997; 272:7578-7581.
52. Ren X-D, Bokoch GM, Traynor-Kaplan A et al. Physical association of the small GTPase Rho with a 68-kDa phosphatidylinositol 4-phosphate 5-kinase in Swiss 3T3 cells. Mol Biol Cell 1996; 7:435-442.
54. Hartwig J H, Bokoch GM, Carpenter CL et al. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell 1995; 82:643-653.
53. Tolias KF, Cantley LC, Carpenter CL. Rho family GTPases bind to phosphoinositide kinases. J Biol Chem 1995; 270:17656-17659.
55. Malcom KC, Ross AH, Qiu R-G et al. Activation of rat liver phospholipase D by the small GTP-binding protein RhoA. J Biol Chem 1994; 269:25951-25954.
56. Kuribara H, Tago K, Yokozeki T et al. Synergistic activation of rat brain phospholipase D by ADP-ribosylation factor and rhoA p21, and its inhibition by *Clostridium botulinum* C3 exoenzyme. J Biol Chem 1995; 270:25667-25671.
57. Hammond SM, Jenco JM, Nakashima S et al. Characterization of two alternatively spliced forms of phospholipase D1. J Biol Chem 1997; 272:3860-3868.
58. Colley WC, Sung, T-C, Roll R et al. Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. Cur Biol 1997; 7:191-201.
59. Kodaki T, Yamashita S. Cloning, expression and characterization of a novel phospholipase D complementary DNA from rat brain. J Biol Chem 1997; 272:11408-11413.

60. Malcom KC, Elliot CM, Exton JH. Evidence for Rho-mediated agonist stimulation of phospholipase D in Rat1 fobroblasts. J Biol Chem 1996; 271:13135-13139.
61. Hess JA, Ross AH, Qui R-G et al. Role odf Rho family proteins in phospholipase D activation by growth factors. J Biol Chem 1997; 272:1615-1620.
62. Baldassare JJ, Jarpe MB, Alferes L et al. Nuclear translocation of RhoA mediates the mitogen-induced activation of phospholipase D involved in nuclear envelope signal transduction. J Biol Chem 1997; 272:4911-4914.
63. Matsui Y, Toh-e A. Isolation and characterization of two novel ras superfamily genes in *Saccharomyces cerevisiae*. Gene 1992; 114:43-49.
64. Adams AE, Johnson DL, Longnecker RM et al. *CDC42* and *CDC43*, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. J Cell Biol 1990; 111:131-142.
65. Johnson DI, Pringle JR. Molecular characterization of *CDC42*, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. J Cell Biol 1990; 111:143-152.
66. Yamochi W, Tanaka K, Nonaka H et al. Growth site localization of Rho1p small GTP-binding protein and its involvement in bud formation in *Saccharomyces cervisiae*. J Cell Biol 1994; 125:213-224.
67. Qadota H, Anraku Y, Botstein D et al. Conditional lethality of a yeast strain expressing human RHOA in place of RHO1. Proc Natl Acad Sci USA 1994; 91:9317-9321.
68. Nonaka H, Tanaka K, Hirano H et al. A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cervisiae*. EMBO J 1995; 14:5931-5938.
69. Levin DE, Bartlettheubusch E. Mutants in the *S. cerevisiae PKC1* gene dispaly a cell cycle-specific osmotic stability defect. J Cell Biol 1992; 116:1221-1229.
70. Roemer T, Paravicini G, Payton MA, Bussey H. Characterization of the yeast (1->6)-β-gluca biosynthetic components, Kre6p and Skn1p, and genetic interactions between the PKC1 pathway and extracellular matrix assembly. J Cell Biol 1994; 127:567-579.
71. Drgonová J, Drgon T, Tanaka K et al. Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. Science 1996; 272:277-279.
72. Qadota H, Python CP, Inoue SB et al. Identification of yeast Rho1 GTPase as a regulatory subunit of 1,3-β-glucan synthase. Science 1996; 272:279-281.
73. Kohno H, Tanaka K, Mino A et al. Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in Saccharomyces cerevisiae. EMBO J 1996; 15:6060-6068.
74. Imamura H, Tanaka K, Hihara T et al. Bni1p and Bnr1p: downstream targets of the rho family small G-proteins which interact with profilin and regulate actin cytoskeleton in Saccharomyces cerevisiae. EMBO J 1997; 16:2745-2755.
75. Evangelista M, Blundell K, Longtine MS et al. Bni1p, a yeast formin linking Cdc42p and the actin cytoskeleton during polarized morphogenesis. Science 1997; 276:118-122.
76. Chang F, Drubin D, Nurse P. cdc12p, a protein required for cytokinesis in fission yeast, is a component for the cell division ring and interacts with profilin. J Cell Biol 1997; 137:169-182.
77. Adam T, Giry M, Boquet P et al. Rho-dependent membrane folding causes Shigella entry into epithelial cells. EMBO J 1996; 15:3315-3321.
78. Watarai M, Kamata Y, Kozaki S et al. rho, a small GTP-binding protein, is essential for Shigella invasion of epithelial cells. J Exp Med 1997; 185:281-292.
79. Nusrat A, Giry M, Turner JR et al. Rho protein regulates tight junctions and perijunctional actin organization in polarized epitelia. Proc Natl Acad Sci USA 1995; 92:10629-10633.

CHAPTER 16

Rac/CDC42 Effectors: ACK, PAK, MRCK, WASP, n-Chimaerin, POR1, Etc.

Sohail Ahmed, Robert Kozma and Louis Lim

Introduction

The Ras/Rho family of GTPases of 21 kDa act as molecular switches controlling a variety of cellular processes (for reviews see refs. 1-3). Most Ras/Rho family members are conserved from yeast to man. There is also isoform diversity and tissue specificity within closely related members of each family. For example, Rac1 is ubiquitous, while Rac2 is only expressed in the immune system and Rac3 is enriched in brain. Ras/Rho GTPases are characterized by the presence at the extreme C-terminal end of an isoprenyl group. This post-translational modification occurs on the cysteine of a CAAX box and gives rise to proteins that associate strongly with the plasma membrane, an association considered to be essential for biological function. These G proteins possess intrinsic GTPase and GDP/GTP exchange activities which allow them to cycle between two conformational states, "on" (GTP-bound) and "off" (GDP-bound). Comparison of the structural features that distinguish the Rho family from the Ras family reveals the presence of an extended loop 8 (extra 14 amino acid residues) in Rho family which provides an additional binding surface for protein-protein interactions.

The use of two mutations of these GTPases in cellular assays has been instrumental in defining their function. The oncogenic Ras mutations, V12 and L61, lead to GTPase-negative forms frozen in the conformational "on" state (GTP-bound) while the dominant negative N17 mutant inhibits cell signaling, possibly by titrating out the GDP dissociation stimulators (GDSs). Using these mutants a clear link has been made between Ras/Rho family members (Rac1, CDC42 and RhoA), the actin cytoskeleton and cell transformation. Rac1, CDC42 and RhoA act downstream of oncogenic Ras in distinct steps of the transformation process, CDC42 and RhoA in morphological transformation, Rac1 in serum-independent growth and all three in anchorage independent growth (chapter 12). Rac1, CDC42 and RhoA also operate immediately downstream of distinct membrane bound receptors to coordinate cellular processes such as changes in the F-actin cytoskeleton, cytokinesis, neutrophil NADPH oxidase and "focal complex" (FC) assembly, Jun kinase (JNK) activity and cell-cell adhesion (Fig. 16.1; chapter 14).

A multitude of factors that regulate the nucleotide state of Rac1, CDC42 and RhoA have been identified over the last five years; these include proteins that stimulate either intrinsic nucleotide GDP/GTP exchange (e.g., DBL) or GTPase activity (e.g., BCR). Three

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

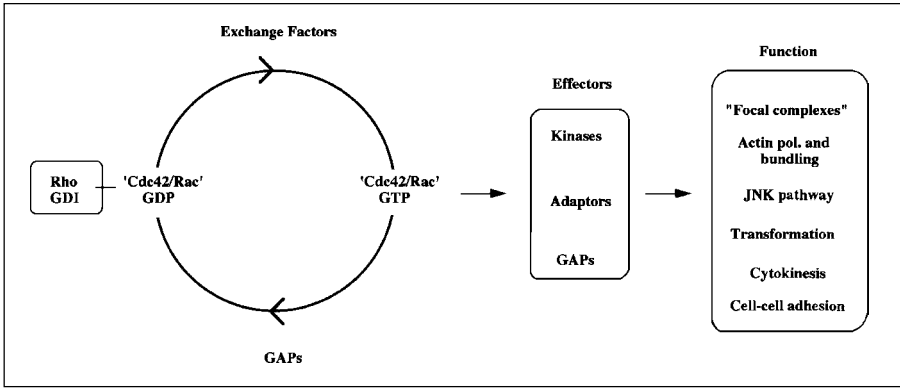


Fig. 16.1. CDC42 and Rac1 GTPase cycle, effectors and functions. CDC42/Rac GTPases cycle between 'on' and 'off' conformations through GTP and GDP binding. The cycle is regulated by exchange factors (GDSs), GTPase activating proteins (GAPs) and GDP dissociation inhibitors (GDIs). The interaction of Rac/CDC42 with effectors induces specific changes in cell function. Effectors full into three distinct classes; kinases, adaptors and GAPs.

distinct isoforms of Rho-GDI that inhibit GDP dissociation and mask the isoprenyl tail of Rho family members (maintaining a cytosolic pool of protein) have been isolated. Sequence analysis of these regulatory factors has revealed that they are multidomain proteins and exchange factors in particular have a complex domain structure and are commonly found with pleckstrin homology (PH) domains (chapter 11). Proteins such as ABR and BCR are of special interest as they consist of domains that confer both GDP/GTP exchange and GTPase stimulating activity. The GTPase specificity of these regulatory proteins is overlapping in vitro.

Effectors can be defined as proteins that are activated by the GTP-bound "on" state of the corresponding GTPases. However, situations can be envisaged in which a cooperative interaction between GTPase and effector occurs, in which case the GTPase is absolutely required for effector function and acts at the same level as the effector. Suppressor analysis in genetically manipulatable systems such as *Caenorhabditis elegans* and *Drosophila melanogaster* was probably the first method used to identify protein effectors in cell signaling pathways. However, this genetic analysis is not possible in mammalian systems. Effector isolation and identification in mammalian systems has been made possible by the establishment of three powerful protein-protein interaction assays: (i) Probing of nitrocellulose immobilized target proteins with labeled protein (the "Far-Western" analysis) combined with column protein purification; (ii) affinity purification using tagged recombinant protein with cell material applied to affinity columns (e.g., GST tag with glutathione agarose columns); and (iii) the "yeast two-hybrid" system in which a bait protein is assessed for its ability to interact with a library of cloned and expressed cDNAs. In the GAL4 two-hybrid system yeast cells grow on histidine only if a bait-library interaction occurs.[4]

Once protein partners have been isolated the binding sites can be identified by deletion analysis and database searches used to identify novel partners using motifs generated from known interacting sites. Rho family effectors isolated using the techniques described above can be placed into three distinct classes; kinases, adaptors and GAPs (Fig. 16.1). The review by Narumiya concerns RhoA effectors while ours will focus on CDC42 and Rac effectors.

ACK

ACK, the activated CDC42-binding kinase, a nonreceptor tyrosine kinase member of the focal adhesion kinase (FAK) family of proteins was cloned using Far Western analysis of

a lambda hippocampal expression library with $Cdc42^{G12V}$ as a probe. ACK was one of the first kinases identified that interacts specifically with a Rho family GTPase.[5] ACK is related to the tyrosine kinase 2 (PYK2, also known as RAFTK or CAK-β or CADTK) and both contain several proline rich C-terminal sequences. Unlike PYK2, ACK contains a SH3 domain and a CDC42-binding domain (CBD). Although ACK was one of the first Cdc42Hs effectors to be isolated, very little information has been published on its cellular function. However, heat shock and changes in osmolarity have been shown to cause its phosphorylation. Thus ACK is presumably activated by cell stress. ACK has been shown to associate with GRB2.[6]

More information is available on its cousin PYK2 which is thought to play an important role in cell signaling pathways in the brain. Activation of G protein-coupled receptors with agents such as acetylcholine or bradykinin results in calcium mobilization and phosphorylation of PYK2, recruitment of the Ras GDS SOS1 via GRB2, and activation of Ras-dependent pathways. Thus PYK2 may form a link between G protein-coupled receptors and subsequent ERK kinase pathway activation.[7] Activation of PYK2 has been reported to require an intact actin cytoskeleton. PYK2 may also be a component of FCs as it binds via its C-terminal polyproline-rich region to paxillin.[8] A dominant negative mutant of PYK2 inhibits phosphorylation of paxillin. PYK2 is also a target of the FYN tyrosine kinase following T cell antigen receptor activation[9] and is activated following β1-integrin stimulation, or B cell antigen receptor activation,[10] as well as by neuronal membrane depolarization.[11] Interestingly, PYK2 overexpression leads to JNK activation, while a dominant negative mutant interfered with JNK activation induced by either UV light or osmotic shock.[12] These results implicate PYK2 as a kinase associated with FCs and the actin cytoskeleton that mediates the activation of MAP kinase cascades downstream of G-protein linked receptors. Whether ACK plays an analogous role is worthy of investigation. The presence in ACK of a CBD motif may suggest that CDC42 is involved in its recruitment to receptor complexes.

PAK

Far Western analysis with CDC42[γ^{32}P]GTP and Rac1[γ^{32}P]GTP as probes of tissue extracts identified a number of interacting proteins.[13] Signals present at 62, 65 and 68 kDa from brain extracts in these overlay assays were due to a family of kinases.[14] Since the kinase activity of the purified 65 kDa protein was activated by CDC42 and Rac1 these brain proteins were named PAKs for p21(CDC42/Rac)-activated kinases. Interestingly, in both CDC42/Rac binding and kinase assays CDC42 was much more potent than Rac1. Peptide sequencing followed by cDNA isolation and characterization revealed that mammalian PAK was homologous to Ste20p, a yeast kinase involved in MAP kinase-like cascades. Preceding the identification of PAK in brain extracts Ste20p had been shown by genetic analysis to be a protein required for yeast budding and mating (i.e., involved in morphological and transcriptional pathways, respectively). The isolation of PAK as a CDC42-binding protein linked *STE20* to *CDC42* pathways in yeast and this has now been confirmed experimentally.[15,16]

Further cloning and sequence analysis has revealed the presence in mammalian cells of at least three isoforms of PAK, α, β and γ, with the latter being ubiquitously expressed.[17-20] Unlike α and γPAK, βPAK once activated (autophosphorylated) no longer binds CDC42 or Rac1.[18,19] As with any other protein family, more PAK members, with different expression patterns, localization characteristics and protein domains, are likely to be identified in future analysis. In fact, the EST database already contains many PAK-like sequences. Neutrophils and macrophages are interesting cell-types since they express all three PAK isoforms at relatively high levels[19,21] (Ahmed, S et al, unpublished data). Proteins that have similarity to the Ste20p/PAK kinase domain alone, the GCK family of kinases, have been identified and are thought to have roles in JNK pathways and in membrane trafficking.[22] PAKs from *C. elegans*[23] and *D. melanogaster*[24] and additional members of the yeast Ste20p family have

been identified. Unique to the yeast PAK like proteins, PAK2, PAKp and Cla4p, is the presence of a PH domain.[22] This rapid explosion in identification and cloning of members of the Ste20p/PAK family of kinases over the last few years opens up the possibility of elucidating their physiological function in the near future.

We define here PAK family proteins as those that contain a CBD in addition to the kinase domain of the STE20 family. A notable feature of the mammalian PAK isoforms are stretches of polyproline flanking the CBD. For example, α and βPAK possess four such polyproline repeats, P1 (1-20), P2 (30-50), P3 (140-160) and P4 (180-200). Intriguingly, γPAK and *Drosophila* PAK do not possess P3 and the yeast proteins Ste20p, Cla4p and Sc-PAK do not have any of these polyproline motifs, although they do have polyproline repeats. If it is assumed that the polyproline motifs P1-P4 are each sites of protein-protein interactions, these N-terminal sequence differences already suggest specific functions for the different PAKs. An additional feature of PAKs is that there is a sequence of about 30 amino acids immediately adjacent to the CBD which is conserved among Ste20p/PAKs from yeast to man but is absent from other CDC42 effectors including ACK and WASP. This sequence in mammalian cells acts as a negative regulatory domain of the kinase (Zhao, ZS et al, unpublished data). The kinase MLK-3 which was identified initially as a protein possessing a CRIB motif (an 18 amino acid sequence present in the CBD) through a database search has a different domain organization compared to PAK.[25] Its kinase domain is in the middle of the protein and the CRIB motif is C-terminal to the kinase domain. Interestingly, MLK-3 has some identity to the polyproline motif P4 near its extreme C-terminal end but does not possess P1-3.

The first (non-GTPase) binding partner reported for PAK was the adaptor protein NCK, a protein that also interacts with WASP[26] (see below). NCK consists of one SH2 domain and three SH3 domains. It is the second SH3 domain of NCK that binds PAK. Further, the second SH3 domain binds the P1 polyproline sequence of PAK and not P2-P4. Therefore NCK will complex with all three PAK isoforms since they all possess P1. PAK phosphorylates NCK although the consequence of this is not clear. More recently, PAK has been found to interact with a number of PAK substrates of molecular sizes between 78-90 kDa through immunoprecipitation experiments. One of these has been cloned, termed PIX (PAK-interacting exchange factor) containing a DBL domain and which catalyses GDP/GTP exchange on Rho family members (Manser E et al, unpublished data). The PIX-PAK interaction occurs via a SH3 domain of PIX and a sequence that includes the polyproline motif P4 of PAK. Other kinase substrates of PAK include two oxidase components p47phox and p67phox (see later section), myosin I heavy chain, myosin II light chain (Fig. 16.2) and PIX. However, the in vivo targets of PAKs have not been established.

The PAK family member we have most information about is Ste20p. As mentioned earlier, this protein is involved in both morphology pathways and MAP kinase-like cascades. Intriguingly, CDC42 binding appears to be essential for localization and morphological activity but not for kinase activation. A CDC42 binding-defective Ste20p can complement a *STE20* mutant as part of the MAP kinase cascade but not in the process of budding.[27,28] PAK is also able to complement *STE20* mutants suggesting overlap in function of these proteins.[16] A *C. albicans* protein related to Ste20p has been identified, Cst20p, that plays a role in mycelial growth and this may have relevance to the pathogenicity of this organism.[29] Interestingly, recent work has shown that mutations in CaCla4p, the homolog of the yeast Cla4p, cause defects in hyphal formation in vitro and gene deletion suppressed *C. albicans* virulence in a mouse model.[30]

The demonstration that both Rac1 and CDC42 induce the activation of JNK and that the T17N (dominant negative) mutants of these GTPases block factor induced activation of JNK/SAPK suggest an important role for these proteins in MAP kinase pathways.[31,32] To-

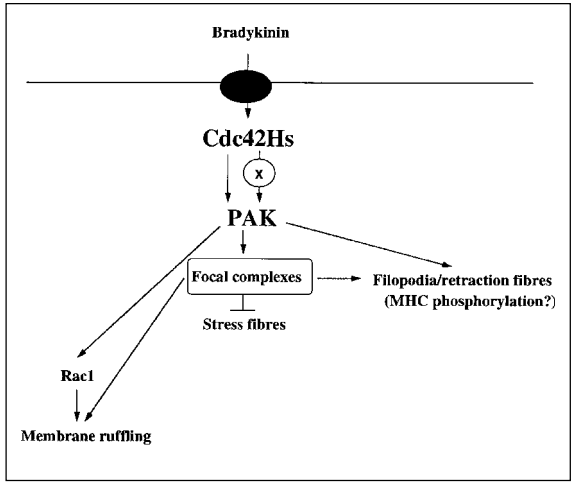


Fig. 16.2. Involvement of PAK in morphology pathways. Bradykinin induces the formation of CDC42-GTP which then activates PAK directly or via other proteins (X) to cause: (i) the formation of filopodia/retraction fibers possibly through phosphorylation of myosin heavy chain (see refs. 48-50); (ii) a change in the turnover of FCs; with inhibition of RhoA FAs and stimulation of Rac1/CDC42 FCs, the former associated with a loss of stress fibers and (iii) the N-terminal polyproline sequences P1-P4 to activate Rac1, a function not requiring the kinase activity of PAK.

gether with the established involvement of *STE20* in MAP kinase-like cascades, these data implicate PAK proteins as links between CDC42/Rac1 and JNK/SAPK in mammalian cells. Some investigators have observed a link between PAK and JNK (using 'dominant negative' [kinase dead] and 'dominant positive' [constitutively active] mutants of PAK;[33-35] while others have not[31] (Abo, A. personal communication). A possible reason for these differing results may be cell type specificity and the particular assay conditions used. Transient transfection assays are the basis of many of these studies where the results may depend on the amounts of DNA, length of expression and the exact construct used. However, this is unlikely to be the complete answer. PAK is clearly a multidomain protein with a sophisticated mechanism of regulation. For example, the polyproline motifs P1-P4 of PAK are likely to interact with different signaling complexes and overexpression of these sequences alone may have effects. Furthermore, knowing the low affinity with which some SH3 domains interact with polyproline sequences, overexpression of PAK may lead to disruption of protein-protein interactions unrelated to PAK pathways. An additional problem with regard to proteins that contain CDC42/Rac binding sites is that in isolation these domains can have 'dominant negative' effects; they have been shown to inhibit CDC42 and Rac from interacting with their targets. This consideration has led to the use of double mutants that are CDC42/Rac-binding defective and either kinase-dead or constitutively-active. The effects of overexpression of these double mutant proteins on cell function are easier to interpret. The possible role of PAK as the effector of CDC42 and Rac1 in JNK pathways has not been resolved and other candidates such as MLK-3 must be considered.[36,37]

CDC42 and Rac1 have been demonstrated to induce specific changes in the actin cytoskeleton. Recent evidence suggest that both p21s induce actin polymerization, i.e., convert actin monomers to F-actin polymers.[38,39] CDC42 and Rac1 activity is also clearly required for the formation or peripheral FCs associated with filopodia, retraction fibers and membrane ruffles[40,41] (Ahmed S et al, unpublished data). [These CDC42- and Rac1-induced FCs are morphologically distinct from one another and from the RhoA-induced focal adhesions (FAs). It would be interesting to establish whether formation of actin microfilaments at the periphery can be dissociated from FC formation and whether these two processes are directly coupled]. Furthermore, in most cells CDC42 activation leads to a rapid activation of Rac1.[40-42] Could PAK have a role in actin polymerization, FC formation or the link between CDC42 and Rac1? The answer appears to be yes in all three cases. Microinjection of

recombinant PAK protein produced in *Escherichia coli*, which is purified as an active kinase, into Swiss 3T3 cells induces filopodia formation.[43] These experiments need to be extended as filopodial activity in serum starved Swiss 3T3 cells is difficult to measure. In similar experiments we have observed that PAK protein induces the formation of retraction fibers and associated FCs followed by membrane ruffling (Ahmed S et al, unpublished). The potential role for PAK in FC formation was first suggested by the observation that the *Drosophila* PAK colocalizes with phosphotyrosine in discrete complexes.[24] The *Drosophila* cells examined were epithelial and involved in the developmental process of dorsal closure. However, it is not clear how these complexes at the leading edge of these cells are related to FCs and FAs seen in mammalian cells.

In fibroblasts, overexpression of kinase dead GTPase binding defective PAK mutants in transfection based assays induces intense membrane ruffling consistent with either activation of Rac1 or an event downstream of Rac1.[43] Interestingly, the ability of PAK to induce membrane ruffling requires the presence of the polyproline sequence P1. There is a correlation between the ability of the N-terminal of PAK (P1) to interact with NCK and its ability to induce actin polymerization.[43] Furthermore, mutation of proline 13 in P1 destroys the ability of this PAK mutant to induce membrane ruffling and to interact with NCK. Thus, apparently, SH3 domain-P1 interactions can drive F-actin formation. It was suggested that PAK is normally folded with the P1-P4 sequences masked.[43] Mutation of the GTPase binding site and/or the kinase domain may expose the polyproline sequences allowing them to interact with SH3 domains of protein partners.

In HeLa cells αPAK is translocated to FCs by both $Cdc42^{G12V}$ and $Rac1^{G12V}$ which themselves colocalize to these sites. PAK can be targeted to Rho FAs only if a kinase-dead form is introduced. The regulatory N-terminal of PAK is responsible for this targeting, including residues 1-60 and 150-250 but not the CBD.[44] As well as FAs, stress fibers are lost on expression of constitutively active forms of PAKs. The ability of PAK to target to CDC42 and Rac1-induced FCs indicates that it may participate in the dynamics of these structures. Since kinase-inactive mutant αPAK^{K298A} is targeted to FAs, this suggests that PAK kinase activity normally negatively regulates binding to these RhoA-dependent structures. It is thus possible that recruitment of αPAK to CDC42/Rac1-dependent FCs results in both activation of PAK and downregulation of binding to these sites, therefore limiting the effects of the kinase.

A constitutively active PAK can be derived by mutation of residues in its N-terminal, C-terminal of the CBD (residues 101-137), implying this to be a negative regulatory region. $\alpha PAK^{83\text{-}149}$ was found to inhibit GTPγS-CDC42 mediated kinase activation of both α and βPAKs (Zhao ZS et al, unpublished data). This PAK inhibitor enabled PAK morphological functions to be examined, without overexpressing the kinase itself or mutant forms which can act as an 'adaptor' potentially recruiting proteins such as NCK (see above). Coexpression of this PAK inhibitor with $Cdc42^{G12V}$ did not apparently affect the formation of CDC42-type FCs but prevented the formation of peripheral actin microspikes and associated loss of Rho FAs and stress fibers normally induced by the CDC42. Coexpression of PAK inhibitor with $Rac1^{G12V}$ prevented loss of stress fibers but not ruffling induced by Rac1. Coexpression of PAK inhibitor also completely abolished the phenotypic effects of hyperactive αPAK^{L107F} in inducing loss of FAs and actin stress fibers (Zhao ZS et al, unpublished data). It seems that PAK facilitates turnover of actin stress fibers and dissolves FAs, and that CDC42 requires PAK kinase activity for production of microspikes (including filopodia) and perhaps cell rounding/retraction.

The experiments described above suggest that PAK is involved in both actin polymerization and FC turnover. Does PAK act directly downstream of CDC42 or Rac1 in inducing changes in morphology? Experiments with point mutations of the effector domain of both CDC42 and Rac1 have tried to address this issue (based on work initially carried out with

Ras, see ref. 45). Mutant C40 of CDC42 and Rac1 do not interact with PAK but still induce morphological activity while the Rac1 A37 mutant can interact with PAK but no longer has morphological activity.[46,47] These observations with GTPase effector mutants makes the assumption that the protein-protein interactions seen in vitro, which in most cases are assays carried out with isolated domains rather than whole proteins, reflects accurately the protein-protein interactions taking place in vivo. It is possible for instance that a 5% binding activity with a target in vitro may be sufficient for interaction in vivo to induce a phenotypic response. Particularly when target interaction may be compartmentalized in vivo, at the plasma membrane for example. These considerations suggest caution must be used in interpreting data from GTPase effector mutant studies. Another possibility is that the Y40C mutants may displace endogenous GTPase from in vivo binding partners, thus freeing these GTPase to interact with their effectors, including PAK.

Whatever the mechanisms, it has recently been shown that the PAK-binding deficient $Cdc42^{G12V}$(Y40C) mutant can still recruit PAK to FCs (Manser E et al, unpublished data). This requires the participation of PIX, the PAK-binding GDS. PIX which is widely expressed and enriched in CDC42 and Rac1 driven FCs was also found to mediate PAK activation by the Y40C mutant. It is possible that PIX may be involved in signaling from CDC42 to Rac1 since this PAK activation is associated with a concurrent production of endogenous Rac1-GTP. By contrast, little CDC42-GTP is formed.

A model for PAKs role in morphology is presented in Figure 16.2. Bradykinin activates CDC42 which induces three separable morphological responses; (i) an immediate formation of filopodia and retraction fibers associated with (ii) a loss of stress fibers and (iii) a delayed activation of Rac1 to induce membrane ruffling. There is evidence as outlined above to support a role for PAK in all these phenotypes. The question of whether CDC42 interacts directly with PAK or via other proteins (X) or both needs to be resolved.

Two recent observations suggest novel roles for PAK. A 65-68 kDa kinase associated with the NEF protein has recently been identified.[51-53] The NEF protein is essential for efficient HIV viral replication. Mutant analysis suggest a correlation between the biological activity of NEF and its association with this kinase. The possibility that this kinase is a member of the PAK family arises from the following observations; (i) it is activated by CDC42/Rac1; (ii) it is detected with some PAK antibodies and (iii) the proteolytic map of the NEF associated kinase is similar to that of PAK proteins. However, since recombinant versions of α, β and γPAK do not interact with NEF in transfected COS cells, it is unlikely that the kinase is one of these three proteins. Nevertheless, the possibility that a protein not present in COS-7 cell, bridges between PAK and NEF, via perhaps SH3/polyproline interactions, cannot be ruled out. This exciting area of research potentially connects PAK proteins with disease states.

FAS induced apoptosis is associated with a decrease in a PAK protein band in Jurkat cells.[54] Further analysis has revealed that the onset of apoptosis, induced by different means (e.g., ceramide and TNFα) leads to caspase mediated proteolysis of PAK2(γ) but not PAK1(α) or 3(β). A cell line expressing a dominant negative mutant of PAK was resistant to Fas induced formation of apoptotic bodies but did not inhibit PS externalization.

MRCK

Recently, another family of CDC42-binding kinases has been cloned by expression screening. These kinases contain a CBD related to those ACK and PAK while its kinase domain is related to that of Rho-binding kinases ROK which is itself related to that of myotonic kinase.[55,56] These ~190 kDa myotonic dystrophy kinase-related CDC42-binding kinases (MRCKα and MRCKβ) have a preferential binding of CDC42 over Rac1.[57] Like ROK, MRCK phosphorylates nonmuscle myosin II light chain at serine 19, crucial in

activating actomyosin-based events, including contractility (chapter 3, 14, 15). Other domains include a cysteine-rich motif related to those of PKC and n-chimaerin and a PH domain.[57]

Not unexpectedly given the similarity in the kinase domain, overexpression of ROKs and MRCKs can result in overlapping morphological activities under certain experimental conditions. MRCKα promoted enhanced formation of stress fibers and FAs, an activity associated with ROK.[56] However, kinase-dead MRCKα did not induce dissolution of these RhoA-dependent structures, unlike kinase-dead (dominant negative) ROKα. MRCKs appear to have a functional role separate from that of ROK. Although phosphorylation of myosin(s) may be a shared activity between ROK and MRCK, it is highly likely that their morphological roles are determined by their site(s) of enzymatic activity, such sites being targeted by their specific domains such as the GTPase-binding or PH domains.

Thus, transfection studies with HeLa cells show MRCKα and $Cdc42^{G12V}$ to colocalize, particularly at the cell periphery. Microinjection studies with plasmid encoding MRCKα and mutants indeed demonstrate a role for MRCK in CDC42-dependent morphological changes. A kinase-dead mutant of MRCKα blocked $Cdc42^{G12V}$-dependent formation of FCs and peripheral microspikes. A kinase-dead and GTPase binding-defective MRCK was an equally effective blocker showing that the these mutants acted as dominant negative inhibitors of kinase activity, rather than through sequestration of endogenous CDC42.

What is the relationship of MRCK to the other CDC42-binding kinase PAK? Activated αPAK dissembles stress fibers FAs in HeLa cells.[44] It has been suggested that the PAK-induced disassembly of these Rho-dependent structures may be required to precede the formation of the CDC42-dependent peripheral structures, perhaps because certain components are common to the different GTPase-dependent structures or to release cellular constraints. Following this dissembly by the activated PAK, there appears to be substantial contraction of the cell.[44] In the studies with MRCK, its coexpression with limiting concentrations of CDC42 led to enhanced formation of microspikes and peculiar restructuring of peripheral segments of the cell; a continual cellular retraction and protrusion was observed.[57] This process led to expansion of part of the cytoplasm. These observations suggest that perhaps MRCK and PAK activities may need to be coordinated to obtain a full CDC42-phenotype. Experiments involving coexpression of both PAK and MRCK may be useful in this regard. The existence of distinct kinase domains with related GTPase-binding domains within MRCK and PAK, and conversely of related kinase domains with different GTPase-binding domains within MRCK and ROK, could be one means of facilitating 'cross-talk' between the different members of the Rho family. This 'cross-talk' at the level of effectors (and other regulatory proteins) may be an essential requirement for integrating cellular activities resulting from signaling by the different GTPases.

n-Chimaerin

n-Chimaerin possesses a protein kinase C (PKC)-like cysteine-rich regulatory domain at the N-terminal and a BCR like domain at the C-terminal.[58] When first isolated, the functions of either domain was not known as the role of the cysteine-rich domain of PKC was unclear (although the N-terminal region of PKC was linked to interaction with lipids and phospholipids). The cysteine-rich domain of n-chimaerin was found to bind phorbol esters with characteristics similar to those observed with PKC δ–ε (i.e., phospholipid-dependent, high-affinity, Ca^{2+}-independent and stereospecific).[59,60] Other proteins with sequence identity to the cysteine-rich regulatory domain of PKC and n-chimaerin include the oncogenes, RAF and VAV, diacylglycerol kinase and the *C. elegans UNC-13* gene product, ROK and MRCK.

The function of the C-terminal BCR related domain of n-chimaerin was resolved when parts of its sequence was found to match peptide sequences obtained from a partially purified

50 kDa protein with RhoA-GAP activity. BCR and n-chimaerin were then shown to be GAPs for Rac1 and CDC42 but not for RhoA.[13,61,62] Subsequent to these initial studies a large number of proteins with sequence identity to the GAP domain and potential GAP activity towards Rho family members have been identified from yeast to man (chapter 11). These include mammalian GAPs active against Rac1 and CDC42 such as ABR,[63,64] β-chimaerin[65] and the Ras GAP binding protein p190.[66] Both p190 and the 50 kDa RhoA-GAP are active against Rac1, CDC42 and RhoA.[67,68] Of particular interest here is the observation that the p85 subunit of PI-3 kinase shares sequence identity with the RhoA-GAP domain but does not possess GAP activity. Mutational analysis of the GAP domain of n-chimaerin has revealed that the GTPase binding can be dissociated from GAP activity. Furthermore, deletion of an invariant arginine 144 in the GAP domain of n-chimaerin was found to have a particularly dramatic effect of GAP activity.[62] From this mutational analysis a motif was identified that linked Ras GAP and Rho-GAP domains centering on this invariant arginine. The GAP activity of full-length n-chimaerin, unlike that of the GAP domain of n-chimaerin, is modulated by phosphatidylserine and phorbol esters.[60] Thus n-chimaerin is a Rac1/(CDC42) GAP that acts as a functional target for both phorbol esters and phospholipids.

Microinjection studies have been used to investigate the cellular function of n-chimaerin.[69] While microinjection of the GAP domain of n-chimaerin alone specifically downregulates Rac1 dependent membrane ruffling and pinocytosis microinjection of the full length protein was able to induce both Rac1 and CDC42 dependent morphologies. These data support the view that GAP proteins can serve as effectors as well as downregulators. In experiments designed to address the mechanism by which n-chimaerin induces changes in cell morphology the following observations were made: (i) the effects of n-chimaerin were dependent upon the presence of active CDC42 and Rac1 as dominant negative mutants (N17) inhibited filopodia and lamellipodia formation, respectively; (ii) mutants of n-chimaerin indicate that its effects were dependent upon its ability to bind to Rac1 and CDC42 rather than its GAP activity; and (iii) full length n-chimaerin was found to associate with the F-actin cytoskeleton via its N-terminal end. Taken together, these results suggest that n-chimaerin may function as a cytoskeletal receptor for Rac1 and CDC42 recruiting protein complexes necessary for remodeling the cytoskeleton (Fig. 16.3).

There is evidence that other GAPs may have roles besides simply being downregulators for their GTPases. The p120 Ras GAP has effector function in blocking atrial potassium channels, and this activity is dependent on its ability to interact with Ras.[70] Also, cells expressing the N-terminal SH2/SH3 containing portion of p120 Ras GAP lose their stress fibers and FAs and show increased ruffling[71] further suggesting effector roles for this Ras GAP. Activation of the GTPase Gq/11 leads to the activation of phospholipase Cβ1, which then acts as a GAP to downregulate its activator Gq/11.[72] IQGAP has a sequence similarity to the Ras GAP domain but does not have Ras GAP activity. Rather IQGAP binds to Rac1 and Cdc42Hs, as well as to calmodulin (see chapter 18). Recently, IQGAP has been shown to cross-link and bundle actin filaments.[73]

Wiskott-Aldrich Syndrome Protein (WASP)

Wiskott-Aldrich Syndrome (WAS) is an X-linked recessive disease of the immune system. WAS is associated with severe thrombocytopenia, eczema, profound immunodeficiency and an increased risk of cancer. A positional cloning strategy was used to clone the WAS gene.[74] DNA mutations in four patients with classical WAS supported the conclusion that aberrations in the cloned gene was responsible for WAS.[74] WAS patients have T and B cells with abnormal cell surface cytoarchitecture, loss of microvilli (the "bald phenotype"), and defective transmembrane signaling.

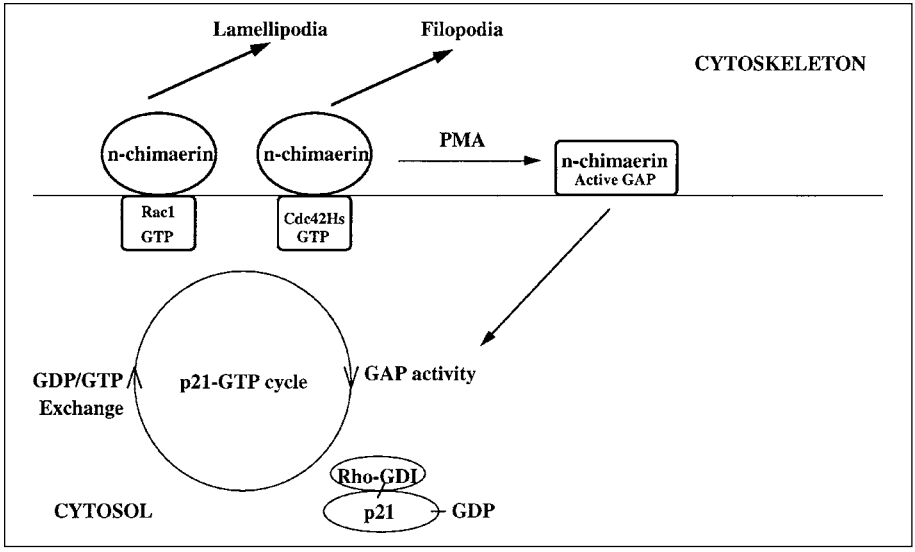


Fig. 16.3. n-Chimaerin as a cytoskeletal receptor. n-Chimaerin recruits Rac1 and CDC42 and associated complexes which induces remodeling of the actin cytoskeleton. PMA activates the GAP activity of n-chimaerin preventing it from recruiting Rac1 and CDC42.

Two groups simultaneously reported that the protein encoded by the WAS gene, WASP, interacted with CDC42; one by two-hybrid analysis[75] the other by protein purification of a CDC42 binding activity from human neutrophils.[76] The CDC42 interaction with WASP is specific, Rac1 binds weakly and RhoA not at all. The WASP interaction also requires CDC42 to be in the GTP bound form.[75,76] The site of WASP that interacts with CDC42 is similar to the CBD found initially in the protein kinase ACK (described above).

An isoform of WASP, N-WASP, has been identified through a search for proteins able to bind the SH3 domain of ASH/GRB2.[77] Unlike WASP, N-WASP is expressed predominantly in the brain, with some expression in the lung, heart, and colon. A yeast protein with sequence identity to WASP has also been identified, a protein known as Las17p, and also called BEE1, through searching of the genome database of *S. cerevisiae*.[78] Sequence analysis of WASP has revealed that it consists of several interesting protein domains. In addition to the CRIB motif, both WASP and N-WASP possess a domain of 100 amino acids composed of polyproline repeats, a PH domain, an IQ motif, and homologies to peptide sequences found in verprolin (17mer) and cofilin (19mer).[76,77] In contrast, although BEE1 has overall structural homology with WASP, it does not possess a CRIB motif or the peptide identity with cofilin. BEE1 is also larger than WASP and N-WASP by approximately 130 amino acids. More distant cousins of WASP include the polyproline containing proteins such as VASP and *Drosophila* ENA.[76,77]

What is the function of these three WASP family proteins? Microinjection of WASP in porcine aortic endothelial (PAE) cells gives rise to the formation of perinuclear "actin clusters". This phenotype for WASP required the activity of CDC42 but not Rac1 or RhoA.[76] From these observations, the physiological link between WASP and F-actin remains unclear. An effector of CDC42 would be predicted to induce the formation of peripheral actin microspikes associated with filopodia and/or retraction fibers. Studies with N-WASP have shown that it has the ability to depolymerize actin filaments and this phenotype was depen-

dent on both a functional PH domain and an actin binding site (as revealed by the use of relevant mutants, ΔVCA and C38W).[77] Interestingly, in N-WASP overexpressing COS7 cells EGF stimulation induced the formation of peripheral actin microspikes.[77] This phenotype was not observed in nontransfected cells or in cells expressing mutants of N-WASP (ΔVCA and C38W). N-WASP appears to associate with the EGF receptor via ASH2/GRB but is not phosphorylated by the EGF receptor. BEE1 is also linked with actin structures. In *S. cerevisiae* the formation of actin patches are intimately associated with the processes of budding and cytokinesis.[78] In BEEl deletion strains of *S. cerevisiae* there are defects in the assembly and organization of actin filaments at the cortex and this translates into defects in budding and cytokinesis. BEE1 is actually localized to actin patches. To gain further insight into their cellular functions it will be important to establish the intracellular localization of WASP and N-WASP in cells of the immune system and nervous system, respectively.

The polyproline stretches present in WASP, N-WASP and BEE1 probably represent sites for protein-protein interactions. In particular, SH3 domains are known to bind certain polyproline motifs. It is not surprising therefore to find that a number of proteins containing SH3 domains have been shown to interact with WASP and BEE1. N-WASP was actually isolated by its ability to interact with the SH3 domain of ASH/GRB2 as described above. WASP has been shown to interact, via SH3 domains, with NCK, SRC family kinases, p85 subunit of PI-3 kinase, phospholipase C γ1, and c-FGR.[79] BEE1 has been shown to interact with the SH3 containing protein of Sla1p. However, it is not clear whether the BEEl-Sla1p interaction occurs via an SH3 domain-polyproline interaction.[78]

These results clearly suggest a role for the WASP, N-WASP and for BEE1 in regulating actin dynamics of mammalian cells and yeast, respectively. In the case of WASP a requirement for the activity of CDC42 has also been demonstrated which suggests that a direct interaction with CDC42 is required for its morphological effects.[76] This conflicts with data obtained with the CDC42 point mutant analysis described above (PAK section).

POR1

POR1 was isolated as a Rac1 interacting protein in yeast two-hybrid screening with Jurkat cDNA libraries.[80] POR1 does not have domain similarity with other proteins present in the database. Functional analysis of POR1 suggests a possible role in membrane ruffling. Firstly, POR1 localizes to regions of the cell periphery undergoing membrane ruffling. Secondly, regions of POR1 can inhibit Rac1 mediated membrane ruffling. Thirdly, POR1 synergizes with Ras in membrane ruffling. Lastly, point mutations of Rac1 that no longer bind POR1 are defective in membrane ruffling. However, overexpression of POR1 itself does not induce membrane ruffling and POR1 does not synergize with Rac1. Taken together these results are supportive of a role for POR1 in Rac1-mediated membrane ruffling although not entirely convincing.

Interestingly, POR1 is virtually identical to a protein that binds Arf1, Arfaptin.[81] The Arf proteins are intimately associated with membrane trafficking. Arfs are regulators of intracellular transport, required for the formation of coated vesicles in the Golgi complex,[82,83] and vesicle transport between the ER and Golgi.[84,85] They are also involved in nuclear vesicle fusion[86] and can activate phospholipase D.[87,88] Recently, Arf6, a protein that induces peripheral actin structures, has been found to bind POR.[89] Arf6 induced cytoskeletal changes appear to be independent of Rac1 activity. It would be interesting to know whether the binding sites in POR1 for Rac1, Afr1 and Arf6 are distinct or whether these GTPases bind to the same site. It is possible that POR1 represents a link between Rac1 and membrane trafficking via the Arfs.

NADPH Oxidase Component p67phox

The NADPH oxidase is an important part of the immune system involved in killing invading microorganisms. It functions primarily by reducing molecular oxygen leading to the formation of superoxide anion by a cytochrome mediated transfer of electrons. Protons also produced during the reduction of the oxidase which may have some function in pH control of phagosomes. Molecular defects in the components of the oxidase are the genetic basis for chronic granulomatous disease (for review see ref. 90).

NADPH oxidase activity can be reconstituted in vitro and this has allowed the minimal characteristics of the enzyme to be investigated.[91] The NADPH oxidase is composed of two integral membrane proteins, subunits of cytochrome b (p22phox and p91phox), and four proteins that translocate between cytosol and plasma membrane, p67phox, p47phox and p40phox and Rac1. The three former proteins are "adaptors" with SH3 domains and polyproline rich regions the only clear domains present. Protein phosphorylation of p47phox and p67phox occurs (for example see refs. 92,93) although the kinases involved have not been clearly identified.

Protein-protein interactions within the complex have been analyzed by immunoprecipitation, two-hybrid analysis, Far Westerns and affinity columns. In the unstimulated neutrophil/macrophage Rac1 exists in a complex of Rho-GDI while the adaptor proteins are present as a 250 kDa complex.[94,95] Immunoprecipitation experiments have shown that there is a 1:1 association between p67phox and p40phox.[95,96] The association of p47phox in the 250 kDa complex is labile. The interaction between p67phox and p40phox does not involve SH3 domains and occurs between the C-terminal of p40phox and the region between the two SH3 domains of p67phox.[97,98] Upon stimulation of neutrophils with agents such as fMLP Rac1 dissociates from Rho-GDI and the p67phox-p40phox-p47phox complex moves to the membrane and binds the p22phox subunit of cytochrome b. The mechanism by which cell stimulation leads to a redistribution of oxidase complex components is under intense investigation. Phosphorylation of p67phox and p47phox and second messenger interaction with these components are likely to be involved. Protein-protein interaction studies have revealed that there is an intramolecular interaction between the N-terminal SH3 domain and C-terminal polyproline region in p47phox and that this can be disrupted by arachidonic acid.[99,100] If this is true in vivo it represents a mechanism by which the SH3 domains of p47phox (p22phox binding sites) are masked until cells are stimulated. The polyproline region of p47phox has been shown to interact with the C-terminal SH3 domain on p67phox[98,101] and the SH3 domain of p40phox can interact with either of the two polyproline regions in p47phox.[102] If and when these interactions occur in vivo is unclear.

Thus the NADPH oxidase represents an exciting opportunity for investigating the molecular mechanisms by which Rac GTPases may influence multimeric protein complexes. Some of the questions that can be posed are: (i) Is Rac1 required for complex assembly, turnover or stability? (ii) Is the role of Rac1 to influence SH3-polyproline interactions, possible through recruiting kinases? (iii) Does Rac1 trigger a conformational change in the complex inducing superoxide formation, enzymic activity?

Rac1 interacts directly with p67phox, but not the other oxidase components, in binding assays.[21,103] However, it cannot be ruled out that Rac1 may bridge between two components, e.g., p67phox and the cytochrome b subunit p22phox. We have localized the Rac1 binding site of p67phox (Ahmed S et al, unpublished). Interestingly, the N-terminal of p67phox which stabilizes the Rac1 interaction is composed of four "tetratricopeptide repeats."[104] These protein domains are thought to form amphipathic helices that may self associate. C-terminal to the Rac1 binding site is a polyproline sequence (SH3 binding site) followed immediately by a SH3 domain (N-terminal). Deletion of the C-terminal of p67phox (192-526), the polyproline sequence (226-236), or the C-terminal SH3 domain (470-526),

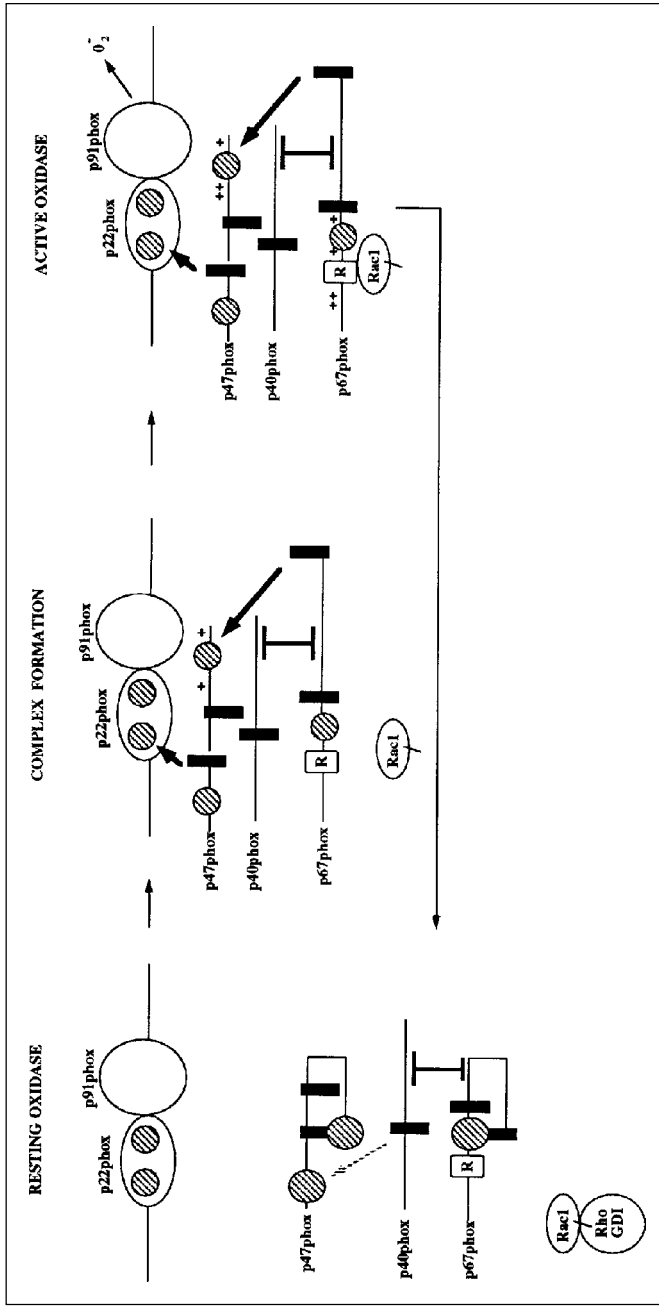


Fig. 16.4. A model for complex formation of the components of the NADPH oxidase. The complex of p47phox, p67phox and p40phox and Rac1 with Rho-GDI is present in resting cells with p22phox and p91phox at the membrane. On cell stimulation a combination of phosphorylation events and second messengers switch intramolecular SH3-polyproline interactions to intermolecular interactions. In particular, p67phox now interacts with p47phox and p47phox can interact with p22phox. These events lead to complex formation at the membrane. Following this additional phosphorylation events and Rac1 binding to p67phox leads to activation of the oxidase. The complex is restored to the resting state by phosphatase activity. Arrows; SH3-polyproline interactions. Bars; protein-protein interaction. Boxes-SH3 domains and circles-polyproline sequences.

Table 16.1. Mammalian CDC42 and Rac1 effectors

GTPase	Effector	Ref.	Function
CDC42	ACK	5	?
	PAK	14	FC's/Actin pol.
	Kinectin	106	?
	WASP	75,76	Actin dynamics
	CIP4	107	Actin dynamics
	CIP5	107	?
	n-Chimaerin	69	Filopodia
	IQGAP	73	Actin dynamics/adhesion
	MRCK	57	Actin dynamics
	MLK-3	36.37	JNK activation
	S6-kinase	108	G1 to S transition
	PI-3 kinase	109	Second messenger syn.
Rac1	POR1	80	Membrane trafficking
	p67phox	21,120	Superoxide formation
	n-Chimaerin	69	Lamellipodia
	Tubulin	110	?
	PAK	14	FC's/Actin pol.
	Kinectin	1067	?
	IQGAP	73	Actin dynamics
	ROK	111,112	FA's/Actin bundling
	Citron	113	?
	MLK-3	36,37	JNK activation
	S67-kinase	108	G1 to S transition
	PLA	114	Second messenger syn.
	PI-3² kinase	115	Second messenger syn.
	PI-4 P kinase	116	Second messenger syn.

The table shows Cdc42Hs and Rac1 effectors and their potential function. Effectors in italics (below dotted line) are those which have not been shown to interact directly with Cdc42Hs or Rac1. FAs-"focal adhesions", FC- "focal complexes", np-not published, pol.- polymerization, syn.-synthesis.

lead to an 8-fold stimulation of Rac1 binding. These results suggest that the cryptic nature of the Rac1 binding site is caused by an intramolecular interaction between the C-terminal SH3 domain and polyproline sequence of p67phox (Ahmed S et al, unpublished data).

PAK kinase has been found to phosphorylate p47phox[105] and we have recently shown that βPAK can phosphorylate p67phox (Ahmed S et al, unpublished). The PAK phosphorylation sites of p67phox are cryptic and map to sites very close to the Rac1 binding site and polyproline region of p67phox. Interestingly, in p47phox the PAK phosphorylation site and the polyproline region are adjacent. We are currently investigating whether PAK phosphorylation of p67phox or p47phox may influence SH3-polyproline interactions or Rac1 binding.

A model incorporating all the above observations is presented in Figure 16.4. The concept that emerges from this model is that switching between intramolecular and intermolecular interactions driven by second messengers and phosphorylation events may provide a mechanism for complex assembly. Rac1 does not appear to be essential for complex as-

sembly. Rather Rac1 may play a role in conformational changes within the complex and/or turnover of the complex. For the NADPH oxidase the stage is now set to elucidate the role of Rac1.

Conclusion

Rac1 and CDC42 are central regulators of cellular processes that work in hierarchies (e.g., downstream of Ras, CDC42-Rac1) but also in antagonism (CDC42 vs. RhoA). This latter phenomenon is most clearly seen in N1E-115 cells where RhoA prevents CDC42 and Rac1 mediated neurite outgrowth and C3 toxin (dominant negative inhibitor of RhoA) induces it.[42] This complex relationship between members of the Ras and Rho families needs to be considered in cellular assays. Isolation of effectors has opened up ways of analyzing Ras/Rho family signaling pathways at the molecular level. From two-hybrid analysis it is clear that there are a large number (possibly 100s) of Rac1 and CDC42 effectors and this confirms their importance in cell biology (see Table 16.1 for current known effectors for Rac1 and CDC42). The use of the techniques discussed to isolate protein partners combined with functional assays will eventually lead to the description of the protein complexes and networks involved in Rho family signaling pathways. These studies will help to elucidate the mechanism by which oncogenic Ras and Rho family proteins cooperate to affect the actin cytoskeleton and cause cell transformation.

Acknowledgments

We thank the Glaxo-Singapore Research Fund for their generous support and our colleagues in the Glaxo-IMCB Group, including Tom Leung, Ed Manser and Zhao Zhuo-Shen whose unpublished work is referred to in this review.

Note added in proof

Some of the unpublished data mentioned have now been published and the details are below.

(Zhao ZS et al, unpublished data) Zhao ZS, Manser E, Chen XQ et al. A conserved negative regulatory region in alpha PAK: Inhibition of PAK kinases reveals their morphological roles downstream of CDC42 and Rac1. Mol Cell Biol 1998; 18:2153-2163.

(Manser et al, unpublished data) Manser E, Loo TH, Koh CG et al. PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol Cell 1998; 1:183-192.

Obermeier A, Ahmed S, Manser E. PAK promotes morphological changes by acting upstream of Rac. EMBO J 1998; 17:101-112.

(Ahmed et al, unpublished data) Ahmed S, Prigmore E, Govind S et al. Cryptic Rac-binding and p21(Cdc42HS/Rac)-activated kinase phosphorylation sites of NADPH oxidase component p67(phox). J Biol Chem 1998; 273:15693-15701.

References

1. Boguski MS, McCormick F. Proteins regulating RAS and its relatives. Nature 1993; 366:643-654.
2. Hall A. Small GTP-binding proteins and regulation of the actin cytoskeleton. Annu Rev Cell Biol 1994; 10:31-54.
3. Symons M. Rho family GTPases: The cytoskeleton and beyond. Trends in Biochem 1996; 4:178-181.
4. Fields S, Sternglanz R. The two-hybrid system: An assay for protein-protein interactions. Trends in Genetics 1994; 10:286-292.
5. Manser E, Leung T, Salihuddin H et al. A non-receptor tyrosine kinase that inhibits the GTPase activity of p21Cdc42. Nature 1993; 363:364-367.

6. Satoh T, Kato J, Nishida K et al. Tyrosine phosphorylation of ACK in response to temperature shift-down, hyperosmotic shock, and epidermal growth factor stimulation. FEBS Letts 1996; 386:230-234.
7. Lev S, Moreno H, Martinez R et al. Protein tyrosine kinase PYK2 involved in Ca2+-induced regulation of ion channel and MAP kinase functions. Nature 1995; 376:737-745.
8. Hiregowdara D, Avraham H, Fu Y et al. Tyrosine phosphorylation of the related adhesion focal tyrosine kinase in megakaryocytes upon stem cell factor and phorbol myristate acetate stimulation and its association with paxillin. J Biol Chem 1997; 272:10804-10810.
9. Qian D, Lev S, Van Oers NSC et al. Tyrosine phosphorylation of Pyk2 is selectively regulated by Fyn during TCR signalling J Exp Med 1997; 185:1253-1259.
10. Astier A, Avraham H, Manie SN et al. The related adhesion focal tyrosine kinase is tyrosine-phosphorylated after β1-integrin stimulation in B cells and binds to p130(cas) J Biol Chem 1997; 272:228-232.
11. Siciliano JC, Toutant M, Derkinderen P et al. Differential regulation of proline-rich tyrosine kinase 2/cell adhesion kinase beta (PYK2/CAK β) and pp125(FAK) by glutamate and depolarization in rat hippocampus. J Biol Chem 1996; 271:28942-28946.
12. Tokiwa G, Dikic I, Lev S et al. Activation of Pyk2 by stress signals and coupling with JNK signalling pathway. Science 1996; 273:792-794.
13. Manser E, Leung T, Monfries C et al. Diversity and versatility of GTPase activating proteins for the p21rho subfamily of ras G proteins detected by a novel overlay assay. J Biol Chem 1992; 267:16025-16028.
14. Manser E, Leung T, Salihuddin H et al. A brain serine/threonine protein kinase activated by CDC42 and Rac1. Nature 1994; 267:40-46.
15. Zhao ZS, Leung T, Manser E et al. Pheromone signalling in *Saccharomyces cerevisiae* requires the small GTP-binding protein Cdc42p and its activator CDC24. Mol Cell Biol 1995; 15:5246-5257.
16. Simon MN, De Virgillio C, Souza B et al. Role of Rho family GTPase CDC42 in yeast-mating pheromone signal pathway. Nature 1995; 376:702-705.
17. Teo M, Manser E, Lim L. Identification and molecular cloning of a p21(CDC42/rac1)-activated serine/threonine kinase that is rapidly activated by thrombin in platelets. J Biol Chem 1995; 270:26690-26697.
18. Manser E, Chong C, Zhao ZS et al. Molecular cloning of a new member of the p21-CDC42/Rac-activated kinase (PAK) family. J Biol Chem 1995; 270:25070-25078.
19. Martin GA, Bollag G, McCormick F et al. A novel serine kinase activated by rac1/CDC42Hs-dependent autophosphorylation is related to PAK65 and Ste20. EMBO J 1995; 14:1970-1978.
20. Bagrodia S, Taylor SJ, Creasy CL et al. Identification of a mouse p21(CDC42/Rac) activated kinase. J Biol Chem 1995; 270:22731-22737.
21. Prigmore E, Ahmed S, Best A et al. A 68-kDa kinase and NADPH oxidase component p67(phox) are targets for CDC42Hs and Rac1 in neutrophils. J Biol Chem 1995; 270:10717-10722.
22. Sells MA, Chernoff J. Emerging from the Pak: The p21-activated protein kinase family. Trends in Cell Biol 1997; 7:162-167.
23. Chen W, Chen S, Seow Fong Y et al. The *Caenorhabditis elegans* p21-activated kinase (CePAK) colocalizes with CeRac1 and CDC42Ce at hypodermal cell boundaries during embryo elongation. J Biol Chem 1996; 271:26362-26368.
24. Harden N, Lee J, Loh HY et al. A *Drosophila* homolog of the Rac- and CDC42-activated serine/threonine kinase PAK is a potential focal adhesion and focal complex protein that colocalizes with dynamic actin structures. Mol Cell Biol 1996; 16:1896-1908.
25. Purbelo PD, Dreschel D, Hall A. A conserved binding motif defines numerous candidate target proteins for CDC42 and Rac GTPases. J Biol Chem 1995; 270:29071-29075.
26. Bokoch GM, Wang Y, Bohl BP et al. Interaction of the Nck adapter protein with p21-activated kinase (PAK1). J Biol Chem 1996; 271:25746-25749.
27. Leberer E, Wu C, Leeuw T et al. Functional characterization of the CDC42p binding domain of yeast Ste20p protein kinase. EMBO J 1997; 16:83-97.

28. Peter M, Neiman AM, Park HO et al. Functional analysis of the interaction between the small GTP binding protein CDC42 and the Ste20 protein kinase in yeast. EMBO J 1996; 15:7046-7059.
29. Leberer E, Harcus D, Broadbent ID et al. Signal transduction through homologs of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*. Proc Natl Acad Sci USA 1996; 93:13217-13222.
30. Lebberer E, Ziegelbauer K, Schmidt A et al. Virulence and hyphal formation of *Candida albicans* require the Ste20p-like protein kinase CaCla4p. Curr Biol 1997; 7:539-546.
31. Minden A, Lin A, Claret FX et al. Selective activation of the JNK signalling cascade and c-Jun transcriptional activity by the small GTPases Rac and CDC42Hs. Cell 1995; 81:1147-1157.
32. Coso OA, Chlariello M, Yu JC et al. The small GTP-binding protein Rac1 and CDC42 regulate the activity of the JNK/SAPK signalling pathway. Cell 1995; 81:1137-1146.
33. Bagrodia S, Derijard B, Davis RJ et al. CDC42 and PAK-mediated signalling leads to Jun kinase and p38 mitogen-activated protein kinase activation. J Biol Chem 1995; 270:27995-27998.
34. Zhang S, Han J, Sells MA et al. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J Biol Chem 1995; 270:23934-23936.
35. Brown JL, Stowers L, Baer M et al. Human Ste20 homologue hPAK1 links GTPases to the JNK MAP kinase pathway. Curr Biol 1996; 6:598-605.
36. Teramoto H, Coso OA, Miyata H et al. Signalling from the small GTP-binding proteins Rac1 and CDC42 to the c- Jun N-terminal kinase/stress-activated protein kinase pathway: A role for mixed lineage kinase 3/protein-tyrosine kinase 1, a novel member of the mixed lineage kinase family. J Biol Chem 1996; 271:27225-27228.
37. Tibbles LA, Ing YL, Kiefer F et al. MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. EMBO J 1996; 15:7026-7035.
38. Zigmond SH, Joyce M, Borleis J et al. Regulation of actin polymerisation in cell-free systems by GTPγS and CDC42. J Cell Biol 1997; 138:363-373.
39. Machesky L, Hall A. role of actin polymerisation and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization J Cell Biol 1997; 138:913-926.
40. Nobes CD, Hall A. Rho, Rac and CDC42 GTPases regulate the assembly of multi-molecular focal complexes associated with actin stress fibres, lamellipodia and filopodia. Cell 1995; 81:53-62.
41. Kozma R, Ahmed S, Best A et al. The ras related protein CDC42Hs and bradykinin promote the formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 1995; 15:1942-1952.
42. Kozma R, Sarner S, Ahmed S et al. Rho family GTPases and neuronal growth cone remodelling; Relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. Mol Cell Biol 1997; 17:1201-1211.
43. Sells MA, Knaus U, Bagrodia DM et al. Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. Curr Biol 1997; 7:202-210.
44. Manser E, Huang HY, Loo TH et al. Expression of constitutively active αPAK reveals effects of the kinase on actin and focal complexes. Mol Cell Biol 1997; 17:1129-1143.
45. White MA, Nicolette C, Minden A et al. Multiple Ras functions can contribute to mammalian cell transformation. Cell 1995; 80:533-541.
46. Lamarche N, Tapon N, Stowers L et al. Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65(PAK) and the JNK/SAPK MAP kinase cascade. Cell 1996; 87:519-529.
47. Westwick JK, Lambert QT, Clark GJ et al. Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. Mol Cell Biol 1997; 17:1324-1335.
48. Brzeska H, Knaus UG, Wang ZY et al. p21-Activated kinase has substrate specificity similar to *Acanthamoeba* myosin I heavy chain kinase and activates *Acanthamoeba* myosin I Proc Natl Acad Sci USA 1997; 94:1092-1095.

49. Wu C, Lee SF, Furmaniak Kazmierczak E et al. Activation of myosin-I by members of the Ste20p protein kinase family. J Biol Chem 1996; 271:31787-31790.
50. Brzeska H, Szczepanowska J, Hoey J et al. The catalytic domain of *Acanthamoeba* myosin I heavy chain kinase. II. Expression of active catalytic domain and sequence homology to p21activated kinase (PAK). J Biol Chem 1996; 271:27056-27062.
51. Nunn MF, Marsh JW. Human immunodeficiency virus type 1 Nef associates with a member of the p21-activated kinase family. J Virology 1996; 70:6157-6161.
52. Lu X, Wu X, Plemenitias A et al. CDC42 and Rac1 are implicated in the activation of the Nef-assocaited kinase and replication of HIV-1. Curr Biol 1996; 6:1677-1684.
53. Sawai ET, Khan IH, Montbriand PM et al. Activation of PAK by HIV and SIV Nef:importance for AIDS in *Rhesus* macaques. Curr Biol 1996; 6:1519-1527.
54. Rudel T, Bokoch GM. Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science 1997; 276:1571-1574.
55. Leung T, Manser E, Tan L et al. A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J Biol Chem 1995; 270:29051-29054.
56. Leung T, Chen XQ, Manser E et al. The p160 RhoA-binding kinase ROKα is a member of a kinase family and is involved in the reorganization of the cytoskeleton. Mol Cell Biol 1996; 16:5313-5327.
57. Leung T, Chen XQ, Tan I et al. Myotonic dystrophy kinase-related CDC42-binding kinase acts as a CDC42 effector in promoting cytoskeletal reorganization. Mol Cell Biol 1998; 18:130-140.
58. Hall C, Monfries C, Smith P et al. Novel human brain cDNA encoding a 34,000 M(r) protein n-chimaerin, related to both the regulatory domain of protein kinase C and BCR, the product of the breakpoint cluster region gene. J Mol Biol 1990; 211:11-16.
59. Ahmed S, Kozma R, Monfries C et al. Human brain n-chimaerin cDNA encodes a novel phorbol ester receptor. Biochem J 1990; 272:767-773.
60. Ahmed S, Lee, J Kozma R et al. A novel functional target for tumor-promoting phorbol esters and lysophosphatidic acid. The p21rac-GTPase activating protein n-chimaerin. J Biol Chem 1993; 268:10709-10712.
61. Diekmann D, Brill S, Garrett MD et al. Bcr encodes a GTPase-activating protein for p21. Nature 1991; 351:400-402.
62. Ahmed S, Lee J, Wen LP et al. Breakpoint cluster region gene product-related domain of n-chimaerin. Discrimination between Rac-binding and GTPase-activating residues by mutational analysis. J Biol Chem 1994; 269:17642-17648.
63. Tan EC, Leung T, Manser E et al. The human active breakpoint cluster region-related gene encodes a brain protein with homology to guanine nucleotide exchange proteins and GTPase- activating proteins. J Biol Chem 1993; 268:27291-27298.
64. Heisterkamp N, Kaartinen V, VanSoest S et al. Human ABR encodes a protein with GAP(rac) activity and homology to the DBL nucleotide exchange factor domain. J Biol Chem 1993; 268:16903-16906.
65. Leung T, How BE, Manser E et al. Germ cell β-chimaerin, a new GTPase-activating protein for p21rac, is specifically expressed during the acrosomal assembly stage in rat testis. J Biol Chem 1993; 268:3813-3816.
66. Settleman J, Albright CF, Foster LC et al. Association between GTPase activators for Rho and Ras families. Nature 1992; 359:153-154.
67. Lancaster CA, TaylorHarris PM, Self AJ et al. Characterization of rhoGAP. A GTPase-activating protein for rho-related small GTPases. J Biol Chem 1994; 269:1137-1142.
68. Barfod ET, Zheng Y, Kuang WJ et al. Cloning and expression of a human Cdc42 GTPase-activating protein reveals a functional SH3-binding domain. J Biol Chem 1993; 268:26059-26062.
69. Kozma R, Ahmed S, Best A et al. The GTPase-activating protein n-Chimaerin cooperates with Rac1 and CDC42Hs to induce the formation of lamellipodia and filopodia. Mol Cell Biol 1996; 16:5069-5080.

70. Yatani A, Okabe K, Polakis P et al. Ras p21 and GAP inhibit coupling of muscarinic receptors to atrial K^+ channels. Cell 1990; 61:769-776.
71. McGlade J, Brunkhorst B, Anderson D et al. The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. EMBO J 1993; 12:3073-3081.
72. Berstein G, Balnk JL, Jhon DY et al. Phospholipase C-β1 is a GTPase activating protein for Gq/11, its physiologic regulator. Cell 1992; 70:411-418.
73. Bashour AM, Fullerton AT, Hart MJ et al. IQGAP1, a Rac- and CDC42-binding protein, directly binds and cross-links microfilaments. J Cell Biol 1997; 137:1555-1566.
74. Derry JMJ, Ochs HD, Francke U. Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. Cell 1994; 78:635-644.
75. Aspenstrom P, Lindberg U, Hall A. Two GTPases, CDC42 and Rac1, bind directly to a protein implicated in the immunodeficiency disorder Wiskott-Aldirch syndrome. Curr Biol 1996; 6:70-75.
76. Symons M, Derry JMJ, Karlak B et al. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. Cell 1996; 84:723-734.
77. Miki H, Miura K, Takenawa, T N. WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. EMBO J 1996; 15:5326-5335.
78. Li R. BEE1, a yeast protein with homology to Wiscott-Aldrich syndrome protein, is critical for the assembly of cortical actin cytoskeleton. J Cell Biol 1997; 136:649-658.
79. RiveroLezcano OM, Marcilla A, Sameshima JH et al. Wiskott-Aldrich syndrome protein physically associates with NCK through SRC homology 3 domains. Mol Cell Biol 1995; 15:5725-5731.
80. Van Aelst L, Joneson T, Bar-Sagi D. Identification of a novel Rac1-interacting protein involved in membrane ruffling. EMBO J 1996; 15:3778-3786.
81. Kanoh H, Williger B-T, Exton JH. Arfaptin 1, a putative cytosolic target protein of ADP-ribosylation factor, is recruited to Golgi membranes. J Biol Chem 1997; 272:5421-5429.
82. Serafini T, Orci L, Amherdt M et al. ADP riboysylation factor is a subunit of the coat of Golgi-derived Cop-coated vesicles, a novel role for a GTP binding protein. Cell 1991; 67:239-253.
83. Orci L, Palmer DJ, Amherdt M et al. Coated vesicle assembly in the golgi requires only coatomer and ARF proteins from the cytosol. Nature 1993; 364:732-734.
84. Balch WE, Kahn RA, Schwaninger R. ADP-ribosylation factor is required for vesicular trafficking between the endoplasmic reticulum and the cis-golgi compartment. J Biol Chem 1992; 267:13053-13061.
85. Dascher C, Balch WE. Dominant inhibitory mutants of ARF1 block endoplasmic reticulum to golgi transport and trigger assembly of the golgi apparatus. J Biol Chem 1994; 269:1437-1448.
86. Boman AL, Taylor TC, Melancon P et al. A role for ADP-ribosylation factor in vesicle dynamics. Nature 1992; 358:512-514.
87. Brown HA, Gutowski S, Moomaw CR et al. ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. Cell 1993; 75:1137-1144.
88. Cockcroft S, Thomas GMH, Fensome A et al. Phopholipase D a downstream effector of ARF in granulocytes. Science 1994; 263:523-526.
89. D'Souza-Schorey C, Boshans RL, McDonough M et al. A role for POR1, a Rac1-interacting protein, in ARF6-mediated cytoskeletal rearrangements. EMBO J 1997; 16:5445-5454.
90. Roos D, Curnutte JT, Hossle JP et al. X-CGDbase: A database of X-CGD-causing mutations. Immunology Today 1996; 17:517-521.
91. Abo A, Boyan A, West I et al. Reconstitution of neutrophil NADPH Oxidase activity in the cell-free system by four components:p67phox, p47phox,p21rac1, and cytochrome b. J Biol Chem 1992; 267:16767-16770.
92. Park JW, Hoyal CR, El Benna J et al. Kinase-dependent activation of the leukocyte NADPH oxidase in a cell-free system. Phosphorylation of membranes and p47(phox) during oxidase activation. J Biol Chem 1997; 272:11035-11043.

93. Dusi S, Rossi F. Activation of NADPH oxidase of human neutrophils involves the phosphorylation and translocation of cytosolic p67phox. Biochem J 1993; 296;367-371.
94. Abo A, Pick E, Hall A et al. Activation of the NADPH oxidase involves the small GTP binding proteinp21rac1. Nature 1991; 353:668-670.
95. Wientjes FB, Hsuan JJ, Totty NF et al. p40(phox), a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains. Biochem J 1993; 296:557-561.
96. Wientjes FB, Panayotou G, Reeves E et al. Interactions between cytosolic components of the NADPH oxidase:p40phox interacts with both p67phox and p47phox. Biochem J 1996; 317:919-924.
97. Fuchs A, Dagher MC, Vignais PV. Mapping the domains of interaction of p40(phox) with both p47(phox) and p67(phox) of the neutrophil oxidase complex using the two-hybrid system. J Biol Chem 1995; 270:5695-5697.
98. Finan P, Shimizu Y, Gout I et al. An SH3 domain and proline-rich sequence mediate an interaction between two components of the phagocyte NADPH oxidase complex. J Biol Chem 1994; 269:13752-13755.
99. Sumimoto H, Kage Y, Nunoi H et al. Role of Src homology 3 domains in assembly and activation of the phagocyte NADPH oxidase. Proc Natl Acad Sci USA 1994; 91:5345-5349.
100. Leto TL, Adams AG, De Mendez I. Assembly of the phagocyte NADPH oxidase: Binding of Src homology 3 domains to proline-rich targets. Proc Natl Acad Sci USA 1994; 91:10650-10654.
101. De Mendez I, Garrett MC, Adams AG et al. Role of p67-phox SH3 domains in assembly of the NADPH oxidase system. J Biol Chem 1994; 269:16326-16332.
102. Ito T, Nakamura R, Sumimoto H et al. An SH3 domain-mediated interaction between the phagocyte NADPH oxidase factors p40(phox) and p47(phox). FEBS Letts 1996; 385:229-232.
103. Diekmann D, Abo A, Johnston C et al. Interaction of Rac with p67(phox) and regulation of phagocytic NADPH oxidase activity. Science 1994; 265:531-533.
104. Ponting CP. Novel domains in NADPH oxidase subunits, sorting nexins, and PtdIns 3-kinases: Binding partners of SH3 domains? Protein Science 1996; 5:2353-2357.
105. Knaus UG, Morris S, Dong HJ et al. Regulation of human leukocyte p21-activated kinases through G protein-coupled receptors. Science 1995; 269:221-223.
106. Hotta K, Tanaka K, Mino A et al. Interaction of the Rho family small G proteins with kinectin, an anchoring protein of kinesin motor. Biochem Biophys Res Commu 1996; 225:69-74.
107. Aspenstrom P. A CDC42 target protein with homology to the non-kinase domain of FER has a potential role in regulating the actin cytoskeleton. Curr Biol 1997; 7:479-487.
108. Chou MM, Blenis J. The 70 kDa S6 kinase complexes with and is activated by the Rho family G proteins CDc42 and Rac1. Cell 1996; 85:573-583.
109. Zheng Y, Bagrodia S, Cerione RA. Activation of phosphoinositide 3-kinase activity by Cdc42Hs binding to p85. J Biol Chem 1994; 269:18727-18730.
110. Best A, Ahmed S, Kozma R et al. The ras-related GTPase Rac1 binds tubulin. J Biol Chem 1996; 271:3756-3762.
111. Ishizaki T, Maekawa M, Fujisawa K et al.The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J 1996; 15:1885-1893.
112. Amano M, Chihara K, Kimura K et al. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 1997; 275:1308-1311.
113. Madaule P, Furuyashiki T, Reid T et al. A novel partner for the GTP-bound forms of rho and rac. FEBS Letts 1995; 377:243-248.
114. Peppelenbosch MP, Qiu RG, Vries-Smits AMM et al. Rac mediates growth factor induced arachidonic acid release. Cell 1995; 81:849-856.
115. Zhang J, King WG, Dillon S et al. Activation of platelet phosphatidylinositide 3-kinase requires the small GTP-binding protein Rho. J Biol Chem 1993; 268:22251-22254.
116. Tolias KF, Cantley LC, Carpenter CL. Rho family GTPases bind to phosphoinositide kinases J Biol Chem 1995; 270:17656-17659.

Section III
Links Between G Proteins and Actin

Chapter 17

Flightless I

Hugh D. Campbell

Mutations in a number of genes are known to impair flight behavior in the fruit fly *Drosophila melanogaster*. Not surprisingly, some of these are mutations in genes for components of muscle. These include, for example, genes for myosin,[1] actin,[2] tropomyosin[3,4] and troponin-T,[5] as well as metabolic enzymes such as α-glycerophosphate dehydrogenase[6,7] and the corresponding membrane-bound respiratory oxidase.[8] Numerous X chromosome linked viable mutations which decrease flight ability were discovered by Homyk and Sheppard and other workers.[9-12] Subsequently[13,14] it was shown that *flightless I*,[3] *flightless O*[2] and *standby*[9-12] are all alleles of a single gene. This gene can be mutated to lethality, as all the above alleles map to the *W-2* lethal complementation group.[15] The gene is now known as *flightless I* (*fliI*), since this was the name given to it in the first report of its discovery.[9]

The viable mutations in *fliI* were shown to result in ultrastructural defects in the indirect flight muscles,[10-12,14] although the direct flight muscles appear normal.[10] In the mutants, the myofibrils are frayed and disorganized, and the Z-bands are absent, split or wavy in appearance. Striated bundles occur, and appear to be aggregates of thin filaments, possibly also including Z-band material, which display electron-dense striations with a regular periodicity of 130 nm.[10] Similar striated bundles occur in other apparently unrelated mutants such as *upheld* (troponin-T)[5,12] and *grounded*.[12]

Embryos homozygous for lethal alleles of *fliI* arrest during development at late larval or pupal stages. Germline clone analysis was used to show that embryos deficient in maternally supplied wild-type *fliI* gene product gastrulate abnormally; cellularization in the case of the most severe alleles such as *W-2* is only partial.[13] Mosaic fate mapping experiments indicate that the focus for *fliI* maps to the anterior ventral region of the blastoderm,[10,16] a region where primordial mesoderm arises. The exact site of the focus and comparison with other known genes indicate that the focus lies in indirect flight muscle primordia rather than in the thoracic nervous system.[10,16]

fliI cDNA Cloning and Protein Structure

Drosophila

The *Drosophila* genomic region spanning *fliI* was cloned by chromosome walking from entry points generated by chromosome microdissection and cloning.[17] Northern analysis was used to detect candidate transcription units that lay between breakpoints of deficiencies that were shown genetically to delimit the position of the *fliI* gene. A 10.2 kb *Xho*I fragment inserted into the genome on a P element vector restored flight ability to *fliI* mutant flies, and restored viability to flies homozygous deficient for the *fliI* genomic region. The single complete transcription unit of ~5 kb present on this 10.2 kb *Xho*I fragment thus

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

was shown to correspond to *fliI*. cDNAs corresponding to this transcription unit were isolated using appropriate genomic fragments as probes. Overlapping cDNAs were analyzed giving a final composite cDNA sequence of 4672 bp plus a polyA tail.[17]

The predicted protein encoded by the cDNA consisted of 1256 amino acid residues. The N-terminal region contained a leucine-rich-repeat (LRR) sequence[18,19] and the C-terminal region showed significant sequence homology to the well-known actin binding protein gelsolin.[20]

C. elegans

Database searches revealed that amino acid sequence with strong homology to the entire fliI LRR region, allowing for several putative introns, was encoded by the *C. elegans* cosmid CELB0303. However, the gelsolin-like region was not present. CELB0303 was one of the first three cosmids sequenced as part of the *C. elegans* genome project.[21] We reasoned that the complete sequence of the *C. elegans fliI* homolog would be useful in achieving the goal of cloning a mammalian homolog. The LRR homology was present near one end of CELB0303, and it appeared plausible that the gelsolin-like portion of the putative homolog would be encoded by adjacent, as yet unsequenced genomic DNA.

C. elegans cDNA libraries were therefore screened using a synthetic DNA probe based on the available CELB0303 cosmid sequence covering part of the predicted LRR coding region. A full-length cDNA was isolated, and complete sequence analysis of this 4529 bp clone revealed an encoded fliI protein homolog of 1257 amino acids, also consisting of an LRR domain and a gelsolin-like domain.[17] Subsequently the sequence of CELB0523, the relevant cosmid adjacent to CELB0303, became available, and as expected contains the remainder of the *fliI* homolog. Thus both the cDNA and genomic sequences for the *C. elegans fliI* homolog have been determined, enabling the complete exon/intron structure of the gene to be deduced.[17]

Human

Alignment of the 1256 amino acid *Drosophila* fliI protein with the 1257 residue *C. elegans* homolog showed they were 49% identical over their entire lengths (69% similarity when conservative substitutions are considered). In a number of regions, significant patches of adjacent amino acids were completely identical. We thought it possible to exploit this to clone the mammalian gene.

A total of 20 oligonucleotide primers were designed from the conserved areas by back-translation of the amino acid sequences using the genetic code. To reduce the complexity of the oligonucleotides, inosine was substituted at some positions of 4-fold (or 3-fold) degeneracy.[22] Nested PCR reactions were then conducted on human DNA samples. One combination of four primers was found to give a strong product band of 850 bp with human genomic DNA as template.[17] Cloning and sequence analysis of this band showed strong homology (>50% identity) of the encoded protein with the *Drosophila* fliI protein, allowing for several small introns. The same primers gave a band of 500 bp with human brain cDNA and sequence analysis of this fragment showed the same homologous region, but without the introns present in the 850 bp band, further confirming the existence of the human homolog.[17] The official names assigned by the relevant nomenclature committees are *FLII* for the human gene and *Fliih* for the mouse gene.

Using the genomic PCR fragment as a probe, human *FLII* cDNAs were isolated from brain cDNA libraries. Sequence analysis yielded a composite cDNA sequence of 4105 bp, most of which was covered by a single clone of 4087 bp lacking 18 bp at the 3′ end.[17] Although these clones did not contain the initiation codon, a cDNA covering this region was subsequently identified, and showed that only two bases of the ATG initiation codon had

been missed.[23] The encoded protein of 1269 amino acids (including the initiating methionine) also contains the LRR and gelsolin-like domains, and is 58% identical (74% similarity allowing for conservative substitutions) to the *Drosophila* protein.[17]

Southern analysis with *FLII* cDNA probes established that the gene is present as a single copy in the haploid human genome.[23,24] There is no evidence for related genes detectable under conditions of reduced hybridization stringency.[23] Northern analysis of polyA$^+$ mRNA showed that the gene is expressed in all tissues tested (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas).[23] The level of *FLII* mRNA in skeletal muscle was elevated 8- to 10-fold, heart 2- to 2.5-fold and lung 1.5- to 2-fold over the relatively uniform level in the remaining tissues. The levels in all tissues tested appear to parallel the expression levels of actin as determined on the same blot with a β-actin probe. Human gelsolin mRNA has a rather different tissue distribution,[25] with very low expression in liver, and elevated expression in placenta, for example. The high level of *FLII* mRNA in skeletal muscle is of particular interest in view of the *Drosophila* muscle phenotype of the viable alleles. It seems possible that homozygous mutations in the human *FLII* gene analogous to the viable *Drosophila* alleles could result in a human genetic muscular disorder.

Protein Structure

A schematic view of the *Drosophila*, *C. elegans* and human proteins, together with schematic views of some other members of the gelsolin family and proteins containing related LRR regions is shown in Figure 17.1. The FLII and related proteins all contain an N-terminal region of almost 400 amino acids made up of 16 copies of a leucine-rich-repeat (LRR) sequence. Similar LRR sequences are involved in protein-protein interactions in many other known cases.[18,19] The LRR region is highly conserved between the *Drosophila*, *C. elegans* and human FLII proteins.[17] This is followed by a region of about 100 amino acids that is less well conserved, and for which significant homology to other known sequences has not been detected. We speculate that this region may act as a linker or spacer region, although it may also have a more specific function. The C-terminal region of about 750 amino acids has significant homology to the actin binding protein gelsolin. This region of the human FLII protein has 31% identity (52% similarity when conservative amino acid substitutions are considered) to human gelsolin.[17,20] The gelsolin-like domain of the *Drosophila* fliI protein has 27% identity (48% similarity) to *Drosophila* gelsolin.[26,27]

Evolution of Gelsolin Gene Family

The discussion of the structure and properties of other gelsolin family members will be brief, as most are covered by other chapters in this book. So far, the mammalian genome appears to contain genes encoding five different gelsolin family members with readily detectable sequence homology. These are gelsolin itself,[20,28] villin,[29,30] Cap G,[31] the FLII protein[17,23] and adseverin or scinderin.[32,33] At the sequence level, gelsolin consists of two large related domains, and both of these contains evidence of a triplication.[34] The 3D structures of portions of gelsolin and related proteins indicates that each of the triplicated units folds into a discrete subdomain.[35-39] Recent evidence indicates that the related proteins destrin and cofilin, while having essentially no primary sequence homology to gelsolin-related proteins, have tertiary structures closely related to the individual triplicated subdomains of gelsolin, villin and severin.[40] Like gelsolin, villin has two copies of the unit containing the triplication, but possesses an unrelated C-terminal extension also involved in actin binding. Adseverin or scinderin appears most similar to gelsolin itself, and contains two copies of the triplication. Cap G has one copy of the unit containing the triplication. These relationships are depicted schematically in Figures 17.1 and 17.4.

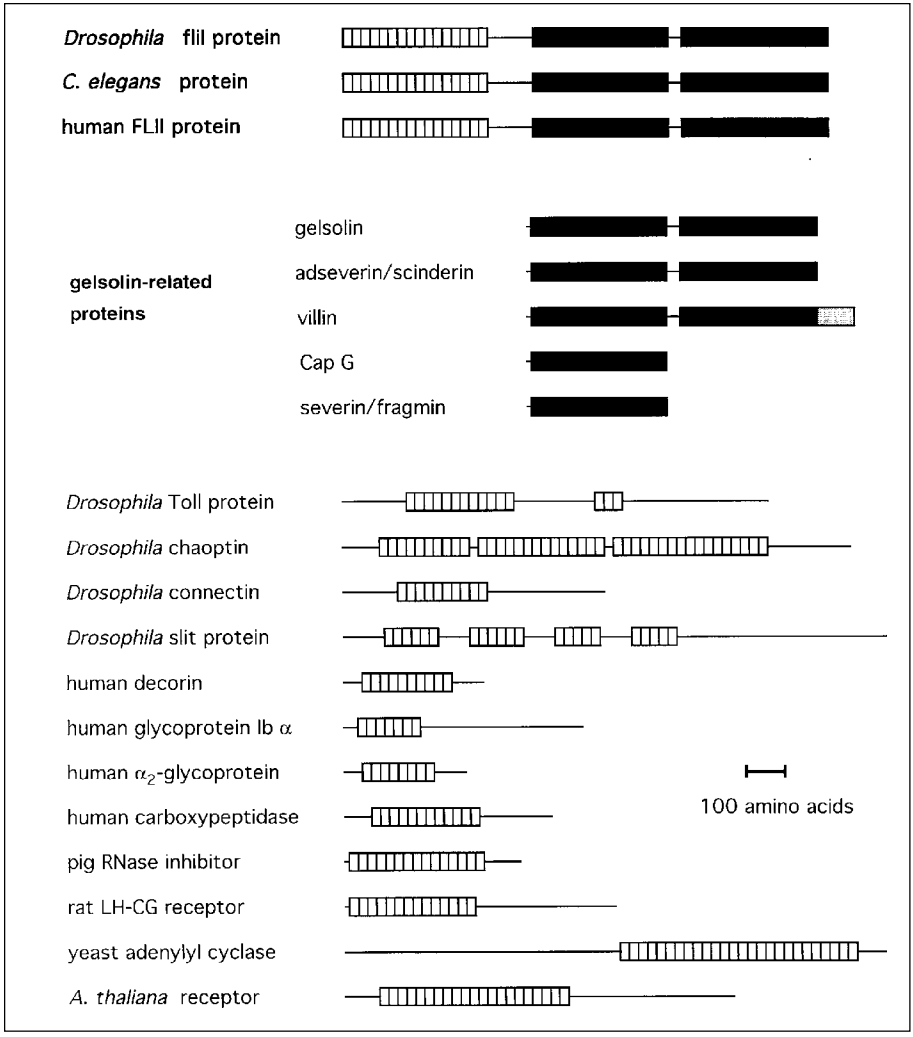


Fig. 17.1. Schematic structures of *Drosophila* fliI protein and homologues from *C. elegans* and human (FLII). The main known classes of gelsolin-related proteins are also depicted. A selection of the more than 60 known LRR-containing proteins is also shown. Open boxes, individual LRR units. Black boxes, domain present as two copies in gelsolins, adseverins, villins and FLII proteins, and as a single copy in Cap G proteins and the fungal proteins severin and fragmin. Note that this domain itself contains a triplication, consisting of 3 related subdomains (See Fig. 17.4). Destrin and cofilin (not shown), while having virtually no primary sequence homology to the gelsolin-related family, have a tertiary structure closely related to individual subdomains of the family. Modified from Campbell HD et al, Proc Natl Acad Sci 1993; 90:11386-11390, copyright (1993) National Academy of Sciences, USA.

Comparison of particular gelsolin family members between human and *Drosophila* indicates that the FLII proteins are much more strongly conserved than either gelsolin or villin. The gelsolin-like domains of the human and *Drosophila* FLII proteins are 59% identical (75% similarity; 9 gaps; GCG Gap program, default parameters). In comparison, human and *Drosophila* cytoplasmic gelsolins are 40% identical (58% similarity; 17 gaps). The gelsolin-like domain of human villin is only 29% identical (50% similarity; 19 gaps) to the gelsolin-like domain of the *Drosophila quail* protein,[41] a villin homolog.

Detailed phylogenetic analysis[23,42] of FLII proteins and other members of the gelsolin gene family indicates that the mammalian capping protein Cap G has arisen by deletion of segment 2 from a dimeric precursor closely related to the gelsolin/adseverin branch of the family. Therefore, it does not represent the precursor molecule from which the duplication was originally generated, as had earlier been thought,[31] but arose subsequent to the duplication. It is possible that the fungal proteins severin and fragmin may have evolved from the hypothetical precursor of the duplication event.

Human *FLII* Gene and Smith-Magenis Syndrome Deletion

Using the human *FLII* cDNA as a probe, initial FISH mapping results showed that *FLII* maps to the short arm of chromosome 17, near 17p11.2. Smith-Magenis syndrome (SMS) deletions also map to 17p11.2. SMS is a human chromosomal microdeletion syndrome characterized by mental retardation and a range of physical, developmental and behavioral abnormalities.[43-45] The *FLII* cDNA was used to isolate three human genomic cosmid clones from a chromosome 17-specific cosmid library, and these cosmids were used in FISH mapping experiments to map the position of *FLII* in relation to SMS deletions.[24] FISH mapping with a mixture of the three cosmids showed that *FLII* mapped to 17p11.2, into the critical region deleted in all SMS patients tested. These results were further confirmed by Southern analysis of DNA from somatic cell hybrid cell lines containing a range of 17p abnormalities including SMS deletions. *FLII* is therefore a candidate gene for SMS.[24]

Using the three *FLII* cosmids described, the *FLII* gene was shown to be deleted in a case where cytogenetic findings had been equivocal.[46] Flow cytometric analysis suggested that the deletion in this case was < 2 Mb.[46] Deletions ranging from < 2 Mb up to 9-10 Mb are known, with there being no apparent correlation between the size of the deletion and the severity of symptoms.[47] Nevertheless, a large number of genes would be deleted from one chromosome 17 even in patients with deletions in the vicinity of 2 Mb. Most likely, haploinsufficiency of one or a small number of genes in this interval is responsible for the syndrome. It is also possible that mutations of the remaining allele of a gene or genes deleted in SMS plays some role in generation of the phenotypic effects, at least in some cases. A number of other genes have been shown to map into the SMS interval, including *LLGL*,[48,49] the human homologue of the *D. melanogaster lethal(2) giant larvae* (*l(2)gl*) gene.[50-53]

Childhood primitive neuroectodermal tumors (PNETs) are the commonest brain tumors in children. Recent work indicates that chromosomal breakpoints involved in the hemizygous loss of 17p commonly involved in PNETs cluster at 17p11.2 in an area which overlaps the SMS critical region.[54,55] A body of evidence, beyond the scope of this review, further indicates the possible location of previously unidentified tumor suppressor genes in this general region. *FLII* and *LLGL* are therefore candidate genes for involvement in the biology of PNETs and possibly other tumors, as well as SMS.

Genomic Structure of *FLII* Genes

Southern blotting suggests that a single copy of the human *FLII* gene is present in the haploid genome, and a single chromosomal location has been shown by FISH analysis. Only one copy of the corresponding gene has so far been detected in available *C. elegans* genomic

sequences, which now constitute almost 70% of the genome. The *Drosophila fliI* gene also appears to be present as a single copy.[17,56]

The structure of the *C. elegans* genomic *fliI* gene homolog has been reported (GenBank entry U01183).[17] As discussed above, this was evaluated by comparing the structure of the *C. elegans fliI*-homologous cDNA which had been cloned and sequenced[17] with the structure of the corresponding genomic DNA in cosmids B0303 (GenBank M77697) and B0523 (GenBank L07143).[21] The *C. elegans* gene consists of 14 exons split by 13 introns. The first intron splits the 5′ untranslated region. It is of interest that the *C. elegans fliI*-homologous gene contains a "gene within a gene", as the previously identified *sup-5* gene[57-59] (GenBank X54122), encoding $tRNA^{Trp}$, lies in intron 9.[17] The functional significance of this arrangement, if any, is unknown.

Comparison of the *Drosophila fliI* cDNA structure with the complete genomic *fliI* sequence from the Oregon R strain of *D. melanogaster*, determined in Canberra, revealed the presence of four exons and three introns, with the first intron of the *Drosophila* gene corresponding exactly in position to intron two of the *C. elegans* gene.[17] The *Drosophila fliI* genomic sequence was also determined in Hawaii for the Canton S strain by G. de Couet and co-workers, and the mutations in two viable and two lethal alleles were analyzed.[56] The lethal allele *l(1)D44* is almost certainly a null, as it contains a deletion of ~400 bp spanning all of exon 1, some 5′ flanking sequence, and 66 bp of intron 1. Two viable alleles both contain substitutions of serine for conserved glycines located in the subdomain equivalent to the S1 domain of gelsolin. These substitutions are on separate loops but located close to one another on the surface; it was stated that no specific conclusions can be drawn from them at this stage.[56] It would be of interest to generate a mouse in which one of these mutations had been inserted in the *Fliih* gene; if affected in muscle tissue, this mouse would represent a model for a potential human genetic disorder of muscle involving *FLII*.

None of the original three *FLII* cosmids (c62F2, c70E2 and c92C10) identified in the study on the mapping of *FLII* into the SMS critical region[24] spanned the entire human *FLII* gene (Fig. 17.2). Therefore a number of additional cosmids were isolated from gridded libraries,[60,61] and a detailed restriction map of the genomic region surrounding *FLII* was constructed. One cosmid, c5C2, which spanned the entire *FLII* gene was chosen for detailed analysis as it contained a vector *Not*I site located adjacent to the 5′ end of the gene. This site enabled convenient subcloning of a 13.7 kb *Not*I fragment containing most of the gene (Fig. 17.2). This 13.7 kb fragment was fully sequenced on both strands, and additional sequencing was done on the adjacent 9 kb *Not*I fragment (Fig. 17.2), yielding the complete structure of the human *FLII* gene.[23]

The human *FLII* gene consists of 30 exons split by 29 introns, spanning 14 kb of genomic DNA,[23] and is schematically depicted in Figure 17.3. Intron 1 of the *FLII* gene corresponds exactly in position to intron 2 of *C. elegans* and intron 1 of *Drosophila*; this intron is located in the N-terminal region of the LRR domain, and is the only intron conserved in position between all three homologs. The third intron of *Drosophila* is conserved in position in human *FLII*, and two other introns are conserved in position between *C. elegans* and human (Fig. 17.4). A number of introns are conserved between various gelsolin family members (Fig. 17.4A), and at least some may have been present prior to the final gene duplication event that gave rise to the dimeric family members (Fig. 17.4B). Some may also have been present prior to the ancestral triplication (Fig. 17.4C). There also appears to be good evidence for intron loss from gelsolin family members. Information from intron positions is contributing to our understanding of gelsolin family evolution.[23]

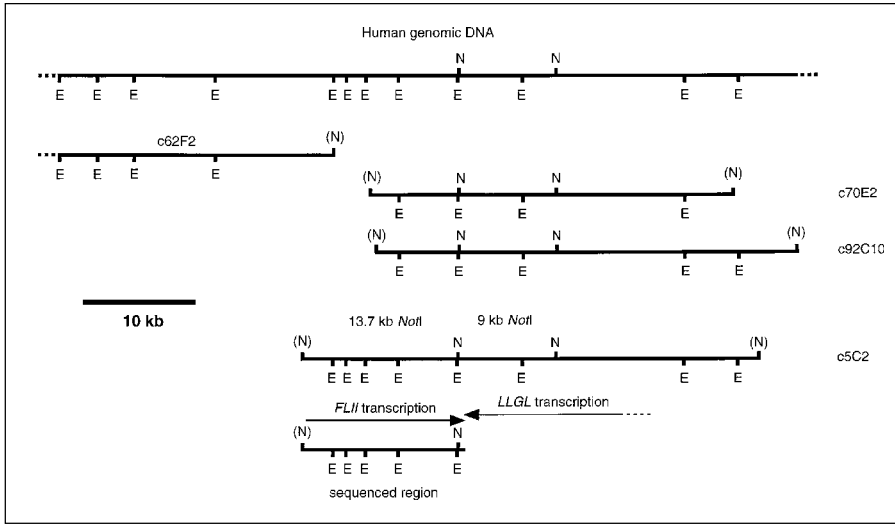


Fig. 17.2. Genomic map and cloning strategy for human *FLII* gene. The top line shows human genomic DNA in the vicinity of *FLII*. Below this are indicated the structures of 4 human genomic cosmids, c62F2, c70E2, c92C10[24] and c5C2.[23] At the bottom is indicated the sequenced region containing the complete *FLII* transcription unit.[23] The direction of *FLII* and *LLGL* transcription are indicated by the arrows. N, *Not*I site and E, *Eco*RI site present in genomic DNA. (N), *Not*I site derived from cosmid polylinker. The scale is indicated.

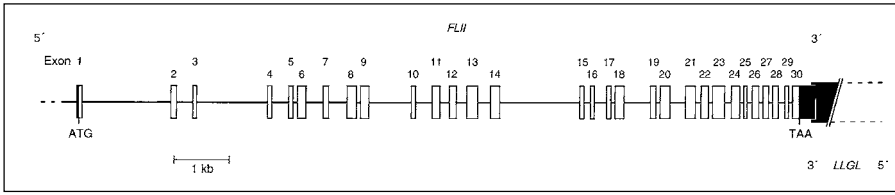


Fig. 17.3. Map of human *FLII* gene showing exon/intron structure. The exons are boxed and numbered 1-30. Shaded portions of boxes indicate untranslated regions and open boxes represent coding sequences. The ATG initiation codon and TAA termination codons are marked. The 3′ end of the human *LLGL* gene and the extent of the overlap with *FLII* are depicted. The scale is indicated. Reprinted with permission from Campbell HD et al, Genomics 1997; 42:46-54.

Overlap of Mammalian *FLII* with *LLGL*

The *Drosophila lethal(2) giant larvae* gene, *l(2)gl* was one of the first tumor suppressor genes to be identified and cloned from any organism.[50-53] *Llglh*, the mouse homolog of *l(2)gl*, was cloned as a potential target gene for the homeobox gene *Hox-C8* by enriching paraformaldehyde-fixed protein-genomic DNA complexes using an anti-Hox-C8 antibody.[62] More recently, *LLGL*, the human homolog of *l(2)gl* has been cloned using the mouse *Llglh* cDNA or oligonucleotides based on it as the probe.[48,49]

While sequencing the *FLII* gene, we sequenced both ends of a 9 kb *Not*I fragment from the *FLII* genomic locus (Fig. 17.2). One end of this contained part of the final exon of *FLII*. The other end matched part of the *LLGL* cDNA sequence,[48,49] indicating that the *FLII* and *LLGL* genes are close together, and in the opposite orientation. In confirmation of the close

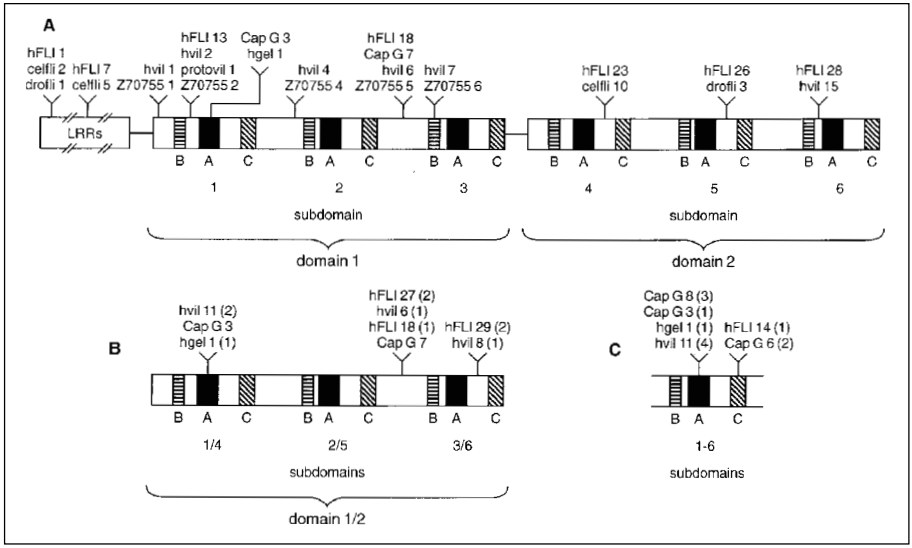


Fig. 17.4. Schematic structure of FLII proteins and conservation of intron position. Domains 1 and 2, subdomains 1-6, and the B, A and C motifs of the subdomains are indicated schematically. hFLI, human *FLII* gene; celfli, *C. elegans fliI* gene; drofli, *D. melanogaster fliI* gene; hvil, human villin gene; protovil, *D. discoideum* protovillin gene; Z70755, *C. elegans* gene encoding reading frame K06A4.3 in GenBank entry Z70755; Cap G, human Cap G gene; hgel, human gelsolin gene. The intron number follows the abbreviation for each gene. Introns conserved within 1 base are indicated. A. Introns conserved between different members of the gelsolin gene family. B. Introns conserved between domains 1 and 2 of family members. The numbers in brackets indicate the domains (1 or 2) within which the introns occur. C. Introns conserved between the subdomains of family members. The numbers in brackets indicate the subdomains (1-6) within which these introns occur. Reprinted with permission from Campbell HD et al, Genomics 1997; 42:46-54.

proximity of these genes, it was found that five cosmids from a gridded chromosome 17 cosmid library[60] hybridized to both the *FLII* cDNA probe and a PCR-generated *LLGL* cDNA probe.[23] A search of the sequence databases with the 3′ end sequence from *FLII* cDNA revealed a short but strong match to the 3′ end (in the opposite orientation) of the cDNA for mouse *Llglh*. The available cDNA sequences for *LLGL*[48,49] are truncated at the 3′ end so it was not possible immediately to compare the 3′ end of *FLII* cDNA with that of human *LLGL* cDNA. However, we had also determined the sequence of mouse *Fliih* genomic DNA at this time, and analysis of 2.5 kb of sequence extending 3′ from within *Fliih* showed that it corresponded exactly to the 3′ end of *Llglh* cDNA, allowing for several introns (H. D Campbell and S. Fountain, unpublished results). This formally established that mouse *Fliih* and *Llglh* overlap.[23]

To verify that human *FLII* and *LLGL* overlap, sequence corresponding to the complete 3′ end of *LLGL* cDNA was required. We designed PCR primers corresponding to unique *LLGL* sequence in the 3′ UTR, and then used these in nested PCR reactions on human brain cDNA libraries to amplify a 600 bp fragment. One end of this fragment matched *LLGL* cDNA, as expected. Unfortunately, the other end did not extend to the *LLGL* polyA site. However, it overlapped an EST carrying the complete 3′ end of *LLGL* cDNA. Comparison of the 3′ end of the human *FLII* cDNA and genomic sequences with this 3′ end sequence for

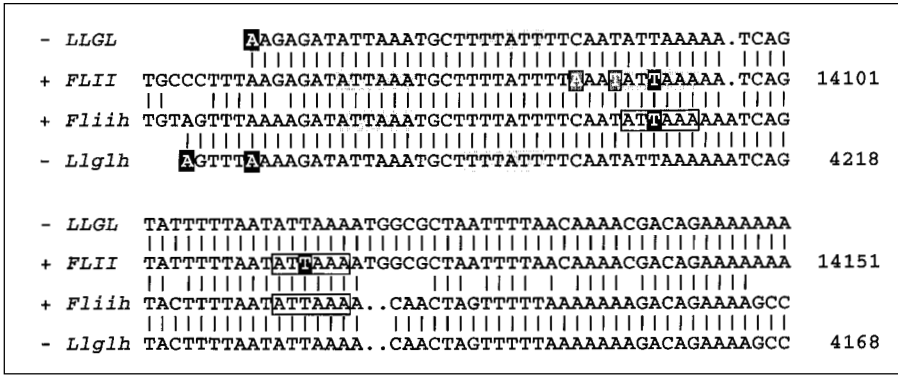


Fig. 17.5. Overlap between human *FLII* and *LLGL*, and mouse *Fliih* and *Llglh* genes. The genes are on opposite strands in a tail-to-tail orientation, with their 3′ ends overlapping. Poly(A) signals for which transcripts have been observed are indicated by shaded blocks of text (ATTAAA for *FLII*/*Fliih* and AATAAA, depicted here as TTTATT on the opposite strand, for *LLGL*/*Llglh*). The major poly(A) sites relating to these poly(A) signals are depicted by solid black boxes, and minor sites by shaded boxes, located 13-22 bases downstream of the respective poly(A) signals. Potential poly(A) signals for which transcripts have not yet been observed are indicated by open boxes. Modified and reproduced with permission from Campbell HD, Genomics 1997; 42:46-54.

LLGL cDNA established that the 3′ ends of the human *FLII* and *LLGL* transcripts overlap.[23] The region of overlap is highly conserved between human and mouse and is depicted in Figure 17.5.

The overlap region contains at least two functional polyA signals for *FLII*, both of which are the variant ATTAAA signal rather than the more usual AATAAA. All of the brain cDNAs we cloned[17] use the most 5′ of these, and the corresponding major polyA site (Fig. 17.5). The majority of human *FLII* EST clones are polyadenylated using the 5′ most polyA signal, mostly at the major site but also at two minor sites (Fig. 17.5). Mouse brain *Fliih* cDNAs are also polyadenylated using the corresponding signal and major site (Fig. 17.5) (H.D. Campbell and S. Fountain, unpublished results). A few *FLII* EST clones use a more 3′ polyA signal (Fig. 17.5). A third conserved ATTAAA sequence is located just downstream (Fig. 17.5). Possibly this may also serve as a polyA signal for *FLII*/*Fliih*. Whether the use in the *FLII* gene of multiple copies of the weaker, variant ATTAAA polyA signal has regulatory significance, and how signal/site selection is governed[63-65] remain to be established. In this context, it is of interest that the *C. elegans fliI*-homologous gene also uses a variant poly(A) signal, a single copy of AATAAT.[17] The *Drosophila fliI* gene uses a single copy of the canonical AATAAA sequence.[17,56]

A single conserved AATAAA polyA signal for *LLGL* is present in the overlap region (Fig. 17.5). Sites of polyadenylation for *LLGL* and *Llglh* as determined from the single available EST cDNA clones for each and the full-length mouse *Llglh* cDNA sequence (Tomotsune et al, 1993) are shown in Figure 17.5. An additional potential polyA signal for *LLGL*/*Llglh* consisting of the variant ATTAAA sequence is also present in the overlap region (Fig. 17.5; not indicated), but it is not known whether this signal is functional. If it is, it would be expected to be utilized at a lower level.

The exact significance, if any, of the overlap of *FLII* with *LLGL* in human, and of *Fliih* and *Llglh* in mice is unclear. One consequence may be that mutations in this region could affect expression of both genes. In *Drosophila*, *l(2)gl* maps to 21A,[50] whereas *fliI* maps to

19F.[14] In *C. elegans*, a homolog of *l(2)gl* is present (GenBank Accession No. U51993, reading frame F56F10.4) and maps to the X chromosome, while the *fliI* homolog maps to chromosome III.[17,21] Therefore it is clear that, at least in some eukaryotic organisms, it is not necessary for the genes to be overlapping, or even on the same chromosome.

However, in the context of the overlap of the genes in humans and mice, it is intriguing that the two genes may both be involved with the actin-based cytoskeleton. *FLII* and its homologu encode members of the gelsolin family of actin-binding proteins, and a subdomain of the FLII protein itself has been shown to bind G actin in a 1:1 stoichiometry, although not to sever F actin filaments as the cognate subdomain of gelsolin does.[66] In *Drosophila*, the fliI protein may be required for the correct distribution of actin during cellularization.[67] The *Drosophila* l(2)gl protein has been shown to be a component of the cytoskeleton[68] and to interact with nonmuscle myosin II.[69,70] Recently, it has been shown that the l(2)gl protein is required for epithelial cell shape changes during development, and it was concluded that it probably plays a role in gastrulation.[71] The association of the l(2)gl protein with nonmuscle myosin appears to be regulated by the activity of a protein kinase which associates with the l(2)gl protein and phosphorylates it on serine residues.[69,70] The human LLGL protein has also been shown to be a component of the cytoskeleton and to interact with nonmuscle myosin II.[49]

Biological Role of FLII Proteins

As noted, the FLII proteins contain a gelsolin-related domain[17] which in the case of the human FLII protein has been shown, as expected, to interact with actin.[66] One of the most closely related LRR sequences is that of yeast adenylate cyclase.[42,72] The yeast adenylate cyclase LRR has been shown to directly interact with the Ras protein,[73] suggesting a similar role for the FLII LRR.[42] The LRR region of FLII is also more closely related to the LRRs of the human and mouse Rsu-1 proteins[74,75] than to other LRR proteins.[42] The mouse *Rsu-1* gene was originally cloned as *Rsp-1*, a suppressor of Ras-mediated transformation, and has been shown to function in vivo as a dominant negative regulator of v-Ras,[74] and as a suppressor of tumorigenicity in the U251 glioblastoma cell line.[76] Recently it has been shown that Rsu-1 interacts with the serine/threonine kinase Raf-1 in vitro, although this interaction may not be mediated via the Rsu-1 LRR region but via the C-terminus.[77] In summary, detailed analysis of FLII protein LRRs in comparison with a number of known LRRs in various proteins including yeast adenylate cyclase and the mammalian Rsu-1 proteins led to the conclusion that the LRRs of FLII proteins may be involved in interaction with a member of the Ras family of proteins.[42]

If this is the case, a role for the FLII protein in modulation of the cytoskeleton by Ras-related signal transduction pathways can be envisaged. Abundant indirect evidence supports the concept that there is some link between Ras-related pathways and regulation of the cytoskeleton. The Ras-related GTPases CDC42, Rac and Rho, for example, are implicated in morphogenesis and cytoskeletal organization.[78-80] The *Drosophila* Rac and CDC42 homologs appear to be involved in muscle development at the myoblast fusion stage and in neuronal development.[81] A dominant inhibitory form of the same *Drosophila* Rac homolog affects dorsal closure during embryogenesis, with disruption of the accumulation of cytoskeletal actin and myosin along the leading edge.[82] In mammals, the Wiskott-Aldrich syndrome protein WASP, involved at least indirectly in modulation of the actin cytoskeleton, has been shown to interact with CDC42.[83]

Mammalian systems probably contain at least 50 Ras-related GTPases.[80] Recent work suggests direct interactions between certain of these Ras-related molecules and components of the cytoskeleton or related molecules. Rad, a novel Ras family member, is most highly expressed in skeletal muscle, heart and lung, and interacts with tropomyosin, a structural

component of muscle and cytoskeletal actin filaments.[84] Two different but closely related unconventional myosins, rat myr 5[85] and human myosin-IXb,[86] have an N-terminal extension homologous to GTPase activating proteins (GAPs) active on the Rho/Rac family of Ras-related proteins. The myr 5 GAP region was expressed and shown to stimulate GTP hydrolysis by recombinant Rho, CDC42, and to a lesser extent, Rac,[85] establishing that a direct interaction between this unconventional myosin and Rho/CDC42/Rac can occur in vitro.

In a recent detailed germline clone study of the defects in cellularization and gastrulation in *fliI* mutant embryos,[67] it was found that during cellularization of the syncytial blastoderm, nuclei in mutant embryos migrate normally to the egg periphery, but then lose their precise cortical positioning. Prior to cellularization, the alignment of the nuclei near the periphery in mutant embryos is slightly less regular than in wild-type embryos. Some nuclei even lose their positioning near the periphery and move towards the center of the egg. In wild-type embryos, the invaginating cleavage furrows that separate the nuclei advance synchronously and in parallel within the entire egg. In contrast, in *fliI* mutant embryos, the cleavage furrows advance in a more irregular fashion, with the furrows running deeper between some nuclei than others. In wild-type embryos, the cleavage furrows are perpendicular to the egg surface, whereas many of the furrows in mutant embryos occur at a variety of angles, giving a disordered appearance in cross-sections. The ends of the advancing cleavage furrows in wild-type embryos terminate in a characteristic tear-drop-shaped area of the furrow canals. In mutant embryos, these structures are not seen, and the ends of the furrow canals appear collapsed. The mutant furrow canals are also associated with small membrane vesicles not found in the wild-type.[67]

Other aspects of the cellularization process are also abnormal in *fliI* mutant embryos.[67] In wild type embryos, gastrulation commences when cellularization is complete. Ventral cells flatten, constrict on their apical side, and the nuclei move basally. In mutant embryos, similar events occur although a significant fraction of the peripheral cytoplasm remains open towards the interior of the blastoderm. In severely affected mutant embryos, nuclei in a ventral position, which normally move away from the apical cell surface, move out of the peripheral layer of cytoplasm towards the interior of the egg. In this case, no ventral furrow is formed. Studies using fluorescent phalloidin to visualize the actin cytoskeleton reveal that in mutant embryos, actin is irregularly distributed along the cleavage furrow membranes, and that like the cleavage furrow membranes themselves, the actin cytoskeleton in the mutant embryos reaches to varying depths. In addition, punctate actin staining, localized to the interior region of the cellularizing blastoderm in the wild-type, reaches into the cellularizing region of *fliI* mutant embryos.[67] These observations support the notion that the fliI protein is intimately involved in some way with the actin cytoskeleton, which is known to be involved in the cellularization process.[87]

Concluding Remarks

Since the FLII proteins are evolutionarily highly conserved throughout both the LRR and gelsolin domains, it seems very likely that important aspects of their function at a biochemical level are highly conserved also. What process(es) in mammals or *C. elegans*, which do not develop by way of a syncytial blastoderm, correspond at a biochemical level to the particular role being played by the fliI protein during cellularization of the *Drosophila* syncytial blastoderm? We have hypothesized that the FLII proteins play a role in regulating the cytoskeleton, possibly mediated via Ras-related signal transduction pathways. Thus, the role of the fliI protein in *Drosophila* cellularization may be an example of the recruitment of a molecule (or pathway) with a more general function into a specialized developmental arena. Information from studies in the variety of experimental organisms already available

(*Drosophila*, *C. elegans*, mouse and human) should enable rapid progress in understanding the exact nature of the function of the FLII proteins.

Acknowledgments

C. Claudianos, M. Crouch, D. Davy, S. Fountain, K. Matthaei and I. G. Young are thanked for comments on the manuscript and valuable discussions.

References

1. Mogami K, O'Donnell PT, Bernstein SI et al. Mutations of the *Drosophila* myosin heavy-chain gene: Effects on transcription, myosin accumulation, and muscle function. Proc Natl Acad Sci USA 1987; 83:1393-1397.
2. Karlik CC, Coutu MD, Fyrberg EA. A nonsense mutation within the *act88F* actin gene disrupts myofibril formation in *Drosophila* indirect flight muscles. Cell 1984; 38:711-719.
3. Karlik CC, Fyrberg EA. An insertion within a variably spliced *Drosophila* tropomyosin gene blocks accumulation of only one encoded isoform. Cell 1985; 41:57-66.
4. Tansey T, Mikus MD, Dumoulin M et al. Transformation and rescue of a flightless *Drosophila* tropomyosin mutant. EMBO J 1987; 6:1375-1385.
5. Fyrberg E, Fyrberg CC, Beall C et al. *Drosophila melanogaster* troponin-T mutations engender three distinct syndromes of myofibrillar abnormalities. J Mol Biol 1990; 216:657-675.
6. O'Brien SJ, MacIntyre RJ. The α–glycerophosphate cycle in *Drosophila melanogaster*. II. Genetic aspects. Genetics 1972; 71:127-138.
7. Kotarski MA, Pickert S, Leonard DA et al. The characterization of α-glycerophosphate dehydrogenase mutants in *Drosophila melanogaster*. Genetics 1983; 105:371-386.
8. Davis MB, MacIntyre RJ. A genetic analysis of the α–glycerophosphate oxidase locus in *Drosophila melanogaster*. Genetics 1988; 120:755-766.
9. Homyk T Jr, Sheppard DE. Behavioral mutants of *Drosophila melanogaster*. I. Isolation and mapping of mutations which decrease flight ability. Genetics 1977; 87:95-104.
10. Koana T, Hotta Y. Isolation and characterization of flightless mutants in *Drosophila melanogaster*. J Embryol Exp Morphol 1978; 45:123-143.
11. Deak II, Rähmi A, Bellamy PR et al. Developmental and genetic studies of the indirect flight muscles of *Drosophila melanogaster*. In: Siddiqui D, Babu P, Hall LM et al, eds. Development and Neurobiology of *Drosophila*. New York: Plenum Press, 1980:183-192.
12. Deak II, Bellamy PR, Bienz M et al. Mutations affecting the indirect flight muscles of *Drosophila melanogaster*. J Embryol exp Morph 1982; 69:61-81.
13. Perrimon N, Smouse D, Miklos GLG. Developmental genetics of loci at the base of the *X* chromosome of *Drosophila melanogaster*. Genetics 1989; 121:313-331.
14. Miklos GLG, de Couet HG. The mutations previously designated as *flightless-I*^{3}, *flightless-O*^{2} and *standby* are members of the *W-2* lethal complementation group at the base of the X-chromosome of *Drosophila melanogaster*. J Neurogenet 1990; 6:133-151.
15. Lifschytz E, Falk R. Fine structure analysis of a chromosome segment in *Drosophila melanogaster*. Mutat Res 1969; 8:147-155.
16. Homyk T. Behavioral mutants of *Drosophila melanogaster*. II. Behavioral analysis and focus mapping. Genetics 1977; 87:105-128.
17. Campbell HD, Schimansky T, Claudianos C et al. The *Drosophila melanogaster flightless-I* gene involved in gastrulation and muscle degeneration encodes gelsolin-like and leucine-rich-repeat domains, and is conserved in *Caenorhabditis elegans* and human. Proc Natl Acad Sci 1993; 90:11386-11390.
18. Kobe B, Deisenhofer J. The leucine-rich repeat: a versatile binding motif. Trends Biochem Sci 1994; 19:415-421.
19. Kobe B, Deisenhofer J. Proteins with leucine-rich repeats. Current Opinion in Structural Biology 1995; 5:409-416.
20. Kwiatkowski DJ, Stossel TP, Orkin SH et al. Plasma and cytoplasmic gelsolins are encoded by a single gene and contain a duplicated actin-binding domain. Nature 1986; 323:455-458.

21. Sulston J, Du Z, Thomas K et al. The *C. elegans* genome sequencing project: A beginning. Nature 1992; 356:37-41.
22. McPherson MJ, Jones KM, Gurr SJ. PCR with highly degenerate primers. In: McPherson MJ, Quirke P, Taylor GR, eds. PCR: A Practical Approach. Oxford: IRL Press, 1991:171-186.
23. Campbell HD, Fountain S, Young IG et al. Genomic structure, evolution and expression of human *FLII*, a gelsolin and leucine-rich-repeat family member: overlap with *LLGL*. Genomics 1997; 42:46-54.
24. Chen K-S, Gunaratne PH, Hoheisel JD et al. The human homologue of the *Drosophila melanogaster* flightless-I gene (*fliI*) maps within the Smith-Magenis microdeletion critical region in 17p11.2. Am J Hum Genet 1995; 56:175-182.
25. Paunio T, Kangas H, Kiuru S et al. Tissue distribution and levels of gelsolin mRNA in normal individuals and patients with gelsolin-related amyloidosis. FEBS Lett 1997; 406:49-55.
26. Heintzelman MB, Frankel SA, Artavanis-Tsakonas S et al. Cloning of a secretory gelsolin from *Drosophila melanogaster*. J Mol Biol 1993; 230:709-716.
27. Stella MC, Schauerte H, Straub KL et al. Identification of secreted and cytosolic gelsolin in *Drosophila*. J Cell Biol 1994; 125:607-616.
28. Kwiatkowski D J, Mehl R, Yin HL. Genomic organization and biosynthesis of secreted and cytoplasmic forms of gelsolin. J Cell Biol 1988; 106:375-384.
29. Bazari, WL, Matsudaira P, Wallek M et al. Villin sequence and peptide map identify six homologous domains. Proc Natl Acad Sci 1988; 85:4986-4990.
30. Pringault E, Robine S, Louvard D. Structure of the human villin gene. Proc Natl Acad Sci 1991; 88:10811-10815.
31. Mishra VS, Henske EP, Kwiatkowski DJ et al. The human actin-regulatory protein Cap G: gene structure and chromosome location. Genomics 1994; 23:560-565.
32. Nakamura S, Sakurai T, Nonomura Y. Differential expression of bovine adseverin in adrenal gland revealed by in situ hybridization. Cloning of a cDNA for adseverin. J Biol Chem 1994; 269:5890-5896.
33. Marcu MG, Rodriguez del Castillo A, Vitale ML et al. Molecular cloning and functional expression of chromaffin cell scinderin indicates that it belongs to the family of Ca(2+)-dependent F-actin severing proteins. Mol Cell Biochem 1994; 141:153-165.
34. Way M, Weeds A. Nucleotide sequence of pig plasma gelsolin. Comparison of protein sequence with human gelsolin and other actin-severing proteins shows strong homologies and evidence for large internal repeats. J Mol Biol 1988; 203:1127-1133.
35. McLaughlin PJ, Gooch JT, Mannherz HG et al. Structure of gelsolin segment 1-actin complex and the mechanism of filament severing. Nature 1993; 364:685-692.
36. Rozycki MD, Myslik JC, Schutt CE et al. Structural aspects of actin-binding proteins. Curr Opin Cell Biol 1994; 6:87-95.
37. Markus MA, Nakayama T, Matsudaira P et al. Solution structure of villin 14T, a domain conserved among actin-severing proteins. Protein Sci 1994; 3:70-81.
38. Markus MA. Solving the atomic resolution structure of a protein in solution: nuclear magnetic resonance studies of villin 14T. Pharm Acta Helv 1996; 71:65-78.
39. Schnuchel A, Wiltscheck R, Eichinger L et al. Structure of severin domain 2 in solution. J Mol Biol 1995; 247:21-27.
40. Hatanaka H, Ogura K, Moriyama K et al. Tertiary structure of destrin and structural similarity between two actin-regulating protein families. Cell 1996; 85:1047-1055.
41. Mahajan-Miklos S, Cooley L. The villin-like protein encoded by the *Drosophila quail* gene is required for actin bundle assembly during oogenesis. Cell 1994; 78:291-301.
42. Claudianos C, Campbell HD. The novel *flightless-I* gene brings together two gene families, actin binding proteins related to gelsolin and leucine-rich-repeat proteins involved in ras signal transduction. Mol Biol Evol 1995; 12:405-414.
43. Smith AC, McGavran L, Robinson J et al. Interstitial deletion of (17)(p11.2p11.2) in nine patients. Am J Med Genet 1986; 24:393-414.
44. Greenberg F, Guzzetta V, Montes de Oca-Luna R et al. Molecular analysis of the Smith-Magenis syndrome: a possible contiguous-gene syndrome associated with del(17)(p11.2). Am J Hum Genet 1991; 49:1207-1218.

45. Greenberg F, Lewis RA, Potocki L et al. Multi-disciplinary clinical study of Smith-Magenis syndrome (deletion 17p11.2). Am J Med Genet 1996; 62:247-254.
46. Juyal RC, Greenberg F, Mengden GA et al. Smith-Magenis syndrome deletion: a case with equivocal cytogenetic findings resolved by fluorescence in situ hybridization. Am J Med Genet 1995; 58:286-291.
47. Trask BJ, Mefford H, van den Engh G et al. Quantification by flow cytometry of chromosome-17 deletions in Smith-Magenis syndrome patients. Hum Genet 1996; 98:710-718.
48. Koyama K, Fukushima Y, Inazawa J et al. The human homologue of the murine *Llglh* gene (*LLGL*) maps within the Smith-Magenis syndrome region in 17p11.2. Cytogenet Cell Genet 1996 72:78-82.
49. Strand D, Unger S, Corvi R et al. A human homologue of the *Drosophila* tumour suppressor gene *l(2)gl* maps to 17p11.2-12 and codes for a cytoskeletal protein that associates with nonmuscle myosin II heavy chain. Oncogene 1995; 11:291-301.
50. Mechler BM, McGinnis W, Gehring WJ. Molecular cloning of *lethal(2)giant larvae*, a recessive oncogene of *Drosophila melanogaster*. EMBO J 1985; 4:1551-1557.
51. Jacob L, Opper M, Metzroth B et al. Structure of the *l(2)gl* gene of *Drosophila* and delimitation of its tumor suppressor domain. Cell 1987; 50:215-225.
52. Lützelschwab R, Klämbt C, Rossa R et al. A protein product of the *Drosophila* recessive tumor gene, *l(2)gl*, potentially has cell adhesion properties. EMBO J 1987; 6:1791-1797.
53. Watson KL, Justice RW, Bryant PJ. *Drosophila* in cancer research: The first fifty tumor suppressor genes. J Cell Sci Suppl 1994; 18:19-33.
54. Scheurlen WG, Seranski P, Mincheva A et al. High-resolution deletion mapping of chromosome arm 17p in childhood primitive neuroectodermal tumors reveals a common chromosomal disruption within the Smith-Magenis region, an unstable region in chromosome band 17p11.2. Genes Chromosomes Cancer 1997; 18:50-58.
55. Wilgenbus KK, Seranski P, Brown A et al. Molecular characterization of a genetically unstable region containing the SMS critical area and a breakpoint cluster for human PNETs. Genomics 1997; 42:1-10.
56. de Couet HG, Fong KSK, Weeds AG et al. Molecular and mutational analysis of a gelsolin-family member encoded by the *flightless I* gene of *Drosophila melanogaster*. Genetics 1995; 141:1049-1059.
57. Waterston RH, Brenner S. A suppressor mutation in the nematode acting on specific alleles of many genes. Nature 1978; 275:715-719.
58. Wills N, Gesteland RF, Karn J et al. The genes *sup-7 X* and *sup-5 III* of *C. elegans* suppress amber nonsense mutations via altered transfer RNA. Cell 1983; 33:575-583.
59. Kondo K, Hodgkin J, Waterston RH. Differential expression of five tRNA(UAGTrp) amber suppressors in *Caenorhabditis elegans*. Mol Cell Biol 1988; 8:3627-3635.
60. Kallioniemi O-P, Kallioniemi A, Mascio L et al. Physical mapping of chromosome 17 cosmids by fluorescence in situ hybridization and digital image analysis. Genomics 1994; 20:125-128.
61. Zehetner G, Lehrach H. The Reference Library System—sharing biological material and experimental data. Nature 1994; 367:489-491.
62. Tomotsune D, Shoji H, Wakamatsu Y et al. A mouse homologue of the *Drosophila* tumour-suppressor gene l*(2)gl* controlled by *Hox-C8* in vivo. Nature 1993; 365:69-72.
63. Proudfoot N. Ending the message is not so simple. Cell 1996; 87:79-781.
64. Wahle E, Keller W. The biochemistry of polyadenylation. Trends Biochem Sci 1996; 21:247-250.
65. Edwalds-Gilbert G, Veraldi KL, Milcarek C. Alternative poly(A) site selection in complex transcription units: means to an end? Nucleic Acids Res 1997; 25:2547-2561.
66. Orloff GJ, Allen PG, Miklos GLG et al. Human flightless-I has actin binding ability. Mol Biol Cell 1995; 6:139.
67. Straub KL, Stella MC, Leptin M. The gelsolin-related *flightless-I* protein is required for actin distribution during cellularisation in *Drosophila*. J Cell Sci 1996; 109:263-270.
68. Strand D, Raska I, Mechler BM. The *Drosophila* lethal(2)giant larvae tumor suppressor protein is a component of the cytoskeleton. J Cell Biol 1994; 127:1345-1360.

69. Strand D, Jakobs R, Merdes G et al. The *Drosophila* lethal(2)giant larvae tumor suppressor protein forms homo-oligomers and is associated with nonmuscle myosin II heavy chain. J Cell Biol 1994; 127:1361-1373.
70. Kalmes A, Merdes G, Neumann B et al. A serine-kinase associated with the p127-l(2)gl tumour suppressor of *Drosophila* may regulate the binding of p127 to nonmuscle myosin II heavy chain and the attachment of p127 to the plasma membrane. J Cell Sci 1996; 109:1359-1368.
71. Manfruelli P, Arquier N, Hanratty WP et al. The tumor suppressor gene, *lethal(2)giant larvae* (*l(2)gl*), is required for cell shape change of epithelial cells during *Drosophila* development. Development 1996; 122:2283-2294.
72. Kataoka T, Broek D, Wigler M. DNA sequence and characterization of the *S. cerevisiae* gene encoding adenylate cyclase. Cell 1985; 43:493-505.
73. Minato T, Wang J, Akasaka K et al. Quantitative analysis of mutually competitive binding of human Raf-1 and yeast adenylyl cyclase to Ras proteins. J Biol Chem 1994; 269:20845-20851.
74. Cutler ML, Bassin RH, Zanoni L et al. Isolation of *rsp-1*, a novel cDNA capable of suppressing v-Ras transformation. Mol Cell Biol 1992; 12:3750-3756.
75. Tsuda T, Cutler ML. Human *RSU1* is highly homologous to mouse *Rsu-1* and localizes to human chromosome 10. Genomics 1993; 18:461-462.
76. Tsuda T, Marinetti MR, Masuelli L et al. The Ras suppressor *RSU-1* localizes to 10p13 and its expression in the U251 glioblastoma cell line correlates with a decrease in growth rate and tumorigenic potential. Oncogene 1995; 11:397-403.
77. Masuelli L, Cutler ML. Increased expression of the Ras suppressor *Rsu-1* enhances Erk-2 activation and inhibits Jun kinase activation. Mol Cell Biol 1996; 16:5466-5476.
78. Tapon N, Hall A. Rho, Rac and CDC42 GTPases regulate the organization of the actin cytoskeleton. Curr Opin Cell Biol 1997; 9:86-92.
79. Lim L, Manser E, Leung T et al. Regulation of phosphorylation pathways by p21 GTPases. The p21 Ras-related Rho subfamily and its role in phosphorylation signalling pathways. Eur J Biochem 1996; 242:171-185.
80. Macara IG, Lounsbury KM, Richards SA et al. The Ras superfamily of GTPases. FASEB J 1996; 10:625-630.
81. Luo L, Liao YJ, Jan LY et al. Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev 1994; 8:1787-1802.
82. Harden N, Loh HY, Chia W et al. A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila*. Development 1995; 121:903-914.
83. Symons M, Derry JMJ, Karlak B et al. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. Cell 1996; 84:723-734.
84. Zhu J, Bilan PJ, Moyers JS et al. Rad, a novel Ras-related GTPase, interacts with skeletal muscle beta-tropomyosin. J Biol Chem 1996; 271:768-773.
85. Reinhard J, Scheel AA, Diekmann D et al. A novel type of myosin implicated in signalling by rho family GTPases. EMBO J 1995; 14:697-704.
86. Wirth JA, Jensen KA, Post PL et al. Human myosin-IXb, an unconventional myosin with a chimerin-like rho/rac GTPase-activating protein domain in its tail. J Cell Sci 1996; 109:653-661.
87. Schejter ED, Wieschaus E. Functional elements of the cytoskeleton in the early *Drosophila* embryo. Annu Rev Cell Biol 1993; 9:67-99.

CHAPTER 18

Mammalian IQGAPs

André Bernards

Introduction

Members of the Ras superfamily of small GTP-binding proteins function as molecular switches by cycling between active GTP and inactive GDP-bound states. This cycling is accompanied by a prominent conformational change in the effector-binding regions of the GTPases and is catalyzed by guanine nucleotide exchange factors or GEFs, which promote GTP for GDP exchange, and by GTPase activating proteins or GAPs, which increase the slow intrinsic GTP hydrolysis rate of Ras superfamily members. In their active GTP-bound state Ras superfamily members are capable of interacting with a variety of effector molecules which mediate their diverse biological responses. For more extensive descriptions of these or other basic aspects of GTPase biology see refs. 1-5.

The Ras superfamily is commonly subdivided into several subfamilies, the most prominent of which are the Arf, Rab, Ras and Rho groups.[1] Although all Ras superfamily members share a similar design, for reasons that remain poorly understood GAPs for members of different subgroups have with few exceptions been unrelated in sequence.[3] Thus, when we first identified a human protein that shared extensive similarity with a fission yeast Ras GAP homolog,[6] our first guess was that IQGAP1 represented a novel GAP for Ras or a close relative. However, IQGAP1 and a related IQGAP2 protein do not appear to be GAPs for any GTPase, but instead interact with and inhibit the intrinsic GTPase activity of Rho family members CDC42 and Rac1. Both IQGAP proteins also interact with calmodulin (CaM), and purified IQGAP1 cross-links actin microfilaments in vitro.[7] Therefore, rather than attenuating Ras signaling, IQGAPs may function as integrators of CaM and CDC42 or Rac-mediated signals in pathways that lead to increased F-actin cross-linking. This chapter summarizes what is known about IQGAPs and discusses possible functions for these proteins in processes controlled by CDC42 and Rac.

What Is Known and What Isn't

Identification, Expression and Structure of IQGAPs

IQGAP1 was identified while sequencing random cDNAs from a human myeloid cell line,[8] and by us as an accidental byproduct in a PCR reaction using human osteosarcoma cDNA as a template.[6] We named the new gene *IQGAP1*, because the predicted 1657 amino acid protein included four IQ motifs upstream of a segment related to a fission yeast Ras GAP. Although recent work indicates that IQGAPs are not GAPs for Ras and may not be GAPs at all, we will continue to use the IQGAP name, based on the understanding that it refers to structural rather than functional similarity.

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

Two partial IQGAP2 cDNAs were identified during low stringency screens of a mouse brain cDNA library. Subsequently isolated human clones predicted a 1575 amino acid protein that shared 62% sequence identity with IQGAP1.[9] Suggesting a tissue-specific function, IQGAP2 mRNA was found to be highly expressed in murine and human liver, with mRNA levels at or below the detection level in several other tissues.[9] In similar surveys IQGAP1 mRNA was found to be abundant in several murine tissues that are rich in epithelial cells, including placenta, lung and kidney, with lower expression levels found in skeletal muscle, heart, pancreas, and liver.[6] Although mouse brain contained very little IQGAP1 mRNA, a protein that is likely to be a species homolog of IQGAP1 has been purified from bovine brain cytosol[10] and from neural crest-derived bovine adrenal gland.[7] Probable homologs of IQGAP1 and IQGAP2 have also been identified in rabbit liver cytosol,[11] and IQGAP1 was found to be the predominant CaM-binding protein in Ca^{2+}-free human breast cell lysates.[12] Thus, whereas IQGAP2 appears to be largely liver specific, IQGAP1 is widely expressed.

Many signal transduction pathways are "hard-wired" and participants in such pathways frequently contain domains that mediate their interaction with other proteins. The structure of IQGAPs certainly suggests a function that requires interaction with other proteins, since outside of their Ras GAP-related C-terminal segments both IQGAPs largely consist of several proven or putative protein interaction motifs (Fig. 18.1). Among these are an N-terminal calponin-homology domain,[13] which may serve as an F-actin binding site (see below), five (IQGAP2) or six (IQGAP1) copies of a so far unique 50 residue IR (IQGAP repeat) motif of unknown function,[9] a single WW domain that may mediate interaction with proline-rich targets,[14] and four consecutive calmodulin binding IQ motifs.[15] Database searches also reveal similarity between IQGAPs and proteins that contain α-helical coiled coil segments.[9] This similarity reflects the presence of several short stretches of coiled-coil-forming heptad repeats throughout both IQGAP proteins (a computer-generated prediction of the coiled-coil potential of IQGAP1 is included in Fig. 18.1).

IQGAPs Are Not RasGAPs

All GAPs for H-, K-, N-, or R-Ras homologs share a loosely conserved approximately 300 amino acid domain required for their catalytic activity.[3] This so called GAP-related domain (GRD) of IQGAPs is most closely related to that of a fission yeast Ras GAP, named either Sar1[16] or Gap1.[17] Within their GRDs IQGAPs and Sar1/Gap1 share several unique sequence and spacing features that set them apart from other RasGAPs.[6] Two *Dictyostelium* Ras GAP homologs, DdRasGAP1 and DdGAPA, which control different aspects of cytokinesis,[18-20] share similar structural features within their GRDs and together with IQGAPs and Sar1/Gap1 form a distinct subfamily of Ras GAP-related proteins. In support of this conclusion, Sar1/Gap1 and the two *Dictyostelium* proteins also share a low level of sequence similarity with the C-terminal halves of IQGAPs outside of their GRDs (Fig. 18.1). However, although Sar1/Gap1 and DdRasgap1 both stimulated Ras-GTP hydrolysis,[19,21] a bacterial fusion protein representing the entire Sar1/Gap1 homologous segment of IQGAP1 did not stimulate the GTPase activity of human H-Ras in vitro.[6] In these experiments the fusion protein did appear to interact with Ras, since in the presence of GST-IQGAP1 approximately twice as much H-Ras-GTP but not RhoA-GTP remained trapped on the filters in nitrocellulose filter binding GAP assays. Thus, we suggested that IQGAP1 may be a Ras binding protein without major GAP activity, at least under the conditions tested.[6]

Evidence arguing against this conclusion was obtained shortly afterwards. In the case of IQGAP2, full length or truncated proteins made in the baculovirus system did not stimulate the GTPase activity of H-Ras, R-Ras1, Rap1a, or RalA, nor of any tested protein outside of the immediate Ras group. No enhanced filter retention was observed with any GTPase

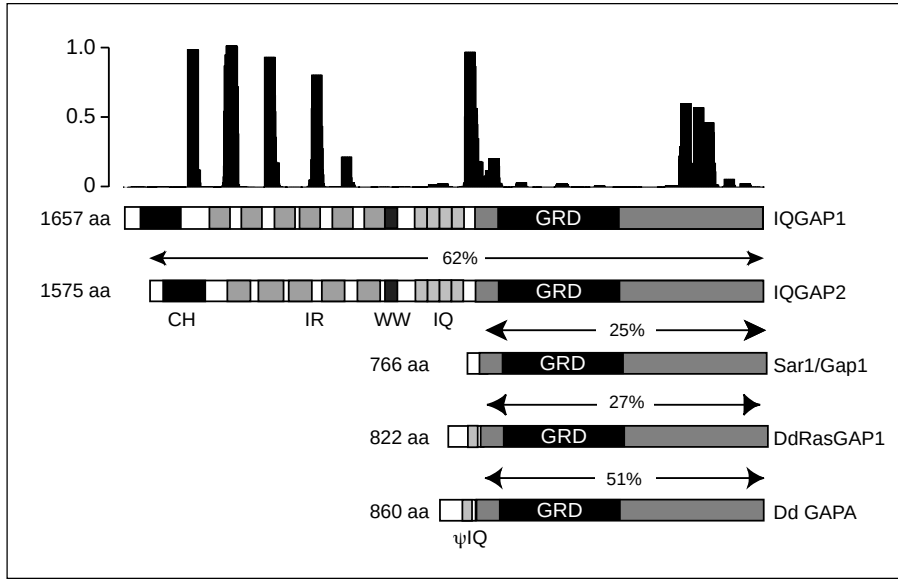


Fig. 18.1. Schematic structure of IQGAPs and related proteins from other organisms. Human IQGAP1 and IQGAP2 are 62% identical over their entire length and share approximately 25% sequence identity with Sar1/Gap1 and similar levels of identity with both *Dictyostelium* proteins.

and in competition experiments a molar excess of full length or truncated IQGAP2 did not inhibit p120GAP-stimulated H-Ras-GTP hydrolysis.[9]

As discussed in more detail below, three groups recently identified mammalian IQGAP1 as a prominent CDC42-binding protein. Similar to our findings, in work by these groups recombinant IQGAP1 did not stimulate the GTPase of H-Ras, K-Ras or R-Ras.[10,21] Moreover, anti-IQGAP1 immunoprecipitates did not contain Ras GAP activity under conditions where p120GAP immunoprecipitates did.[21] This latter result argues against a scenario in which IQGAP1 has latent Ras GAP activity that can be activated by unknown cofactors. Similar to what we found for IQGAP2, but unlike what we reported for IQGAP1, in binding and competition experiments full length or truncated IQGAP1 showed no evidence of interacting with Ras.[10,21] Thus, the increased filter retention of H-Ras in the presence of IQGAP1 in our initial experiments may have been an artifact, perhaps caused by the poor solubility of the truncated GST-IQGAP1 fusion protein.

IQGAP Proteins Bind Calmodulin and Cross-Link F-Actin

The role of CaM in signal transduction is not unlike that of GTPases. Like Ras superfamily members, CaM functions as a molecular switch by cycling between active Ca^{2+}-bound and inactive Ca^{2+}-depleted states. A conformational change upon Ca^{2+}-binding exposes hydrophobic amino acids that allow CaM to interact with amphiphilic α-helical segments of a variety of effector proteins, among which are protein kinases, adenylyl cyclases, and numerous other proteins.[22] As in the case of GTPases, CaM binds most of its targets only when activated, but interacts with other proteins in a Ca^{2+}-independent manner. The binding sites implicated in this latter type of interaction are termed IQ motifs and consist of 23-25 amino acids with an IQXXXRGXXXR consensus.[15] However, it is important to note

that depending on their exact sequence IQ motifs can also mediate Ca^{2+}-dependent CaM binding.[23,24]

Not surprising given the presence of four IQ motifs, both IQGAPs interact with CaM. Thus, a prominent 17 kDa protein that was recognized by an anti-CaM monoclonal antibody coprecipitated with full length IQGAP2, but not with a truncated protein that lacked the IQ motifs. The complex between IQGAP2 and the 17 kDa protein was stable in 1% Triton X-100 and 0.6 M NaCl, and the coprecipitating protein and CaM showed the same characteristic Ca^{2+}-dependent mobility shift in SDS gels.[9] CaM was also the most prominent interactor in yeast two-hybrid screens with baits that included the IQ motifs of IQGAP1,[9] and CaM associated with full length IQGAP1 and with truncated proteins that included the IQ motifs.[21] Moreover, IQGAP1 was recently purified by CaM affinity chromatography and shown to bind CaM-Sepharose either in the presence or absence of calcium.[12] It is still unknown whether all four IQ motifs of IQGAPs contribute to CaM binding. Relevant to this question may be that purified IQGAP1 contained only substoichiometric CaM (a 1:15 CaM to IQGAP1 molar ratio in the preparation containing the highest amount of CaM). However, this low ratio may have reflected loss of CaM during purification.[7]

Both IQGAPs also include an N-terminal approximately 100 residue calponin homology (CH) domain (Fig. 18.1). Named after the calponin family of Ca^{2+}-, CaM- and actin-binding smooth muscle proteins, similar domains function as F-actin binding sites in members of the spectrin, fimbrin and filamin families.[13] The IQGAP CH domains may also serve as F-actin binding sites. Thus, in microinjected Swiss 3T3 cells both IQGAP1[10] and IQGAP2 (S. Brill and AB, unpublished) colocalize with phalloidin-stained F-actin at membrane ruffles, but not at Rho-induced stress fibers. By contrast, in MDCK kidney epithelial cells IQGAP1 colocalizes with cortical actin at regions of cell to cell contact,[10] and the same is true for IQGAP2 in some hepatocyte cell lines (S. Brill, S. Li, and AB, unpublished). Beyond colocalizing with specific F-actin-rich structures, bovine IQGAP1 cosediments with in vitro polymerized actin microfilaments.[7] Since actin polymerization and actin microfilament cross-linking are important events during the generation of actin structures, and because actin cross-linking requires more than one F-actin binding site, Bashour and co-workers also tested whether IQGAP1 had a tendency to form multimers in solution. Suggestive of the existence of dimers, purified IQGAP1 (calculated MW 189 kDa) sedimented with a molecular mass of 360-400 kDa. Whether dimerization of IQGAP1 involves its relatively short potential coiled-coil segments remains to be determined, but consistent with the existence of dimers, addition of purified IQGAP1 converted soluble F-actin into a gel consisting of irregular, interconnected actin microfilaments.[7]

IQGAPs Bind CDC42 and Rac1

Three groups recently identified (species homologs of) IQGAP1,[10,21] or of both IQGAP1 and IQGAP2[11] as prominent CDC42 binding proteins. In the first of these studies, Hart and co-workers identified a 195 kDa COS cell protein that bound immobilized GST-CDC42 and GST-Rac in a GTPγS-dependent manner. Microsequencing identified the protein as IQGAP1.[21] A similar approach by Kuroda and colleagues identified a 170 kDa bovine brain cytosolic protein that bound GST-CDC42-GTPγS, but not GST-CDC42-GDP. A protein of this size also bound Rac1-GTPγS, but not RhoA, H-Ras, or RalA. Again, microsequencing and detection by an antiserum against human IQGAP1 suggested the protein was the bovine homolog of IQGAP1.[10] Finally, McCallum and co-workers found 180 and 175 kDa CDC42-binding proteins in rabbit liver cytosol and identified these proteins as probable homologs of IQGAP1 and IQGAP2, respectively.[11]

We found that IQGAP2 binds CDC42 and Rac1 while screening a panel of GST-GTPase fusion proteins for potential IQGAP substrates. In these experiments GTPγS-loaded CDC42

and Rac1, but not RhoA or several other GTPases, bound recombinant or endogenous IQGAP2. Moreover, similar to results obtained with IQGAP1,[21] epitope-tagged CDC42 coprecipitated with IQGAP2 from transfected cells.[9]

Proteins that interact with GTPases are usually classified either as regulators (GEFs, GAPs or GDIs) or as effectors, although several GTPase binding proteins are believed to combine both functions. Examples include mammalian Raf-1, which in addition to being a Ras effector may also regulate Ras by enhancing its GTPase activity or by preventing its interaction with GAPs,[5,25,26] and mammalian p120GAP, which is a potent Ras GAP, but which also has functions not obviously related to its role as a Ras regulator.[27] Thus, the question whether IQGAPs are regulators or effectors of CDC42 and Rac1 may be overly simplistic. What appears clear, however, is that IQGAPs are not GAPs for CDC42 and Rac1.

Evidence in support of this conclusion was obtained by several groups. In the case of IQGAP2, even a molar excess of full length or truncated recombinant protein did not stimulate the GTPase activity of CDC42 or Rac1. Rather, all recombinant proteins that were capable of interacting with CDC42 and Rac1 had the opposite effect, causing a dosage-dependent inhibition of the intrinsic activity of these GTPases. Moreover, these proteins also caused a dosage-dependent inhibition of RhoGAP-stimulated CDC42-GTP and Rac1-GTP hydrolysis.[9] Similar results have been reported for IQGAP1. Thus, a recombinant protein representing the C-terminal half of IQGAP1 did not stimulate GTP hydrolysis by CDC42, but inhibited both the intrinsic and p190RhoGAP-stimulated rate of CDC42 GTP hydrolysis.[21] Moreover, although IQGAP1 binds CDC42-GTPγS but not CDC42-GDP, and although CDC42 has a relatively high intrinsic GTP hydrolysis rate, a prominent CDC42-IQGAP1 complex remained detectable after prolonged preincubation.[11] Although IQGAPs, therefore, are not GAPs for CDC42 and Rac1, they may still regulate these GTPases by maintaining them in an active state.

A conformational change in the effector binding regions of GTPases upon GTP hydrolysis explains why many effectors interact preferentially with activated GTPases.[28] However, while its preferential interaction with GTP-bound CDC42 or Rac is compatible with an effector role for IQGAP1,[10,11,21] in similar in vitro experiments IQGAP2 bound equally well to GTP-bound, GDP-bound, or nucleotide free CDC42 or Rac.[9,11] Why IQGAP1 and IQGAP2 differ in this respect, whether the in vitro binding results reflect the in vivo situation, and whether the different binding properties reflect functional differences remains unknown.

IQGAPs do not contain so-called CRIB domains that mediate binding of CDC42 and Rac to several potential effectors.[29-31] To test whether the GRDs of IQGAPs were responsible for CDC42 and Rac binding, several groups performed binding studies with truncated proteins.[9,10,21] In our work a baculovirus protein representing the C-terminal half of IQGAP2 but that lacked the first 30 or so amino acids of the Sar1/Gap1 homologous region did not bind, whereas a protein that included everything downstream of the second IQ motif did interact.[9] Similarly, Hart and co-workers found that a protein representing theSar1/Gap1-related segment of IQGAP1 did bind, but that relatively small N-terminal or C-terminal truncations abolished interaction. Moreover, although an internal deletion that removed the GRD prevented binding, the GRD itself was not sufficient for binding.[21] Therefore, either widely spaced residues throughout the Sar1/Gap1-related segments of IQGAPs are required for interaction, or the conformation of the CDC42 and Rac-binding domain of IQGAPs is particularly sensitive to deletion of flanking protein segments.

Rather than identifying the GTPase binding domain of IQGAPs, McCallum et al analyzed which parts of CDC42 mediate IQGAP binding. When added to rabbit liver cytosol, a catalytically active truncated form of CDC42-GAP (a.k.a. RhoGAP) did not prevent the binding of either IQGAP1 or IQGAP2 to a GTPase defective GST-Cdc42^{leu61} protein,

suggesting that the IQGAP and RhoGAP binding regions of CDC42 do not overlap.[12] The potent inhibition by IQGAPs of RhoGAP- or p190RhoGAP-stimulated CDC42-GTP hydrolysis therefore may not involve a simple competition mechanism. In similar experiments a truncated protein containing the CRIB domain of mPAK-3 did strongly compete with IQGAP1 for CDC42-GTPγS binding, and to a lesser extent also weakened IQGAP2 binding. These data suggest that IQGAPs and mPAK-3 interact with similar regions of CDC42.[11]

The same group also analyzed binding of three CDC42 effector domain mutants modeled on Ras mutants that fail to interact with Ras GAP or with effectors. One mutant (CDC42-D38E) showed no change in binding, while the others (CDC42-Y32K and CDC42-T35A) no longer interacted with IQGAP1. The former mutant did retain the ability to bind IQGAP2, but this interaction now became GTPγS-dependent.[11] Since two effector domain mutants failed to interact with IQGAP1, this part of CDC42 is likely to be an important component of the IQGAP1 binding site.

Putting It All Together

Clues as to why IQGAPs do not interact with Ras and why they are not GAPs for CDC42 or Rac1 have been provided by the recent determination of the Ras-Ras GAP complex structure.[32] In this work Scheffzek and co-workers found that arginine-789 (R789) in the so-called "finger loop" of p120GAP plays a critical role in catalyzing Ras.GTP hydrolysis by projecting into the active site of the enzyme. Eight other p120GAP residues (labeled by plusses in Fig. 18.2) directly interacted with Ras and residues at corresponding positions are likely to be important determinants of the target specificity of Ras GAP related proteins.

All proteins with confirmed Ras GAP activity have either an arginine or a lysine at the position corresponding to R789 in p120GAP (indicated with a filled circle in Fig. 18.2). However, both IQGAPs have threonines and *Dictyostelium* GAPA has an alanine at this position. These proteins would thus be predicted to lack GAP activity. Moreover, several of the eight p120GAP residues that directly contact Ras are not conserved in IQGAPs, which may explain why these proteins interact with CDC42 and Rac1 rather than with Ras. Consecutive lysine and glutamic acid residues in the so-called variable loop of p120GAP are predicted to be especially important for stabilizing the Ras-Ras GAP interaction[32] and are absent in IQGAPs (Fig. 18.2). Thus it might be interesting to see whether replacing the variable loop of IQGAPs with that of, e.g., neurofibromin, would alter the GTPase binding properties of IQGAPs. Similarly, it would be interesting if a mutant IQGAP with an arginine at the position corresponding to p120GAP R789 would show GAP activity towards CDC42 and Rac1.

Fig. 18.2 (opposite). Alignment of the putative catalytic domains of Ras GAP homologs based on a similar alignment published by Scheffzek et al.[32] A closed circle identifies R789 in the $L1_c$ so-called finger loop of p120GAP. Plusses label p120GAP residues that directly contact Ras.[32] Among proteins in this alignment, human p120GAP, human neurofibromin (hsNF1), murine Gap1 (mmGAP1), *S. pombe* Sar1/Gap1 (spGap1), and *Dictyostelium discoideum* RasGAP1 (ddRasGAP1) have Ras GAP activity, whereas *Dictyostelium* GAPA (ddGAPA) and both IQGAPs have either not been tested (ddGAPA) or do not stimulate (IQGAP1&2) Ras.GTP hydrolysis. The drawing above the sequence identifies α-helical segments and intervening loops in p120GAP. The $L2_c$ loop in spGap1, ddRasGAP1, ddGAPA, and both IQGAPs is longer than the corresponding loop in other RasGAPs. To improve the presentation, some amino acids in loop $L2_c$ have been left out of this alignment (the number in brackets indicates the number of residues that were excluded; see ref. 32 and the chapter by Klaus Scheffzek in this book for more details).

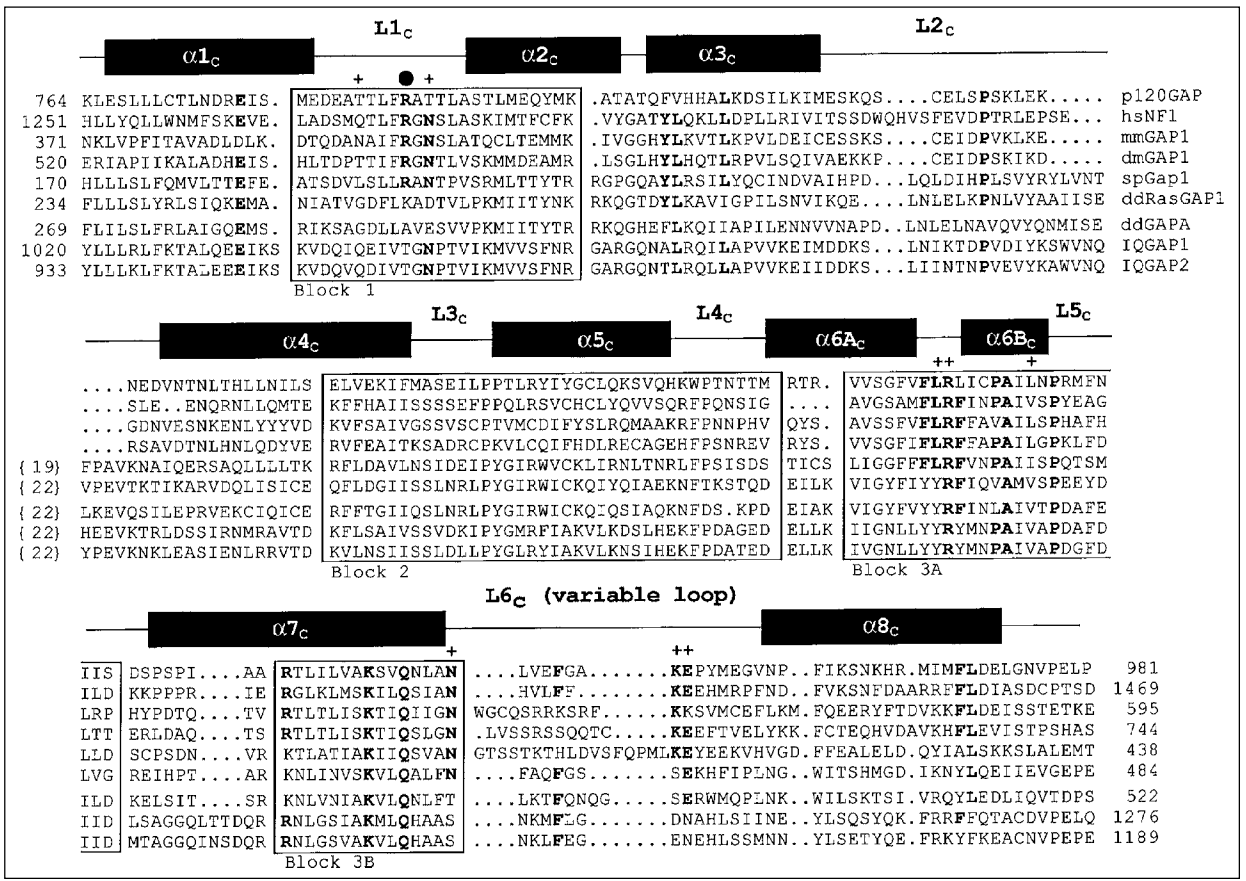

L1c
L2c
α1c
α2c
α3c
764 KLESLLLCTLNDREIS. MEDEATTLFRATTLASTLMEQYMK .ATATQFVHHALKDSILKIMESKQS....CELSPSKLEK..... p120GAP
1251 HLLYQLLWNMFSKEVE. LADSMQTLFRGNSLASKIMTFCFK .VYGATYLQKLLDPLLRIVITSSDWQHVSFEVDPTRLEPSE... hsNF1
371 NKLVPFITAVADLDLK. DTQDANAIFRGNSLATQCLTEMMK .IVGGHYLKVTLKPVLDEICESSKS....CEIDPVKLKE..... mmGAP1
520 ERIAPIIKALADHEIS. HLTDPTTIFRGNTLVSKMMDEAMR .LSGLHYLHQTLRPVLSQIVAEKKP....CEIDPSKIKD..... dmGAP1
170 HLLLSLFQMVLTTEFE. ATSDVLSLLRANTPVSRMLTTYTR RGPGQAYLRSILYQCINDVAIHPD...LQLDIHPLSVYRYLVNT spGap1
234 FLLLSLYRLSIQKEMA. NIATVGDFLKADTVLPKMIITYNK RKQGTDYLKAVIGPILSNVIKQE....LNLELKPNLVYAAIISE ddRasGAP1
269 FLILSLFRLAIGQEMS. RIKSAGDLLAVESVVPKMIITYTR RKQGHEFLKQIIAPILENNVVNAPD..LNLELNAVQVYQNMISE ddGAPA
1020 YLLLRLFKTALQEEIKS KVDQIQEIVTGNPTVIKMVVSFNR GARGQNALRQILAPVVKEIMDDKS...LNIKTDPVDIYKSWVNQ IQGAP1
933 YLLLKLFKTALEEEIKS KVDQVQDIVTGNPTVIKMVVSFNR GARGQNTLRQLLAPVVKEIIDDKS...LIINTNPVEVYKAWVNQ IQGAP2
Block 1
L3c
L4c
L5c
α4c
α5c
α6Ac
α6Bc
....NEDVNTNLTHLLNILS ELVEKIFMASEILPPTLRYIYGCLQKSVQHKWPTNTTM RTR. VVSGFVFLRLICPAILNPRMFN
....SLE..ENQRNLLQMTE KFFHAIISSSSEFPPQLRSVCHCLYQVVSQRFPQNSIG .... AVGSAMFLRFINPAIVSPYEAG
....GDNVESNKENLYYYVD KVFSAIVGSSVSCPTVMCDIFYSLRQMAAKRFPNNPHV QYS. AVSSFVFLRFFAVAILSPHAFH
....RSAVDTNLHNLQDYVE RVFEAITKSADRCPKVLCQIFHDLRECAGEHFPSNREV RYS. VVSGFIFLRFFAPAILGPKLFD
{19} FPAVKNAIQERSAQLLLLTK RFLDAVLNSIDEIPYGIRWVCKLIRNLTNRLFPSISDS TICS LIGGFFFLRFVNPAIISPQTSM
{22} VPEVTKTIKARVDQLISICE QFLDGIISSLNRLPYGIRWICKQIYQIAEKNFTKSTQD EILK VIGYFIYYRFIQVAMVSPEEYD
{22} LKEVQSILEPRVEKCIQICE RFFTGIIQSLNRLPYGIRWICKQIQSIAQKNFDS.KPD EIAK VIGYFVYYRFINLAIVTPDAFE
{22} HEEVKTRLDSSIRNMRAVTD KFLSAIVSSVDKIPYGMRFIAKVLKDSLHEKFPDAGED ELLK IIGNLLYYRYMNPAIVAPDAFD
{22} YPEVKNKLEASIENLRRVTD KVLNSIISSLDLLPYGLRYIAKVLKNSIHEKFPDATED ELLK IVGNLLYYRYMNPAIVAPDGFD
Block 2
Block 3A
L6c (variable loop)
α7c
α8c
IIS DSPSPI....AA RTLILVAKSVQNLAN ....LVEFGA.....KEPYMEGVNP..FIKSNKHR.MIMFLDELGNVPELP 981
ILD KKPPPR....IE RGLKLMSKILQSIAN ....HVLFF.....KEEHMRPFND..FVKSNFDAARRFFLDIASDCPTSD 1469
LRP HYPDTQ....TV RTLTLISKTIQIIGN WGCQSRRKSRF.....KKSVMCEFLKM.FQEERYFTDVKKFLDEISSTETKE 595
LTT ERLDAQ....TS RTLTLISKTIQSLGN .LVSSRSSQQTC.....KEEFTVELYKK.FCTEQHVDAVKHFLEVISTPSHAS 744
LLD SCPSDN....VR KTLATIAKIIQSVAN GTSSTKTHLDVSFQPMLKEYEEKVHVGD.FFEALELD.QYIALSKKSLALEMT 438
LVG REIHPT....AR KNLINVSKVLQALFN ....FAQFGS.....SEKHFIPLNG..WITSHMGD.IKNYLQEIIEVGEPE 484
ILD KELSIT....SR KNLVNIAKVLQNLFT ....LKTFQNQG.....SERWMQPLNK..WILSKTSI.VRQYLEDLIQVTDPS 522
IID LSAGGQLTTDQR RNLGSIAKMLQHAAS ....NKMFLG.....DNAHLSIINE..YLSQSYQK.FRRFFQTACDVPELQ 1276
IID MTAGGQINSDQR RNLGSVAKVLQHAAS ....NKLFEG.....ENEHLSSMNN..YLSETYQE.FRKYFKEACNVPEPE 1189
Block 3B

Fig. 18.2.

A reasonable hypothesis based on current evidence is that IQGAPs somehow function as integrators of CaM and CDC42/Rac signals in pathways that lead to increased F-actin cross-linking. Relevant to this hypothesis may be that CaM appears to regulate the interaction between CDC42 and IQGAP1. Thus, whereas IQGAP1 binds CaM-Sepharose both in the presence or absence of Ca^{2+}, Ca^{2+}/CaM but not CaM alone inhibited the interaction between GST-CDC42 and IQGAP1 in a dosage dependent manner.[12] This suggests a model in which Ca^{2+} induces a conformational change in a preexisting CaM-IQGAP-CDC42 (or Rac) complex leading to the release of the (active) GTPase. This may inactivate IQGAP-mediated signals, while at the same time allowing the released GTPase to interact with proteins such as phosphoinositol 4-phosphate-5 kinase,[33,34] WASP homologs,[31,35,36] or Por1,[37] which among others have been implicated as potential effectors of Rac or CDC42-induced cytoskeletal changes (see refs. 38 and 39 for reviews). Whether the putative Ca^{2+}-induced conformational shift also affects the actin binding or cross-linking properties of IQGAPs remains an open question, but it is interesting to note that exogenous CaM inhibited F-actin binding by purified IQGAP1.[7]

Caveats and Future Work

Microinjection of activated Rho, Rac or CDC42 into Swiss 3T3 fibroblasts stimulates the formation of actin-rich stress fibers, lamellipodia and membrane ruffles, and filopodia, respectively.[40-43] Chapter 14, other chapters in this book, and several recent reviews discuss how Rho family GTPases might control localized G-actin polymerization and F-actin cross-linking that underlie the formation of these structures.[38,39,44,45] While it is an attractive idea that IQGAPs may somehow be involved in this process, it is important to note that CDC42 and Rac promote different types of actin reorganization in different cell types,[46,47] and also function in processes that do not involve actin reorganization.[48,49] Thus, pathways downstream of CDC42 and Rac including those leading to actin reorganization are likely to be complex and undoubtedly involve proteins other than IQGAPs.

Moreover, whilst the localization of IQGAP1 and IQGAP2 to membrane ruffles in microinjected fibroblasts suggests a role as a mediator of the cytoskeletal effects of Rac, competition binding experiments indicate that IQGAP1 has a much higher affinity for CDC42,[11,21] and the same is probably true for IQGAP2.[9] Thus, IQGAPs may primarily function in pathways involving CDC42. Relevant to this issue may also be that whereas both IQGAPs localize to membrane ruffles in overexpressing fibroblasts, endogenous IQGAP1 in epithelial MDCK cells,[10] and endogenous IQGAP2 in hepatocytes (our unpublished work) localize to areas of cell to cell contact. We note in this respect that recent work suggests roles for Rho and Rac in the control of cadherin-based cell adhesion[50] and for Drosophila CDC42 in epithelial cell morphogenesis.[46]

Important clues to the function of new proteins can often be obtained by analyzing homologs in organisms amenable to genetic analysis. However, although fission yeast Sar1/Gap1 and two *Dictyostelium* Ras GAP homologs share similarity with the C-terminal halves of IQGAPs, no obvious IQGAP homologs containing all domains indicated in Figure 18.1 have been identified to date. This may change because a partially sequenced *Caenorhabditis elegans* cDNA (GenBank accession numbers D33786 and D36706) predicts a protein with IQ motifs upstream of a Sar1/Gap1-related segment. It should be interesting to determine whether this protein also includes homology to the N-terminal segments of IQGAPs. A protein identified by the *Saccharomyces cerevisiae* genome project (GenBank accession number Z73598) also appears distantly related to IQGAPs. This 1495 residue protein includes an N-terminal CH domain, at least four IQ motifs in the central region of the protein, and a low level of similarity to the extreme C-terminal segments of IQGAPs. However, no obvious GRD is present in this protein.

Finally, since regions outside of the GRD or IQGAPs may contribute to CDC42 binding it is interesting to note that DdRasGAP1, which like the related GAPA protein controls different aspects of cytokinesis in *Dictyostelium*,[18-20] was identified in a two-hybrid screen with human H-Ras^{Val12} as a bait. Interestingly, the domain responsible for the interaction did not include the GRD, but mapped close to the C-terminus of the protein.[19] Since sequences near the C-terminus are conserved between IQGAPs, Sar1/Gap1, both *Dictyostelium* proteins, as well as the nematode and yeast proteins, it should be interesting to determine whether this sequence binds Ras or other GTPases directly or indirectly through a third protein.

Acknowledgments

Work in my laboratory is supported by a grant from the National Cancer Institute (1-R01-CA70234). I am grateful to Lawrence Weissbach, Suzanne Brill and Shihong Li for their comments.

Note Added in proof:

IQGAP-related proteins from *S. cerevisiae* (Iqg1/Cyk1p) and *S. pombe* (Rng2p) direct the formation of a contractile actomyosin ring between mother and daughter cells during cytokinesis (Epp JA, Chant J (1997). An IQGAP-related protein controls actin-ring formation and cytokinesis in yeast. (Curr Biol 7; 921-929; Lippincott J, Li R). Sequential assembly of myosin II, and IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. (J Cell Biol 1998; 140:355-366. Eng K, Naqvi NI, Wong KCY, Balasubramanian MK.) Rng2p, a protein required for cytokinesis in fission yeast, is a component of the actomyosin ring and the spindle pole body. (Curr Biol 1998; 11:611-621). It remains to be determined whether mammalian IQGAPs have similar roles or have evolved to serve different functions.

References

1. Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: A conserved switch for diverse cellular functions. Nature 1990; 348:125-132.
2. Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: Conserved structure and molecular mechanism. Nature 1991; 349:117-127.
3. Boguski MS, McCormick F. Proteins regulating Ras and its relatives. Nature 1993; 366:643-654.
4. Lowy DR, Willumsen BM. Function and regulation of ras. Annu Rev Biochem 1993; 62:851-891.
5. Katz ME, McCormick F. Signal transduction from muliple Ras effectors. Curr Opin Genet Dev 1997; 7:75-79.
6. Weissbach L, Settleman J, Kalady MF et al. Identification of a human rasGAP-related protein containing calmodulin-binding motifs. J Biol Chem 1994; 269:20517-20521.
7. Bashour A-M, Fullerton AT, Hart MJ, Bloom GS. IQGAP1, a Rac- and CDC42-binding protein, directly binds and cross-links microfilaments. J Cell Biol 1997; 137:1555-1566.
8. Nomura N, Nagase T, Miyajima N et al. Prediction of the coding sequences of unidentified human genes. II. The coding sequences of 40 new genes (KIAA0041-KIAA0080) deduced by analysis of cDNA clones from human cell line KG-1. DNA Res 1994; 1:223-229.
9. Brill S, Li S, Lyman CW et al. The Ras GTPase-activating-protein-related human protein IQGAP2 harbors a potential actin binding domain and interacts with calmodulin and Rho family GTPases. Mol Cell Biol 1996; 16:4869-4878.
10. Kuroda S, Fukata M, Kobayashi K et al. Identification of IQGAP as a putative target for the small GTPases, CDC42 and Rac1. J Biol Chem 1996; 271:23363-23367.

11. McCallum SJ, Wu WJ, Cerione RA. Identification of a putative effector for Cdc42Hs with high sequence similarity to the RasGAP-related protein IQGAP1 and a Cdc42Hs binding partner with similarity to IQGAP2. J Biol Chem 1996; 271:21732-21737.
12. Joyal JL, Annan RS, Ho Y-D et al. Calmodulin modulates the interaction between IQGAP1 and CDC42. J Biol Chem 1997; 272:15419-15425.
13. Castresana J, Saraste M. Does Vav bind to F-actin through a CH domain? FEBS letters 1995; 374:149-151.
14. Sudol M. The WW module competes with the SH3 domain? Trends Biochem Sci 1996; 21:161-3.
15. Cheney RE, Mooseker MS. Unconventional myosins. Curr Opin Cell Biol 1992; 4:27-35.
16. Wang Y, Boguski M, Riggs M, Rodgers L, Wigler M. *Sar1*, a gene from *Schizosaccharomyces pombe* encoding a protein that regulates ras1. Cell Regul 1991; 2:453-465.
17. Imai Y, Miyake S, Hughes DA, Yamamoto M. Identification of a GTPase-activating protein homolog in *Schizosaccharomyces pombe*. Mol Cell Biol 1991; 11:3088-3094.
18. Faix J, Dittrich W. DGAP1, a homologue of rasGTPase activating proteins that controls growth, cytokinesis, and development in *Dictyostelium discoideum*. FEBS Letters 1996; 394:251-257.
19. Lee S, Escalante R, Firtel RA. A Ras GAP is essential for cytokinesis and spatial patterning in *Dictyostelium*. Development 1997; 124:983-996.
20. Adachi H, Takahashi Y, Hasebe T, Shirouzu M, Yokoyama S, Sutoh K. *Dictyostelium* IQGAP-related protein specifically involved in the completion of cytokinesis. J Cell Biol 1997; 137:891-898.
21. Hart MJ, Callow M, Souza B, Polakis P. IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs. EMBO J 1996; 15:2997-3005.
22. Cohen P, Klee C. Calmodulin. Molecular Aspects of Cell Regulation. Vol. 5. Amsterdam: Elsevier, 1988:371.
23. Houdusse A, Cohen C. Target sequence recognition by the calmodulin superfamily: Implications from light chain binding to the regulatory domain of scallop myosin. Proc Natl Acad Sci USA 1995; 92:10644-10647.
24. Rhoads AR, Friedberg F. Sequence motifs for calmodulin regulation. FASEB J 1997; 11:331-340.
25. Warne PH, Viciana PR, Downward J. Direct interaction of ras and the amino-terminal domain of raf-1 in vitro. Nature 1993; 364:352-355.
26. Zhang X-F, Settleman J, Kyriakis JM et al. Normal and oncogenic $p21^{ras}$ proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 1993; 364:308-313.
27. Hall A. ras and GAP-who's controlling whom? Cell 1990; 61:921-923.
28. Milburn MV, Tong L, deVos AM et al. Molecular switch for signal transduction: structural differences between active and inactive forms of protooncogenic *ras* proteins. Science 1990; 247:939-945.
29. Manser E, SYM, Zhao Z-S, Lim L. A brain serine/threonine protein kinase activated by CDC42 and Rac1. Nature 1994; 367:40-46.
30. Burbelo PD, Drechsel D, Hall A. A conserved binding motif defines numerous candidate target proteins for both CDC42 and Rac GTPases. J Biol Chem 1995; 270:29071-29074.
31. Symons M, Derry JMJ, Karlak B et al. Wiskott-Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. Cell 1996; 84:723-734.
32. Scheffzek K, Ahmadian MR, Kabsch W et al. The Ras-RasGAP complex: Structural basis for GTPase activation and its loss in oncogenic Ras mutants. Science 1997; 277:333-338.
33. Tolias KF, Cantley LC, Carpenter CL. Rho family GTPases bind to phophoinositide kinases. J Biol Chem 1995; 270:17656-17659.
34. Hartwig JH, Bokoch GM, Carpenter CL et al. Thombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell 1995; 82:643-653.
35. Aspenström P, Lindberg U, Hall A. Two GTPases, CDC42 and Rac, bind directly to a protein implicated in the immunodeficiency disorder Wiskott-Aldrich syndrome. Curr Biol 1996; 6:70-75.

36. Miki H, Miura K, Takenawa T. N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangeement in a PIP2-dependent manner downstream of tyrosine kinases. EMBO J 1996; 15:5326-5335.
37. Van Aelst L, Joneson T, Bar-Sagi D. Identification of a novel Rac1-interacting protein involved in membrane ruffling. EMBO J 1996; 15:3778-3786.
38. Hall A. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu Rev Cell Biol 1994; 10:31-54.
39. Tapon N, Hall A. Rho, Rac and CDC42 GTPases regulate the organization of the actin cytoskeleton. Curr Opin Cell Biol 1997; 9:86-92.
40. Ridley AJ, Hall A. The small GTP-binding protein *rho* regulates assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992; 70:389-399.
41. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A. The small GTP-binding protein *rac* regulates growth factor-induced membrane ruffling. Cell 1992; 70:401-410.
42. Nobes CD, Hall A. Rho, Rac, and CDC42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stess fibers, lamellipodia, and filipodia. Cell 1995; 81:53-62.
43. Kozma R, Ahmed S, Best A, Lim L. The Ras-related protein CDC42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 1995; 15:1942-1952.
44. Ridley AJ. Membrane ruffling and signal transduction. Bioessays 1994; 16:321-327.
45. Zigmond SH. Signal transduction and actin filament organization. Curr Opin Cell Biol 1996; 8:66-73.
46. Eaton S, Auvinen P, Luo L, Jan YN, Simons K. CDC42 and Rac1 control different actin-dependent processes tn the Drosophila wing disc epithelium. J Cell Biol 1995; 131:151-164.
47. Nusrat A, Giry M, Turner JR et al. Rho protein regulates tight junctions and perijunctional actin organization in polarized epithelia. Proc Natl Acad Sci USA 1995; 92:10629-10633.
48. Bokoch GM. Regulation of the human neutrophil NADPH oxidase by the Rac GTP-binding proteins. Curr Opin Cell Biol 1994; 6:212-218.
49. Vojtek AB, Cooper JA. Rho family members: Activators of MAP kinase cascades. Cell 1995; 82:527-529.
50. Braga VMM, Machesky L, Hall A, Hotchin NA. The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. J Cell Biol 1997; 137:1-11.

Section IV
New Anticancer Drugs

CHAPTER 19

Farnesyltransferase Inhibitors: Agents for the Treatment of Human Cancer

Kenneth S. Koblan and Nancy E. Kohl

Introduction

Mutationally activated *ras* genes are the oncogenes most frequently found in human tumors.[1] Approximately 30% of human cancers, including 90% of pancreatic cancers and 50% of colon cancers,[2,3] harbor oncogenically mutated *ras* genes. These mutations are most commonly found in the Ki- and N-*ras* genes, but are also present in some Ha-*ras* genes. These three *ras* genes encode four highly homologous Ras proteins, Ha-, N-, Ki4A- and Ki4B-Ras. They are monomeric G proteins of 21 kDa that function in the regulation of the transduction of proliferative or differentiating signals from the membrane to the nucleus depending on the cell type (see ref. 1). Cycling of Ras between the active, GTP-bound and inactive, GDP-bound forms is accomplished by both GAPs that stimulate the intrinsic GTPase activity of Ras and GDSs that stimulate the GDP dissociation (GDP/GTP exchange). Mutations in Ras which abolish the GAP-sensitivity, such as those found in human tumors, result in constitutively active proteins.

The Ras proteins are produced in the cytoplasm as precursor molecules and must undergo a series of post translational modifications to generate membrane localized, biologically active proteins (reviewed in ref. 4). The first and obligatory step in this series is the addition of the 15-carbon isoprenoid, farnesyl, to the C-terminal cysteine residue in a reaction catalyzed by the enzyme farnesyl:protein transferase (FPTase). This cysteine, located four amino acids from the carboxy terminus of the protein, is part of the CA_1A_2X motif found in all FPTase protein substrates, where C is cysteine, A_1 and A_2 are usually aliphatic amino acids and X is usually serine, methionine, glutamine, alanine or cysteine. Following farnesylation, the A_1A_2X residues are proteolytically cleaved and the now C-terminal cysteine is methylated. For all Ras proteins except Ki4B-Ras, palmitate groups are then added to cysteine residues upstream of the farnesylated cysteine.

Genetic analyses in both yeast and mammalian cells have demonstrated that farnesylation is essential for the transforming activity of the Ras oncoproteins. Oncogenically mutated Ras proteins in which the farnesyl acceptor cysteine is mutated such that farnesylation can not occur are no longer able to transform cells.[5-8] Genetic ablation of FPTase in yeast renders oncogenic Ras mutants harboring mutations analogous to those found in oncogenic mammalian Ras unable to display the phenotypes characteristic of these

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

proteins.[9,10] Importantly, yeast in which FPTase is ablated are viable.[11] These observations suggest that inhibitors of FPTase might be effective in the treatment of some human cancers.

FPTase, a ubiquitously expressed, cytosolic enzyme, is a heterodimer comprised of two subunits, a 45 kDa α subunit and a 48 kDa β subunit.[4] FPTase requires both Zn^{2+} and Mg^{2+} for activity: the Zn^{2+} is required for binding of the protein substrate and during catalysis appears to be coordinated to the cysteine thiol of the CA_1A_2X substrate.[12] In addition to the Ras proteins, other substrates of FPTase include the nuclear lamins; three proteins of the visual transduction system, transducin, cGMP phosphodiesterase and rhodopsin kinase; skeletal muscle phosphorylase kinase; the peroxisomal protein, Pxf; the cell regulatory phosphatases PTP1 and PTP2; and RhoB.[13,13a] CA_1A_2X tetrapeptides can serve as substrates for farnesylation, suggesting that the minimal recognition sequence of these proteins by FPTase is the CA_1A_2X tetrapeptide.[14,15] However, for some protein substrates, additional elements, such as the polylysine domain just upstream of the CA_1A_2X motif in Ki4B-Ras,[16] are also important determinants for interaction with the enzyme.

FPTase is one of a family of prenyltransferases found in mammalian cells. Two other enzymes, geranylgeranyl-protein transferase (GGPTase) types I and II, catalyze the addition of a 20-carbon isoprenoid, geranylgeranyl, to the C-terminal cysteine residues of substrate proteins. GGPTase-I preferentially recognizes CA_1A_2X-containing proteins in which X is a leucine or phenylalanine. However, the specificity of FPTase and GGPTase-I is not absolute. For example, proteins that are preferentially farnesylated, such as N-Ras, Ki4A-Ras and Ki4B-Ras, in which the X residue is a methionine, can be substrates for geranylgeranylation by GGPTase-I,[17-19] particularly when FPTase activity is inhibited.[11,20,21] GGPTase-II transfers the geranylgeranyl group to both cysteines of proteins that have C-terminal sequences CXC, CC or CCXX.[4]

Several methods have been used to identify small molecule inhibitors of FPTase, including random screening of defined chemicals, natural products and combinatorial libraries, and rational design based on the isoprenoid and protein substrates of the farnesylation reaction. All of these methods have yielded potent inhibitors of the enzyme. This review will highlight the development and biological properties of each of these classes of FPTase inhibitors (FTIs).

CA_1A_2X Analogs

Mechanistic and biochemical studies of FPTase have demonstrated that, although the enzyme utilizes polypeptides as substrates in the cell, tetrapeptides corresponding to the C-terminal tetrapeptide from the Ras proteins (CA_1A_2X box) contain the essential determinants for enzyme recognition and, thus, can be farnesylated.[14] The substrate-like tetrapeptides are potent inhibitors of the enzyme with IC_{50} values similar to the K_M values for the intact proteins. Structure-activity studies demonstrated that a wide range of amino acids are tolerated within the central dipeptide ("A_1A_2" of the CA_1A_2X) while the carboxyl terminal X residue is a critical prenylation determinant.[18,22,23]

In cell culture models, CA_1A_2X-based tetrapeptides failed to affect the post-translational processing of Ras proteins or alter the growth characteristics of *ras*-transformed cells. The lack of biological activity for tetrapeptides in culture is most likely due to instability from proteolytic degradation, poor membrane permeance, and inactivation via FPTase-catalyzed farnesylation. Tetrapeptides in which the penultimate residue (A_2) was replaced with an aromatic amino acid (e.g., CVFM) yielded potent nonsubstrate inhibitors.[24] Since the nonsubstrate peptides CVFM and CIFM were also inactive in tissue culture, subsequent synthetic efforts focused on stabilization of the backbone and membrane permeability.

The incorporation of nonhydrolyzable, isosteric replacements for the amide bonds of CA_1A_2X tetrapeptides can lead to increases in FPTase inhibition (in vitro potency against

the enzyme). Incorporation of the reduced amide (ψ-CH_2NH) linkage at various points in the CA_1A_2X sequence (e.g., B581 IC_{50}= 21 nM; L-731,735 IC_{50}=20 nM; Fig. 19.1) increased intracellular stability and resulted in potent compounds. Isosteric replacements afforded compounds that were selective, nonsubstrate inhibitors that were active in cell culture models. The lactone, L-731,734 (a prodrug for L-731,735) as well as the doubly reduced CIFM analog, B581 (Eisai), inhibited Ha-Ras farnesylation in intact cells with an IC_{50} value of 50 μM.[25,26] At a concentration of 1 mM, L-731,734 blocked the growth in soft agar of Ha-*ras* transformed cells, a hallmark of a cancer cell's transformed phenotype.

The CA_1A_2X tetrapeptide motif has served as a template for replacement of the central hydrophobic residues with aminobenzoic acid or aminobenzodiazepine scaffold and results in compounds with nanomolar IC_{50}'s and specificity for FPTase over GGPTase-I.[27,28] Hamilton and co-workers (University of Pittsburgh) have utilized the aminobenzoic acid scaffold to develop potent inhibitors which block Ha-Ras farnesylation in transformed cells at 1 μM and reduce the growth of Ras tumors in nude mouse xenografts (i.e., FTI-277). Brown and Goldstein and collaborators (Genentech, University of Texas) have developed a series of benzodiazepine analogs of the CA_1A_2X tetrapeptide designed on a model in which the CA_1A_2X adopts a beta turn when bound at the active site of FPTase (experimental transfer NOE data supports the beta turn hypothesis[29,30]). The benzodiazepine analog BZA-2B (IC_{50} = 0.3 nM) is a potent and selective FPTase inhibitor and an ester prodrug, BZA-5B, slows the growth of Ha-*ras*-transformed cells in monolayer and increases the life span of nude mice following intraperitoneal implantation of HT1080 cells (a human fibrosarcoma line).[31,32] Development and refinement of existing nonpeptidal FPTase analogs may benefit from the recent elucidation of the crystal structure of FPTase.[33]

Work in our laboratory has focused on a peptidomimetic, L-739,750, which was based on the tetrapeptide Cys-Ile-Phe-Met (Fig. 19.1). The backbone of L-739,750, contains an oxymethylene peptide bond replacement and substitutes a methionine sulfone in the X position. The compound is a potent FPTase inhibitor (IC_{50} = 1.8 nM) and a prodrug form (L-739,749), inhibits Ras farnesylation and membrane association in cells at low micromolar concentrations (half-maximal effect 0.1-1 μM). The terminal carboxylate is masked by esterification to enhance cell penetration; in the presence of intracellular esterases the labile ester is converted to the free acid form L-739,750. Importantly, the geranylgeranylation of proteins in these cells was unaffected by this compound and thus remained FPTase-specific. The ester L-739,749 also reduces the colony formation of Ha-*ras*-transformed cells in soft agar at concentrations in the range of 1 to 2 μM and blocks the growth of Ha-*ras*-dependent tumor xenografts in nude mice. The tumor growth suppression by L-739,749 appears to result from inhibition of either Ras or RhoB pathways and not from nonspecific cytotoxicity, as the growth of cells transfected by either v-*mos* or v-*raf*, proteins which function downstream of Ras, were unaffected.[33a]

Cell culture studies utilizing many different FPTase inhibitors indicate that this class of compounds would act primarily as cytostatic agents. However, recent studies conducted in transgenic mice that overexpress the v-Ha-*ras* oncogene demonstrate that tumor regression can be induced by inhibiting protein farnesylation.[34] Complete regression of 100% of small established tumors and partial regression (partial with respect to the number of respondents and degree of response) of large mammary and salivary tumors was achieved with the isopropyl ester of L-739,750 (L-744,832, Fig. 19.1). At a dose of 40 mg/kg/day, subcutaneously, tumors ranging in size from 50 to 2000 mm^3 were observed to undergo nearly complete regression within a few weeks of initiating treatment. Remarkably, mice treated for up to 11 weeks with this compound showed no apparent signs of toxicity to the gastrointestinal tract, bone marrow, or retina, tissues which are known to contain farnesylated proteins or

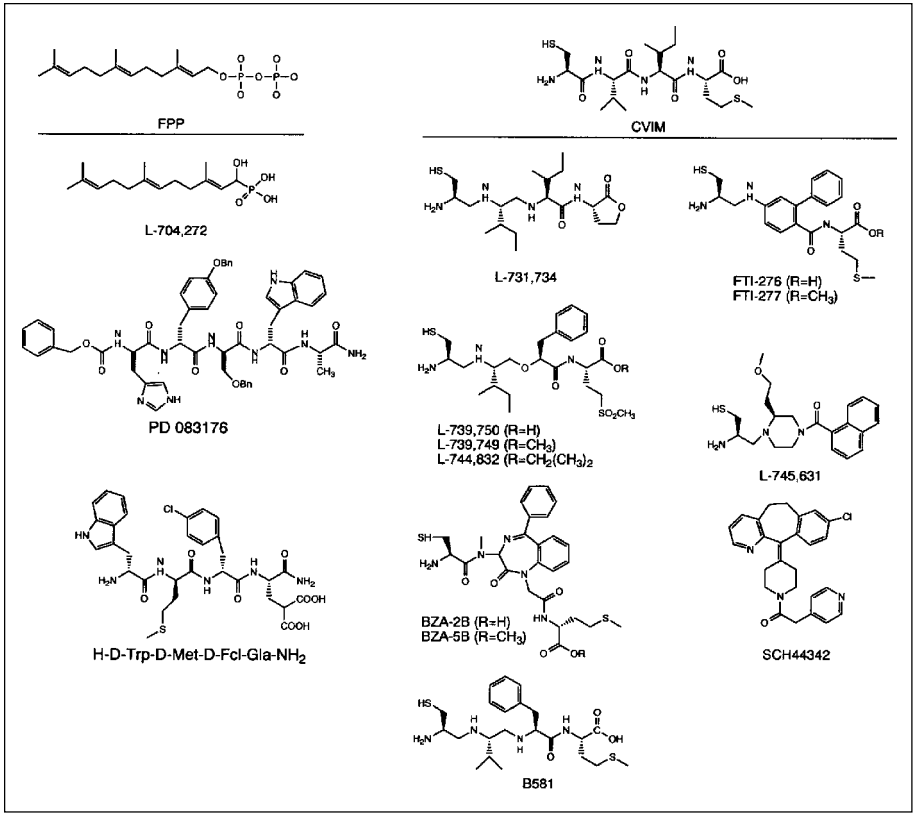


Fig. 19.1. Substrate-based inhibitors of farnesyl:protein transferase.

are dependent upon rapid growth and differentiation of cells. Thus, in the rodent systems, as in the tissue culture models, the potential for a high therapeutic index is implied.

Although the strategy of incorporating a prodrug at the carboxyterminus has yielded cell active inhibitors, a more attractive alternative would be deletion of the X residue all together. Several cysteinyl dipeptide amides which are potent nonsubstrate FPTase inhibitors have been reported.[35] Remarkably these dipeptidomimetics retain >100-fold selectivity for FPTase over GGPTase-I in spite of lacking the X residue which seems to determine specificity of prenylation for CA_1A_2X peptides. Hamilton and co-workers have demonstrated that deletion of the carboxylic acid from their biphenyl inhibitors results in only a modest 5-fold loss in activity. Several piperazine derivatives (e.g., L-745,631), lacking the carboxy terminal portion of the CA_1A_2X sequence are potent FPTase inhibitors (IC_{50}=1-10 nM) and block the growth of Ha-*ras*-transformed Rat1 cells (47% inhibition at a dose of 45 mg/kg/day i.p.) in mouse xenograft studies.[36]

In addition to modification of the carboxy terminus of the CA_1A_2X peptidomimetics, development of a surrogate for the cysteine residue because of its potential for thiol-based toxicity in animals has been pursued by a number of groups. Brystol-Myers Squibb has described a number of compounds in which histidine or imidazole has served as a surrogate for cysteine in CA_1A_2X-based inhibitors.[37] The imidazole structures have been designed to provide a ligand for an active site zinc ion which has been suggested to coordinate to the

thiol group of the CA_1A_2X sequence during catalysis. Patent applications have described other heterocyclic cysteine surrogates which include pyroglutamyl and imidazolyl compounds although the activity in animal models has not been disclosed.[38] The report by Schering-Plough of a tricyclic inhibitor SCH44342 (an analog of loratadine) as a 160 nM FPTase inhibitor, which is competitive with CA_1A_2X binding, was the first compound to lack both a thiol and carboxylic acid of the earlier analogues.[39] The compound inhibits Ha-Ras farnesylation in cell culture at concentrations between 1 and 3 μM. Alterations in the morphology of cells transformed by Ha-*ras* alleles were reported at concentrations of SCH44342 ranging from 5-10 μM. The relationship between SCH44342 and molecules which are known to have advantageous pharmacokinetic profiles rendering them useful in humans (e.g., cyproheptadine) may bode well for the clinical utility of this class.

FPP Analogs and Antagonists

A number of farnesyl phosphonic acids have been reported which are purely competitive inhibitors of FPTase rather than substrates in the enzymatic reaction.[23,40] These compounds, which lack the pyrophosphate leaving group of FPP, are potent inhibitors of FPTase and are remarkably selective (>1000-fold) in their inhibition of FPTase over other isoprenoid utilizing enzymes in vitro. The (α-hydroxyfarnesyl)phosphonic acid derivative (L-704,272 IC_{50} = 5 nM, Fig. 19.1) partially suppresses Ha-Ras farnesylation in cell culture at micromolar concentrations and was one of the first compounds to display biological activity.[41] Compounds which contain a lipophilic diacid motif or polycarboxylic acids (e.g., chaetomellic acid and zaragozic acids) are a recurring theme in FPTase inhibitors that compete with FPP (see below).[42-44] In general FPP analogs display some selectivity for FPTase in vitro. However, in vivo the precise effect on other FPP-utilizing enzymes (e.g., squalene synthase, FPP synthase) is unclear and therefore so are the cellular effects.

A series of inhibitors derived via a synthetic peptide combinatorial library approach have been reported which differ substantially from the typical CA_1A_2X sequence.[45] The C-terminal residue is the amide of gamma-carboxyglutamic acid, while the other amino acids are typically in the D-configuration (e.g., H-D-Trp-D-Met-D-Fcl-Gla-NH_2, Fig. 19.1). Surprisingly, it appears that the tetrapeptides compete with FPP in the farnesylation reaction, presumably via the dicarboxylate of the C-terminus interacting at the FPP binding site on FPTase. Several peptide-based inhibitors which compete with FPP binding have recently been described by Parke-Davis (e.g., PD083176).[46] The potency of this series of compounds is affected by inclusion of phosphate ions in the assay buffer and may enable the design of more potent and specific FPTase inhibitors.

Microbial-Derived FTIs

In parallel with the design of FTIs based on the protein and isoprenoid substrates of the farnesylation reaction, there has been a considerable effort directed toward the identification of natural product-derived inhibitors of FPTase. The natural product-derived FTIs reported to date can be grouped into four classes based on their kinetic mechanism: (1) compounds competitive with FPP; (2) compounds competitive with the protein acceptor; (3) compounds competitive with neither substrate; and (4) compounds for which the kinetic mechanism is not known. Compounds in class 1 include manumycin, zaragozic acids, chaetomellic acids, actinoplanic acids, organic acid and RPR113228. The majority of these compounds resemble FPP in structure, having a polar head group, consisting of one or more carboxylic acid moieties, and a hydrophobic tail. It is hypothesized that the carboxyl carbons of these compounds can superimpose onto the phosphorus atoms and the hydrophobic chain onto the isoprene groups of FPP. Molecular modeling studies have demonstrated for chaetomellic acid A that such a superimposition is possible.[42]

Manumycin (Fig. 19.2), perhaps the most thoroughly studied of the natural product FTIs, was identified in a microbial screen using a yeast strain with a deficiency in the GPA1 gene that codes for the α subunit of yeast guanine nucleotide-binding protein.[44] When dissociated from the α subunit, the βγ subunit of the G-protein functions as a growth inhibitor. Inhibition of the function of the βγ subunit by inhibition of farnesylation of the γ subunit restores growth to gpa1 mutants. Analysis of derivatives of manumycin suggest that the cyclohexenone epoxide moiety is required for activity but that the triene chain and the amide-bound C_5N moiety are not.[44] Manumycin inhibits FPTase with an IC_{50} value of 5-35 μM and is selective with respect to the related prenyltransferase GGPTase-I (IC_{50}=180 μM).[44] Manumycin has been shown to inhibit the anchorage-dependent growth of the human hepatoma cell line, Hep G2, which harbors an oncogenically mutated N-*ras* gene, in a dose- and time-dependent manner.[47] Inhibition of cell growth correlated with inhibition of DNA synthesis. Manumycin inhibited N-Ras farnesylation and Map kinase activity in the Hep G2 cells but had no effect on processing of the GGPTase-I substrate, Rap1. Importantly, manumycin did not affect HMGCoA activity or cholesterol biosynthesis in these cells.

Manumycin has shown anti-tumor activity in vivo against a variety of xenografts, including a Ki-*ras*-transformed murine fibrosarcoma, K-BALB, and two human tumor cell lines, HT1080, a fibrosarcoma that harbors an activated N-*ras* gene, and MIA PaCa-2, a pancreatic carcinoma that harbors a Ki-*ras* oncogene.[44,48] Furthermore, manumycin has been shown to suppress the multivulval phenotype in *Caenorhabditis elegans* resulting from a *let-60 ras* mutation.[49] In *C. elegans*, vulval cell differentiation is controlled by the Ras MAP kinase pathway. *Let-60 ras* encodes a Ras protein that is 83% identical to human N-Ras, and a gain-of-function mutation in this gene that is similar to *ras* mutations detected in many human cancers results in an abnormally high number of vulval cells (the multivulval phenotype). Manumycin is able to suppress this phenotype in a dose-dependent manner without other significant effects on the organism.

The zaragozic acids (A, B, C, D and D_2, Fig. 19.2) were originally isolated as potent inhibitors of the FPP-utilizing enzyme squalene synthase[43,50] and were later shown to be inhibitors of FPTase.[41,43] The usefulness of these compounds as FTIs is limited since they are more potent inhibitors of squalene synthase (IC_{50} = 0.2-6 nM) than of FPTase (IC_{50} =100-1000 nM).[43] However, zaragozic acid A exhibits a 2- to 3-fold selectivity for FPTase versus GGPTase-I.[41] Improvement in potency and selectivity has been achieved with a semisynthetic analog of zaragozic acid A, zaragozic acid A analog (Fig. 19.2), indicating that it might be possible to design a potent and selective inhibitor in this series. Neither zaragozic acid A nor the analog are active in cell-based processing assays at concentrations up to 100 μM, presumably because of poor membrane permeability due to their charge.[41]

Chaetomellic acids A and B (Fig. 19.2) are both potent (IC_{50} values for chaetomellic acids A and B are 55 and 185 nM, respectively), reversible inhibitors of FPTase that are selective as compared to GGPTase-I, GGPTase-II and squalene synthase.[41,51] The inactivity of chaetomellic acid A in a cell based processing assay at concentrations up to 100 μM[41] suggests that the utility of these compounds may be limited.

Actinoplanic acids A and B (Fig. 19.2) are potent inhibitors of FPTase (IC_{50} values of 230 nM and 50 nM, respectively) and exhibit good selectivity as compared to squalene synthase and GGPTase-I (IC_{50} >>1 μM for both compounds).[52,53] Organic acid (Fig. 19.2) is a yet more potent inhibitor of FPTase (IC_{50} = 14 nM) that similarly shows good selectivity as compared to GGPTase-I (IC_{50} = 60 μM).[54,55] As is true of the structurally similar chaetomellic acids, organic acid is inactive at concentrations up to 10 μM in whole cell processing assays, presumably due to the charged nature of the compound.

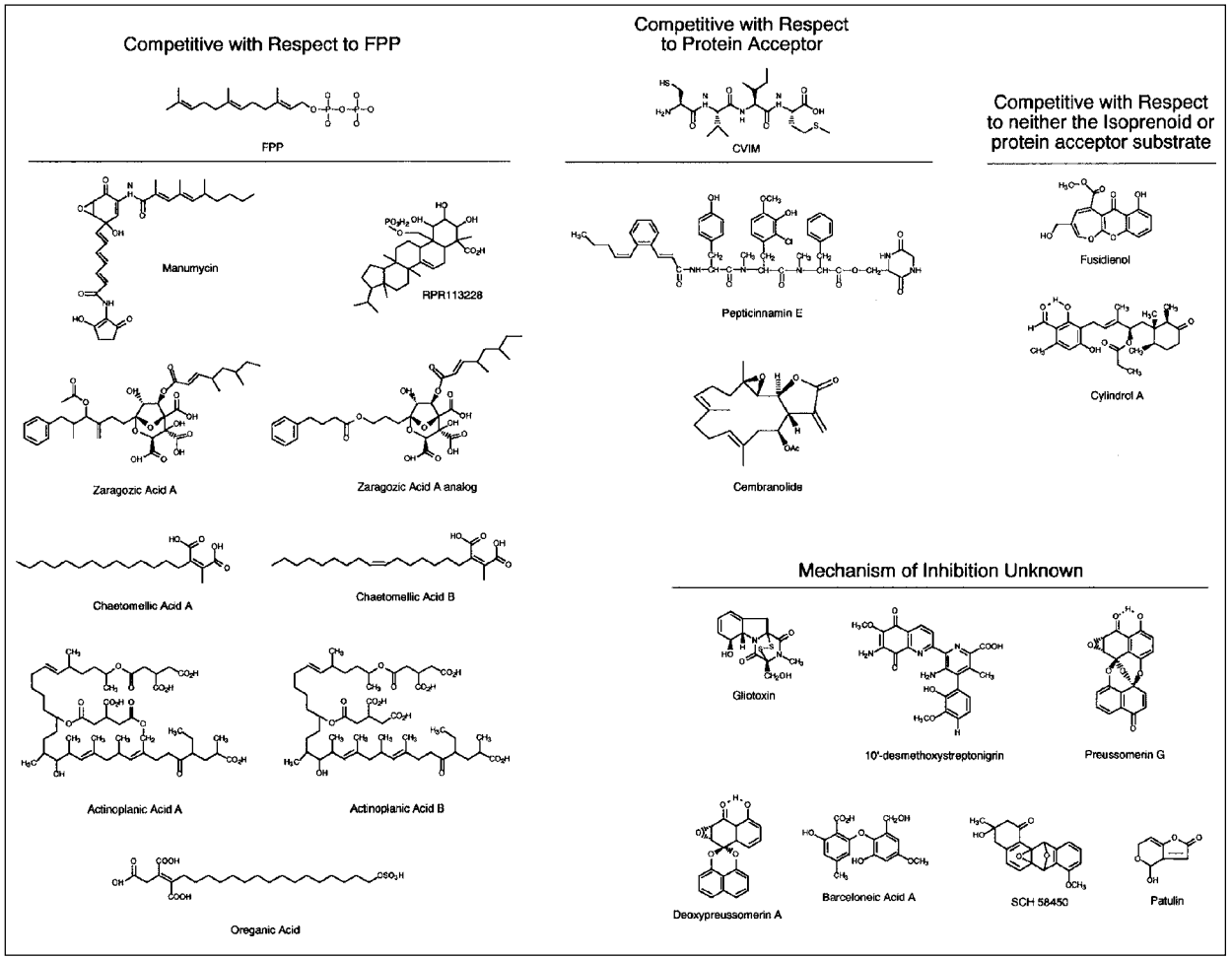


Fig. 19.2. Microbially-derived inhibitors of farnesyl:protein transferase.

RPR113228 (Fig. 19.2), isolated from the fermentation broth of the fungus *Chrysosporium lobatum*, inhibits FPTase with an IC_{50} value in the range of 0.8-2 μM.[56] This compound is selective for FPTase as compared to GGPTase-I (IC_{50} = 59 μM) and squalene synthase (inactive).

Compounds which are competitive with respect to the protein acceptor in the farnesylation reaction include the pepticinnamins and cembranolide (Fig. 19.2).[57-59] The pepticinnamins, a series of peptides produced by *Streptomyces* sp. OH-4652, inhibit FPTase with IC_{50} values in the range of 0.1-1.0 μM.[57] Cembranolide, isolated from a marine soft coral *Lobophytum cristagalli*, is a potent (IC_{50} = 0.15 μM) and selective (IC_{50} vs. GGPTase-I = 5.3 μM) inhibitor of FPTase.[59] While this compound shows weak activity in whole cell processing assays (26% inhibition of processing of Ha-Ras at 5.3 μM), cellular toxicity precludes the use of higher concentrations.

Both fusidienol and cylindrol A fail to show kinetic competition with either the protein or isoprenoid substrate in the farnesylation reaction.[60-62] Fusidienol, isolated from the fungus *Fusidium griseum*, inhibits bovine brain FPTase with an IC_{50} value of 300 nM, but is less potent an inhibitor of human FPTase (IC_{50}= 2.7 μM).[60] This compound is inactive against squalene synthase and GGPTase-I. Cylindrol A is a less potent inhibitor of FPTase (IC_{50}=2.2 μM) and is similarly inactive against squalene synthase and GGPTase-I.[61] Structure-activity analyses indicate that the bicyclic ring system as well as the aldehyde and ketone are all important for biological activity.[62] Small changes in the molecule have profound effects on FPTase activity.

Compounds for which the mechanism of action are not known include gliotoxin, 10'-desmethoxystreptonigrin, preussomerins and deoxypreussomerins, barcelonic acid A, SCH58450 and patulin (Fig. 19.2). Gliotoxin was isolated from a fermentation broth of a fungus by direct assay of FPTase,[63] although the compound also scores in the yeast assay.[49] Gliotoxin inhibits FPTase with an IC_{50} value of 1.1 μM.[63] It is likely that the active form of the compound is the dithiol form in which the disulfide bridge is reduced, since the FPTase assays include DTT. While the exact mechanism of inhibition of gliotoxin has not been defined, the compound is noncompetitive with respect to the protein acceptor and is not itself a substrate. Similar to manumycin, gliotoxin shows antitumor activity against the K-BALB and HT1080 cell lines in xenograft models and suppresses the multivulval phenotype in *C. elegans*.[49]

10'-desmethoxystreptonigrin, a novel analog of streptonigrin produced by *Streptomyces albus*, is a broad spectrum antibiotic with modest FPTase inhibitory activity (IC_{50}=21 μM).[64] When evaluated in vivo in the P388 leukemia model, this compound did not show antitumor activity at nontoxic doses. The preussomerins and deoxypreussomerins inhibit FPTase with IC_{50} values in the range of 1.2-17 μM.[65] Selectivity for FPTase over GGPTase-I has been demonstrated for one member of this family, preussomerin G (FPTase IC_{50}=1.2 μM; GGPTase-I IC_{50}=20 μM). Maximal activity requires that the compound contain a conjugated ketone in the lower half of the preussomerin molecule and either an epoxide or at least a 1,2-dioxygen in the upper half of the molecule. Loss of one of these structural features compromises activity, while loss of both features abolishes activity. Barcelonic acid A is a relatively weak FPTase inhibitor, exhibiting an IC_{50} value of 40 μM.[66] SCH 58450, isolated from a *Streptomyces sp.*, inhibits FPTase with an IC_{50}=29 μM and exhibits 25-fold selectivity for FPTase over GGPTase-I.[67] Patulin is a weak inhibitor of FPTase (IC_{50} =290 μM).[68] While this compound inhibits incorporation of radiolabeled mevalonate, an isoprenoid precursor, into cellular proteins (50% inhibition at 5 μM), it is difficult to attribute this effect to inhibition of FPTase.

Issues

Numerous structurally diverse analogs of the two substrates of the farnesylation reaction, FPP and CA_1A_2X tetrapeptides, have been identified that are potent and selective inhibitors of FPTase in vitro. A subset of these compounds show anti-tumor activity in in vivo models of cancer.[34,36,44,48,49,69-73] When investigated, no signs of clinical toxicity have been reported in treated animals.[34,48,69] While such a therapeutic index bodes well for the usefulness of the FTIs in the clinic, it is difficult to explain the apparent lack of toxicity to untransformed tissues. The wild type Ras present in normal tissues requires farnesylation for biological activity as does oncogenic Ras. It has been proposed that untransformed cells contain a form of Ras, N-Ras or Ki-Ras, whose prenylation is not inhibited by the FTIs.[74] Indeed, tetrapeptides analogous to the CA_1A_2X sequences of N- and Ki-Ras bind more tightly to the enzyme than a Ha-Ras CA_1A_2X tetrapeptide.[22] This observation suggests that it may be more difficult to inhibit the farnesylation of N- and Ki-Ras than Ha-Ras in cells. In this regard, it has been shown that higher concentrations of a CA_1A_2X peptidomimetic are required to inhibit the anchorage independent growth of human tumor cells harboring a mutant N- or Ki-*ras* gene than those lines harboring a mutant Ha-*ras* gene.[70]

Another group has demonstrated that inhibition of the anchorage independent growth of human tumor cells is independent of the mutational status of *ras*:[75] approximately half of the cells with wild type *ras* and half of the cells harboring mutant Ki-*ras* alleles were sensitive to the FTI. These results suggest that the specificity of the FTI is not limited to cells whose aberrant growth is driven by Ras. In some of the cells with wild type *ras*, sensitivity to the FTI may result from activation of the Ras pathway by alteration of proteins upstream of Ras. Another possible explanation is that farnesylated proteins other than Ras also play a role in the biology of the FTIs. Known farnesylated proteins that could be important for growth include RhoB, RhoE, PTP1 and PTP2.[13,76]

It has recently been demonstrated that N- and Ki-Ras, but not Ha-Ras, are modified by a geranylgeranyl group when FPTase activity is ablated.[20,21] In mammalian cells, geranylgeranylated forms of oncogenic Ras exhibit the same level of transforming activity as the farnesyl-modified Ras.[77] These observations raise the possibility that use of a nonselective inhibitor or a combination of an FTI and a GGPTase-I inhibitor (GGTI) might be more effective than a highly selective FTI against human tumors in which the most commonly mutated *ras* alleles are Ki- and N-*ras*. However, it has recently been reported that cotreatment of mice bearing tumors harboring a mutant K-Ras oncoprotein with an FTI and a GGTI did not enhance the antitumor activity of the FTI.[78]

It is clear that many important issues remain to be addressed in the preclinical development of FTIs and work continues in the laboratory to gain additional insight into the biology of these compounds. Nevertheless, the anti-tumor activity of these compounds in the absence of toxic side effects in animal models is encouraging. However, the ultimate utility of the FTIs against human cancers will have to await evaluation in the clinic.

Acknowledgments

We thank Drs. Jay Gibbs and Sam Graham for critical reading of the manuscript.

References

1. Barbacid M. *ras* Genes. Ann Rev Biochem 1987; 56:779-827.
2. Bos JL. The *ras* gene family and human carcinogenesis. Mutation Res 1988; 195:255-271.
3. Bos JL. *ras* oncogenes in human cancer: a review. Cancer Res 1989; 49:4682-4689.
4. Zhang FL, Casey PJ. Protein prenylation: molecular mechanisms and functional consequences. Ann Rev Biochem 1996; 65:241-269.

5. Willumsen BM et al. Harvey murine sarcoma virus p21 *ras* protein: Biological and biochemical significance of the cysteine nearest the carboxy terminus. EMBO J 1984; 3:2581-2585.
6. Kato K et al. Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. Proc Natl Acad Sci USA 1992; 89:6403-6407.
7. Jackson JH et al. Farnesol modification of Kirsten-ras exon 4B protein is essential for transformation. Proc Natl Acad Sci USA 1990; 87:3042-3046.
8. Hancock JF et al. All ras proteins are Polyisoprenylated but Only Some are Palmitoylated. Cell 1989; 57:1167-1177.
9. Powers S et al. RAM, a gene of yeast required for a functional modification of RAS proteins and for production of mating pheromone a-factor. Cell 1986; 47:413-422.
10. Goodman LE et al. Mutants of Saccharomyces cerevisiae defective in the farnesylation of Ras proteins. Proc Natl Acad Sci USA 1990; 87:9665-9669.
11. Trueblood CE, Ohya Y, Rine J. Genetic evidence for in vivo cross-specificity of the CaaX-box proteinprenyltransferases farnesyltransferase and geranylgeranyltransferase-I in Saccharomyces cerevisiae. Mol Cell Biol 1993; 13:4260-4275.
12. Reiss Y, Brown MS, Goldstein JL. Divalent cation and prenyl pyrophosphate specificities of the protein farnesyltransferase from rat brain, a zinc metalloenzyme. J Biol Chem 1992; 267:6403-6408.
13. Gibbs JB, Oliff A. The potential of farnesyltransferase inhibitors as cancer chemotherapeutics. Ann Rev Pharmacol Toxicol 1997; 37:143-166.
13a. Lebowitz PF et al. Franesyltransferase inhibitors alter the prenylation and growth-stimulating function of RhoB. J Biol Chem 1997; 272:15591-15594.
14. Reiss Y et al. Inhibition of purified $p21^{ras}$ farnesyl:protein transferase by Cys-AAX tetrapeptides. Cell 1990; 62:81-88.
15. Schaber MD et al. Polyisoprenylation of Ras in vitro by a farnesyl-protein transferase. J Biol Chem 1990; 265:14701-14704.
16. James GL, Goldstein JL, Brown MS. Polylysine and CVIM sequences of K-RasB dictate specificity of prenylation and confer resistance to benzodiazepine peptidomimetic in vitro. J Biol Chem 1995; 270:6221-6226.
17. Zhang FL et al. Characterization of Ha-Ras, N-Ras, Ki-Ras4A, and Ki-Ras4B as in vitro substrates for farnesyl protein transferase and geranylgeranyl protein transferase type I. J Biol Chem 1997; 272:10232-10239.
18. Moores SL et al. Sequence dependence of protein isoprenylation. J Biol Chem 1991; 266(22):14603-14610.
19. James G, Goldstein JL, Brown MS. Resistance of K-RasBV12 proteins to farnesyltransferase inhibitors in Rat1 cells. Proc Natl Acad Sci USA 1996; 93:4454-4458.
20. Rowell CA et al. Direct demonstration of geranylgeranylation and farnesylation of Ki-Ras in vivo. J Biol Chem 1997; 272:14093-14097.
21. Whyte DB et al. K- and N-Ras are geranylgeranylated in cells treated with farnesyl protein transferase inhibitors. J Biol Chem 1997; 272:14459-14464.
22. Reiss Y et al. Sequence requirement for peptide recognition by rat brain $p21^{ras}$ protein farnesyltransferase. Proc Natl Acad Sci USA 1991; 88:732-736.
23. Pompliano DL et al. Steady-state kinetic mechanism of Ras farnesyl:protein transferase. Biochemistry 1992; 31:3800-3807.
24. Goldstein JL et al. Nonfarnesylated tetrapeptide inhibitors of protein farnesyltransferase. J Biol Chem 1991; 266(24):15575-15578.
25. Garcia AM et al. Peptidomimetic inhibitors of Ras farnesylation and function in whole cells. J Biol Chem 1993; 268:18415-18418.
26. Kohl NE et al. Selective inhibition of ras-dependent transformation by a farnesyltransferase inhibitor. Science 1993; 260:1934-1937.
27. Nigam M et al. Potent inhibition of human tumor $p21^{ras}$ farnesyltransferase by A_1A_2-lacking $p21^{ras}$ CA_1A_2X peptidomimetics. J Biol Chem 1993; 268:20695-20698.
28. Qian Y et al. Design and structural requirements of potent peptidomimetic inhibitors of p21ras farnesyltransferase. J Biol Chem 1994; 269:12410-12413.

29. Stradley SJ, Rizo J, Gierasch LM. Conformation of a heptapeptide substrate bound to protein farnesyltransferase. Biochemistry 1993; 32:12586-12590.
30. Koblan KS et al. NMR studies of novel inhibitors bound to farnesyl-protein transferase. Protein Science 1995; 4:681-688.
31. James GL et al. Benzodiazepine peptidomimetics: Potent inhibitors of Ras farnesylation in animal cells. Science 1993; 260:1937-1942.
32. Genentech, Inc. and University of Texas, WO9426723 1994.
33. Park H-W et al. Crystal structure of protein farnesyltransferase at 2.25 angstrom resolution. Science 1997; 275:1800-1804.
33a. Lebowitz PF, Davide JP, Prendergast GC. Evidence that farnesyltransferase inhibitors suppress Ras transformation by interfering with Rho activity. Mol Cell Biol 1995; 15:6613-6622.
34. Kohl NE et al. Inhibition of farnesyltransferase induces regression of mammary and salivary carcinomas in ras transgenic mice. Nature Med 1995; 1:792-797.
35. deSolms SJ et al. Pseudodipeptide inhibitors of protein farnesyltransferase. J Med Chem 1995; 38:3967-3971.
36. Williams TM et al. 2-Substituted piperazines as constrained amino acids. Application to the synthesis of potent, non carboxylic acid inhibitors of farnesyltransferase. J Med Chem 1996; 39:1345-1348.
37. Leftheris K et al. Peptide based p21ras farnesyl transferase inhibitors: Systematic modification of the tetrapeptide CA1A2X motif. Bioorganic and Medicinal Chemistry Letters 1994; 4:887-892.
38. Merck & Co., Inc., WO9509001 1995.
39. Schering Corp., WO9510514 1995.
40. Patel DV et al. Phosphinyl acid-based bisubstrate analog inhibitors of Ras farnesyl protein transferase. J Med Chem 1995; 38:435-442.
41. Gibbs JB et al. Selective inhibition of farnesyl-protein transferase blocks Ras processing in vivo. J Biol Chem 1993; 268:7617-7620.
42. Singh SB et al. Isolation and structure of chaetomellic acids A and B from Chaetomella acutiseta: Farnesyl pyrophosphate mimic inhibitors of Ras farnesyl-protein transferase. Tetrahedron 1993; 49:5917-5926.
43. Dufresne C et al. Zaragozic acids D and D2: Potent inhibitors of squalene synthase and of ras farnesyl-protein transferase. J Nat Prod 1993; 56:1923-1929.
44. Hara M et al. Identification of Ras farnesyltransferase inhibitors by microbial screening. Proc Natl Acad Sci USA 1993; 90:2281-2285.
45. Wallace A et al. Selection of potent inhibitors of farnesyl-protein transferase from a synthetic tetrapeptide combinatorial library. J Biol Chem 1996; 271:31306-31311.
46. Leonard DM et al. Structure-activity relationships of cysteine-lacking pentapeptide derivatives that inhibit ras farnesyltransferase. J Med Chem 1997; 40:192-200.
47. Nagase T et al. Inhibition of cell growth of human hepatoma cell line (Hep G2) by a farnesyl protein transferase inhibitor: a preferential suppression of ras farnesylation. Internatl J Cancer 1996; 65:620-626.
48. Ito T et al. Suppression of human pancreatic cancer growth in BALB/c nude mice by manumycin, a farnesyl:protein transferase inhibitor. Japanese J Cancer Res 1996; 87:113-116.
49. Hara M, Han M. Ras farnesyltransferase inhibitors suppress the phenotype resulting from an activated ras mutation in Caenorhabditis elegans. Proc Natl Acad Sci USA 1995; 92:3333-3337.
50. Bergstrom JD et al. Zaragozic acids: A family of fungal metabolites that are picomolar competitive inhibitors of squalene synthase. Proc Natl Acad Sci USA 1993; 90:80-84.
51. Lingham RB et al. Chaetomella acutiseta produces chaetomellic acids A and B which are reversible inhibitors of farnesyl-protein transferase. Applied Microbiol Biotechnol 1993; 40:370-374.
52. Singh SB et al. Actinoplanic acid A: A macrocyclic polycarboxylic acid which is a potent inhibitor of Ras farnesyl-protein transferase. J Amer Chem Soc 1994; 116:11606-11607.
53. Singh SB et al. Structure, chemistry, and biology of actinoplanic acids: Potent inhibitors of Ras farnesyl-protein transferase. J Organic Chem 1995; 60(24):7896-7901.

54. Jayasuriya H et al. Oreganic acid: A potent novel inhibitor of Ras farnesyl-protein transferase from an endophytic fungus. Bioorganic and Medicinal Chemistry Letters 1996; 6:2081-2084.
55. Silverman KC et al. Oreganic acid, a potent inhibitor of Ras farnesyl-protein transferase. Biochem Biophys Res Com 1997; 232:478-481.
56. Van der Pyl D et al. RPR113228, a novel farnesyl-protein transferase inhibitor produced by Chrysosporium lobatum. J Antibiotics 1995; 48:736-737.
57. Omura S et al. Pepticinnamins, new farnesyl-protein transferase inhibitors produced by an actinomycete. J Antibiotics 1993; 46:222-228.
58. Shiomi K et al. Pepticinnamins, new farnesyl-protein transferase inhibitors produced by an actinomycete II. Structural elucidation of pepticinnamin E. J Antibiotics 1993; 46:229-234.
59. Coval SJ et al. A cembranolide diterpene farnesyl protein transferase inhibitor from the marine soft coral Lobophytum cristagalli. Bioorganic and Medicinal Chemistry Letters 1996; 6:909-912.
60. Singh SB et al. Fusidienol: A novel inhibitor of Ras farnesyl-protein transferase from Fusidium griseum. Tetrahedron Letters 1994; 35:4693-4696.
61. Singh SB et al. Cylindrol A: A novel inhibitor of Ras farnesyl-protein transferase from Cylindrocarpon lucidum. Tetrahedron Letters 1995; 36:4935-4938.
62. Singh SB et al. Chemistry and biology of cylindrols: Novel inhibitors of Ras farnesyl-protein transferase from Cylindrocarpon lucidum. J Organic Chem 1996; 61:7727-7737.
63. Van der Pyl D et al. Inhibition of farnesyl-protein transferase by gliotoxin and acetylgliotoxin. J Antibiotics 1992; 45:1802-1805.
64. Liu WC et al. 10'-desmethoxystreptonigrin, a novel analog of streptonigrin. J Antibiotics 1992; 45:454-457.
65. Singh SB et al. Preussomerins and deoxypreussomerins: Novel inhibitors of Ras farnesyl-protein transferase. J Organic Chem 1994; 59:6296-6302.
66. Jayasuriya H et al. Barceloneic acid A, a new farnesyl-protein transferase inhibitor from a Phoma species. J Nat Prod 1995; 58:986-991.
67. Phife DW et al. SCH 58450, a novel farnesyl protein transferase inhibitor possessing a 6a,12a:7,12-diepoxybenz[a]anthracene ring system. Tetrahedron Letters 1995; 36(39): 6995-6998.
68. Miura S, Hasumi K, Endo A. Inhibition of protein prenylation by patulin. FEBS Letters 1993; 318:88-90.
69. Kohl NE et al. Farnesyltransferase inhibitors block the growth of ras-dependent tumors in nude mice. Proc Natl Acad Sci USA 1994; 91:9141-9145.
70. Nagasu T et al. Inhibition of human tumor xenograft growth by treatment with the farnesyl transferase inhibitor B956. Cancer Res 1995; 55:5310-5314.
71. Sun J et al. Ras CAAX peptidomimetic FTI 276 selectively blocks tumor growth in nude mice of a human lung carcinoma with K-ras mutation and p53 deletion. Cancer Res 1995; 55:4243-4247.
72. Leftheris K et al. Development of highly potent inhibitors of Ras farnesyltransferase possessing cellular and in vivo activity. J Med Chem 1996; 39:224-236.
73. Mallams AK et al. Antitumor 8-chlorobenzocycloheptapyridines: A new class of selective, nonpeptidic, nonsulfhydryl inhibitors of Ras farnesylation. Bioorganic and Medicinal Chem 1997; 5:93-99.
74. James GL et al. Benzodiazepine peptidomimetic BZA-5B interrupts the MAP kinase activation pathway in H-Ras-transformed Rat-1 cells, but not in untransformed cells. J Biol Chem 1994; 269:27705-27714.
75. Sepp-Lorenzino L et al. A peptidomimetic inhibitor of farnesyl:protein transferase blocks the anchorage-dependent and -independent growth of human tumor cell lines. Cancer Res 1995; 55:5302-5309.
76. Murphy C et al. Endosome dynamics regulated by a Rho protein. Nature 1996; 384:427-432.
77. Cox AD et al. Specific isoprenoid modification is required for function of normal, but not oncogenic, Ras protein. Mol Cell Biol 1992; 12:2606-2615.
78. Lerner EC, Hamilton AD, Sebti SM. Inhibition of Ras prenylation: A signaling target for novel anti-cancer drug design. Anti-Cancer Drug Design 1997; 12:229-238.

CHAPTER 20

SCH51344: An Inhibitor of RAS/RAC-Mediated Membrane Ruffling

C. Chandra Kumar

Introduction

Twenty-three years ago, Klaus Weber and his colleagues reported that actin filaments are reorganized from an ordered bundle state in normal cells into a diffused mesh in transformed cells.[1,2] Since then, the biochemical and molecular basis of this phenomenon and its importance for malignant transformation has been studied in great detail.[3-5] It is now clearly established that reorganization of actin filaments is a hallmark of many transformed cells. At the biochemical level, actin filament reorganization is reflected in the loss of synthesis of several proteins including smooth muscle (sm) forms of myosin light chain-2 and α-actin, vinculin, gelsolin, α-actinin, tropomyosin I.[6-11] The importance of these changes for malignant transformation is confirmed by the observation that if the normal levels of these proteins are restored, transformation is inhibited and cells revert to normal phenotype.[12-15] These studies suggest that reorganization of the actin cytoskeleton is an essential component of malignant transformation.

In fibroblast cells, actin filaments exist principally in three major structures, the actin stress fibers and the cell surface protrusions including membrane ruffles and microspikes, in addition to the contractile ring formed during mitosis.[3,16] Stress fibers emanate from distinct areas of the plasma membrane known as focal adhesions and span across the cell. A great deal of progress has been made in characterizing the proteins and pathways regulating actin filament organization.[16-21] A major breakthrough during the last few years has been the discovery of the RHO family GTPases whose function is to regulate actin filament organization in eukaryotic cells. RHO controls stress fiber formation, whereas RAC regulates the formation of lamellipodia and membrane ruffles.[22-24] A large number of exchange factors for RHO family members has been identified over the past few years, by virtue of their oncogenic potential in NIH 3T3 cells, suggesting a role for these GTPases in cell transformation.[25-27] However, the exact mechanism(s) whereby actin filaments are reorganized in transformed cells still remains to be elucidated.

Most of the studies concerning the role of oncogenes in bringing about the morphological and cytoskeletal changes, have concentrated on RAS oncogene.[28] RAS is one of the most frequently mutated oncogenes in human cancer, being found in 40% of all human tumors.[29,30] RAS proteins play an essential role in receptor-mediated signal transduction

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

pathways that control cell proliferation and differentiation.[31,32] The three mammalian RAS genes produce four proteins H-, K-(A and B)-, and N-RAS that are membrane localized guanine nucleotide binding proteins.[31] RAS proteins are active in the GTP-bound state and inactive in the GDP-bound state.[31,32] RAS in its GTP-bound state couples the signals of activated growth factors to downstream mitogenic effectors. The high frequency of mutations of RAS in human tumors and its critical function in downstream signaling by oncogenic receptor and cytoplasmic tyrosine kinases renders RAS, its regulators and effectors as promising targets for the development of anticancer drugs.

The first evidence indicating the involvement of RAS in actin filament organization was provided by Dafna Bar-Sagi and Jim Feramisco.[33] They showed that microinjection of RAS V12 protein into REF-52 cells stimulated membrane ruffling and fluid phase pinocytosis. Further studies revealed that RAC, a member of the RHO family, is one of the essential downstream targets for RAS mediating the stimulation of membrane ruffles.[23,34] Recent genetic and biochemical studies indicate that RAS interacts with multiple targets in the cell and has at least two functions in mammalian cells, one regulating gene expression and the other controlling actin cytoskeletal organization.[34,35] The first signaling pathway, referred to as the extracellular-signal regulated kinase (ERK) pathway involves a series of cytoplasmic serine/threonine kinases that eventually leads to phosphorylation of specific transcription factors and activation of immediate early genes. The second pathway referred to as the cell morphology pathway is mediated by members of the RHO family of proteins. These two pathways diverge at the level of RAS and act synergistically to cause transformation.[34-36] These findings open up a new array of drug targets for RAS-mediated malignancies.[37]

A number of approaches have been taken to identify drugs that inhibit the transforming activity of RAS protein either directly or indirectly.[37,38] One direct approach has been to inhibit the activity of the farnesyl transferase (FTase) that is responsible for the transfer of a farnesyl group to the cysteine residue located in a carboxyl-terminal tetrapeptide sequence of the RAS protein.[38] As discussed in detail in the preceding chapter by Nancy Kohl and her colleague, a number of potent compounds that inhibit the FTase activity have been developed.[39-41] Several of these compounds have been shown to reverse critical aspects of RAS transformation and inhibit the growth of RAS transformed cells in nude mice.[42] As discussed in the following chapter, two distinct antibiotics (radicicol and trichostatin A) that upregulate gelsolin gene also can reverse RAS transformation at the cell culture levels.[42a,42b] In addition, a number of other compounds such as azatyrosine, oxanosine and antipain that revert RAS-transformed cells to a normal phenotype have been described.[43-45] However, the mechanism by which these compounds induce phenotypic reversion is not well understood. Because of the importance of the RAS oncogene for the development of malignant tumors in humans, it would be desirable to identify novel targets or mechanisms by which RAS transformation can be inhibited.

Smooth Muscle α-Actin Promoter Activity as a Marker for RAS-Transformation

The most prominent single molecular event among malignantly transformed rat and mouse fibroblasts is a total shut down of sm α-actin synthesis.[7,46] Mammalian actin family comprises six isoforms of which β- and γ- nonmuscle actins are expressed in all cells, whereas smooth muscle α-actin is normally restricted to smooth muscle cells and fibroblasts.[47,48] Sm α-actin expression is regulated by growth hormones, and altered by pathological conditions including oncogenic transformation and atherosclerosis.[49-51] In fibroblasts, sm α-actin comprises only ~14% of the total actin compared with up to 60-70% in vascular smooth muscle cells.[47,48] β-actin predominates in the highly motile lamellipodia, whereas sm α-actin is re-

stricted to the less motile stress fibers.[52,53] Recent studies have shown that the expression of sm α-actin leads to decreased motility, leading to the proposal that an important function of α-actin is to immobilize cells.[54] Since sm α-actin expression is totally shut down in transformed fibroblasts, it is possible that lack of α-actin in transformed cells may be responsible for their constitutive enhanced motility.[54]

We have used the smooth muscle (sm) α-actin gene as a paradigm to understand the molecular mechanisms involved in repression of these cytoskeletal markers in RAS-transformed cells and also for the development of a novel reporter gene-based assay system to identify agents that inhibit RAS-transformation.[55,56] As a first step, we used plasmids containing 5'-upstream sequences of the human sm α-actin gene fused to different reporter genes to demonstrate that changes in the α-actin mRNA and protein levels are due to changes at the transcriptional level. The results of a transient transfection analysis using plasmid pαACAT containing human sm α-actin promoter (-894 to +12 relative to transcription start site) linked to Chloramphenicol acetyl transferase (CAT) reporter gene, revealed that sm α-actin promoter activity is repressed selectively in RAS-transformed DT cells compared to the parental normal NIH3T3 cells. Its activity is restored in C11 revertant cells, indicating that this promoter activity is a sensitive marker to follow phenotypic changes in fibroblasts.[57] The human sm α-actin promoter contains two serum response elements (SREs) that show homology to *c-fos* SRE and bind to serum response factor (SRF) with different affinities.[56,58] We have shown that the two SREs are required to mediate repression of the α-actin promoter in RAS-transformed cells and the two SREs synergize with each other to confer RAS-responsiveness to heterologous promoters.[56] SRF can activate α-actin promoter activity in RAS-transformed cells and YY1, which also binds to SRE, antagonizes this activation.[56] These results suggest the involvement of negative factors that either bind to SRE sequences directly or interact with SRF to mediate repression of the α-actin promoter upon RAS-transformation.

α-Actin Promoter Based Reporter Gene Assay System

We have exploited the phenomenon of actin filament reorganization in transformed cell lines, to develop a novel reporter gene-based assay system to identify agents that inhibit RAS-induced transformation.[55] The rationale for the development of this assay system was based on the observations that human smooth muscle forms of myosin light chain-2 (MLC-2) and α-actin mRNA and protein levels are repressed in RAS-transformed fibroblasts.[6,7,56] Revertants of RAS-transformed cells showed normal levels of MLC-2 gene expression suggesting that the expression of these cytoskeletal markers is modulated by RAS-transformation.[6] A schematic illustration of the α-actin promoter based reporter gene assay system is shown in Figure 20.1. Stable fibroblast cell lines expressing human growth hormone or β-galactosidase (β-gal) under the control of the α-actin promoter were derived. Transformation of these stable cells by RAS resulted in repressed reporter-gene activity. Lastly, the RAS-transformed cells carrying the α-actin promoter-β-gal reporter construct, were used to identify agents that revert RAS-transformation, by their ability to activate or derepress reporter gene activity. This assay system was validated in the following ways: (1) Derepression of the reporter was demonstrated in revertants of RAS-transformed cells;[6] (2) inhibitors of RAS-transformation, such as FTase inhibitors, were found to derepress α-actin promoter driven reporter gene activity in RAS-transformed cells (unpublished observations); (3) a transdominant deletion mutant of v-jun lacking the activation domain which can suppress the RAS-transformed phenotype was shown to derepress the α-actin promoter in RAS-transformed cells (unpublished observations).[59] A major advantage of this system is that cytotoxic agents such as nonspecific inhibitors of DNA, RNA and protein synthesis will

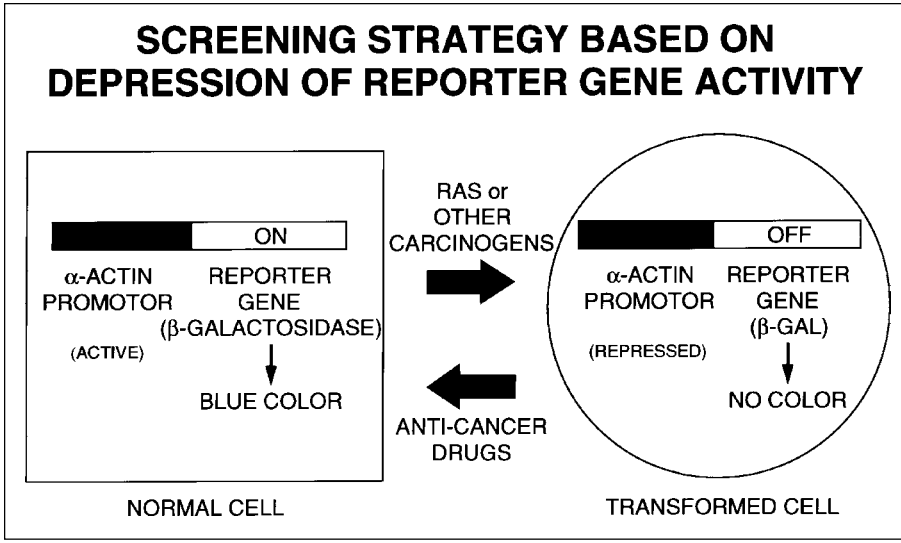


Fig. 20.1. Schematic representation of the strategy for setting up a system for screening drugs based on derepression of reporter-gene activity. See the text for details.

not be scored as false positives, since derepression of reporter gene activity requires the cells to actively synthesize mRNA and protein. Using this cell-based reporter gene assay system, we identified a novel class of molecules known as pyrazolo-quinolines, among the library of compounds at the Schering-Plough Research Institute.[60] A representative compound in this series (SCH51344) was used to validate the efficacy and determine the mechanism by which these compounds inhibit RAS-transformation.

Activation of sm α-Actin Promoter Activity and Restoration of Actin Bundles in RAS-Transformed Cells by SCH51344

The structure of SCH51344 (6-methoxy-4-[2-[(2-hydroxyethoxyl)-ethyl]amino]-3-methyl-1M-pyrazolo [3,4]quinoline) that can derepress α-actin promoter driven CAT activity in RAS-transformed cells is shown in Figure 20.2. Following transfection of plasmid pαACAT into RAS-transformed Rat-2 cells, cells were washed with PBS and a fresh medium containing different concentrations of SCH51344 was added to cells. As a control, the plasmid pBLCAT2 containing minimal thymidine kinase promoter linked to the CAT reporter gene was used. Treatment of RAS-transformed Rat-2 cells with increasing concentrations of SCH51344 led to a significant increase (3- to 50-fold) in α-actin promoter driven CAT activity, whereas it had very little effect on TK promoter driven CAT activity.[60] Next, we wanted to assess the organization of actin filaments in RAS-transformed Rat-2 cells following treatment with SCH51344. Actin filaments were visualized by immunofluorescence microscopy using rhodamine-conjugated phalloidin, which avidly binds filamentous actin. As shown in Figure 20.3, Rat-2 cells exhibited organized actin stress fibers extending throughout the cell from end to end. In contrast, in RAS-transformed cells, the actin cables are diffuse. Treatment of RAS-transformed cells with SCH51344 significantly increased the number of organized stress fibers. Thus activation of sm α-actin promoter activity in RAS-transformed cells by SCH51344 is accompanied by the restoration of organized actin bundles.

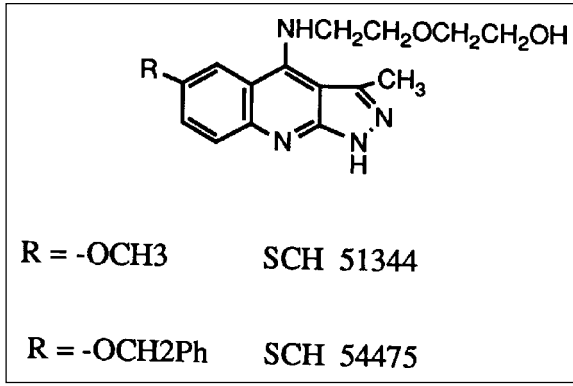


Fig. 20.2. Structure of SCH51344 and SCH 54475, a benzyloxy analog of SCH51344. Reprinted with permission from Cancer Reseaerch 1995; 55:5106-5117.

SCH51344 Inhibits Anchorage-Dependent and -Independent Growth of RAS-Transformed Cells

We have examined the anchorage-dependent and -independent growth of RAS-tranformed cells following treatment with SCH51344.[60] To follow the anchorage-dependent growth, two treatment regimens were employed. Cells were treated continuously with DMSO (control), once with SCH51344, or continuously with SCH51344 by replenishment at each 2-day feeding interval. Single treatment or continuous treatment with SCH51344 (40 μM) caused 56% and 66% inhibition of growth of RAS-transformed cells, respectively. This inhibition of growth of RAS-transformed cells was dose dependent. The drug treatment was also effective in inhibiting the anchorage-independent growth of K-RAS transformed NIH 3T3 cells (DT). In contrast, SCH 54473, a derivative of SCH51344 which was inactive in the α-actin reporter gene assay had very little effect. In addition, we have shown that this compound is effective in inhibiting the anchorage-independent growth of human colon and pancreatic tumor lines including, Panc-1, SW-480 and DLD-1. These tumor lines contain multiple genetic alterations in addition to activated RAS genes. SCH51344 was also effective in inhibiting RAS V12-induced maturation of Xenopus oocytes.[60]

One of the interesting features about SCH51344 is the apparent lack of toxic effect on normal cells. Treatment of PC 12 cells with SCH51344 for 3 days did not inhibit nerve growth factor-induced neurite outgrowth, suggesting that SCH51344 has very little effect on normal signaling pathways mediated by RAS.[60] In addition, pretreatment of Rat-2 fibroblast cells with SCH51344 did not abolish serum-induced activation of *c-fos* promoter activity (unpublished observations). These studies demonstrate that SCH51344 selectively suppresses RAS-transformation without affecting normal cell functions.

SCH51344 Has No Effect on RAS-Induced ERK1/ERK2 Activation

In order to understand the mechanism by which SCH51344 inhibits RAS-transformation, we have sought to determine whether SCH51344 disrupts the signaling pathway that activates extracellular-signal regulated kinase (ERK) activity in normal and RAS transformed cells. Rat-2 fibroblast cells were deprived of serum and pretreated for 24 h in the presence of SCH51344 before stimulation with EGF for 15 min. MAP kinase activity was assessed by measurement of the ability of immunoprecipitated ERK1 and ERK2, which are members of the MAP kinase family, to phosphorylate myelin basic protein (MBP) in vitro. Pretreatment of cells grown in normal or serum-starved medium with SCH51344 for 24 h had very little effect on basal MAP kinase activity. Furthermore, EGF-stimulated MAP kinase activity was

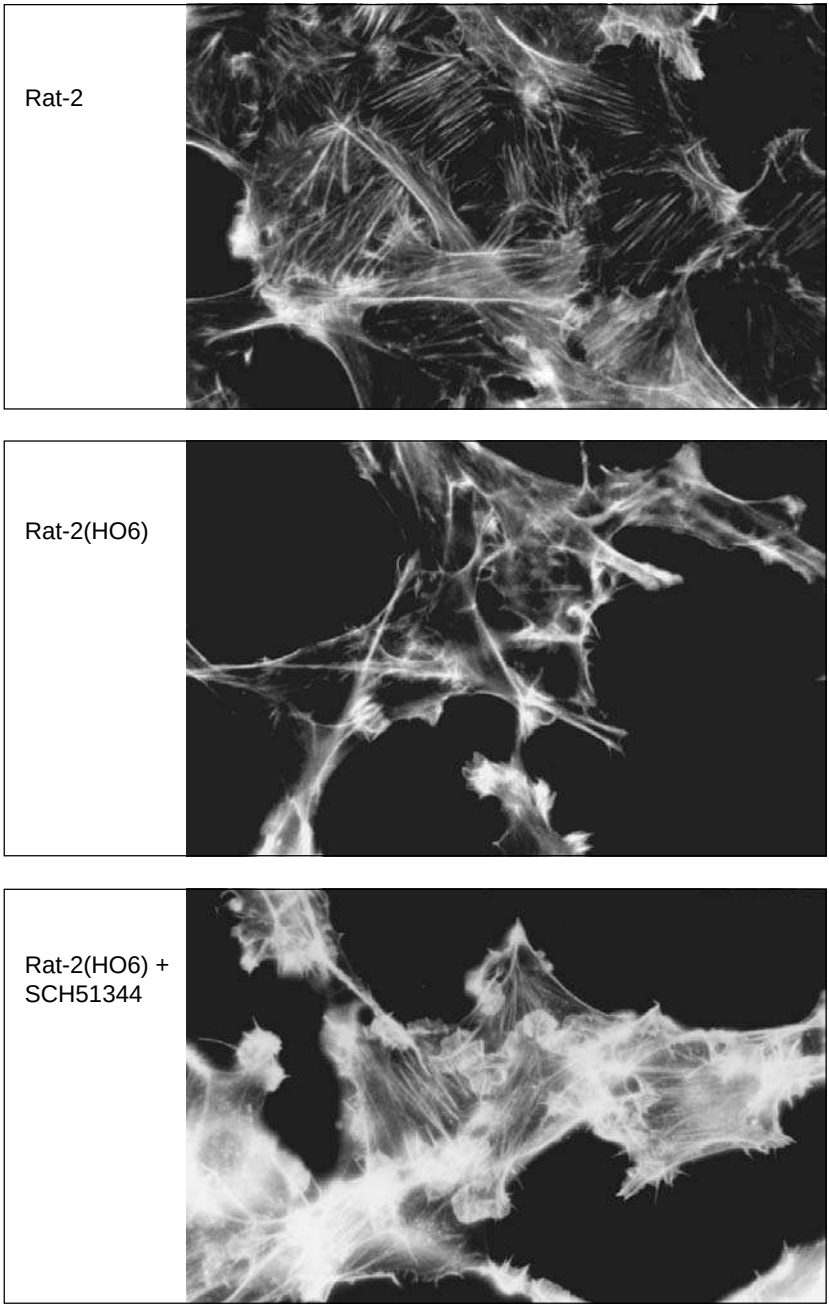


Fig. 20.3. SCH51344 treatment induces actin stress fibers in RAS-transformed cells. Rat-2 (HO6) cells were plated on coverslips one day before treatment with either DMSO (control) or SCH51344 (25 μM). Following a two day treatment, cells were fixed and stained with rhodamine-conjugated phalloidin and processed for fluorescence microscopy. Reprinted with permission from Cancer Reseaerch 1995; 55:5106-5117.

also not inhibited by pretreatment of cells with SCH51344. Next, we determined whether the drug had any effect on the activation of ERK in response to the expression of the three isoforms of oncogenic RAS proteins. Expression of the activated forms of H-, K- and N-RAS resulted in significant stimulation of ERK-2 activity in Rat-2, COS and NIH 3T3 cells, and treatment with SCH51344 did not block this stimulation. The drug treatment had very little effect on the expression levels of transiently expressed epitope-tagged ERK-2 under these conditions. These results clearly show that the drug treatment has very little effect on the activation of ERK signaling cascade in response to either growth factor stimulation or oncogenic RAS expression.[60,60a]

Treatment of normal fibroblast cells with SCH51344 did not block the EGF or serum-induced activation of MEK and p90RSK activities.[60] The drug treatment had very little effect on the activities of ERK in RAS-transformed DT and Rat-2 (HO6) cells. These results suggested that SCH51344 inhibits RAS-transformation by a novel mechanism and acts on a signaling pathway distinct from ERK-dependent signaling pathway.[60]

Inhibition of Cell Morphology Pathway by SCH51344

Recent studies have shown that RAS activates the membrane ruffling pathway and ERK pathway through distinct effectors and that input from both pathways is required for the transforming activity of RAS.[35,61] The next logical step was to analyze if SCH51344 inhibited the cell morphology pathway activated by RAS. In collaboration with Dr. Dafna Bar-Sagi's group, we have shown that this is indeed the case.[60a] In these studies, REF-52 cells were pretreated with diluent or SCH51344 for 24 h prior to microinjection with a plasmid encoding T7-tagged H-RAS V12. Induction of membrane ruffling was analyzed by phase contrast microscopy. In parallel, REF-52 cells were fixed and immunostained for the expression of H-RAS V12 protein in the cells. Microinjection of H-RAS V12 resulted in the induction of membrane ruffles. However, pretreatment of the cells with SCH51344 completely inhibited RAS-induced membrane ruffling (Fig. 20.4, top panel). Immunofluorescent staining of the injected cells confirmed that the drug treatment had no apparent effect on the expression and the subcellular distribution of the H-RAS V12 protein. Pretreatment of the cells with 25 μM SCH51344 for 5 h prior to microinjection was sufficient to inhibit membrane ruffling induced by H-RAS V12. In addition, the inhibitory effect was reversible, since membrane ruffles could be readily seen within 45 min following removal of the drug. The rapid reversibility of the inhibitory effects of the drug rules out the possibility that these effects reflect a general toxic effect of the drug. H-RAS V12 induced membrane ruffling was inhibited in a concentration dependent manner by SCH51344 and greater than 90% inhibition was seen at 6.25 μM. SCH51344 was found to be effective in inhibiting the membrane ruffling induced by activated forms of H-RAS, K-RAS and N-RAS.[60a]

SCH51344 Inhibits RAC-Induced Membrane Ruffling, but not JNK Activation

Previous studies using Swiss 3T3 fibroblasts have shown that microinjection of the RAC protein induces polymerization of actin leading to the formation of membrane ruffles and RAS-induced membrane ruffling depends on RAC activity.[34,35] As shown in Figure 20.4 (lower panel), pretreatment of REF-52 cells with SCH51344 inhibited membrane ruffling induced by RAC V12 and the drug treatment had no effect on the expression or subcellular localization of RAC V12 under these conditions. The inhibition of RAC V12-induced membrane ruffling by SCH51344 was concentration dependent, similar to H-RAS V12 and the concentration required for half-maximal inhibition was less than 6.25 μM. These results indicate that the drug interferes with the ability of RAS to induce membrane ruffling at a point downstream from RAC. These observations are consistent with the recent work of

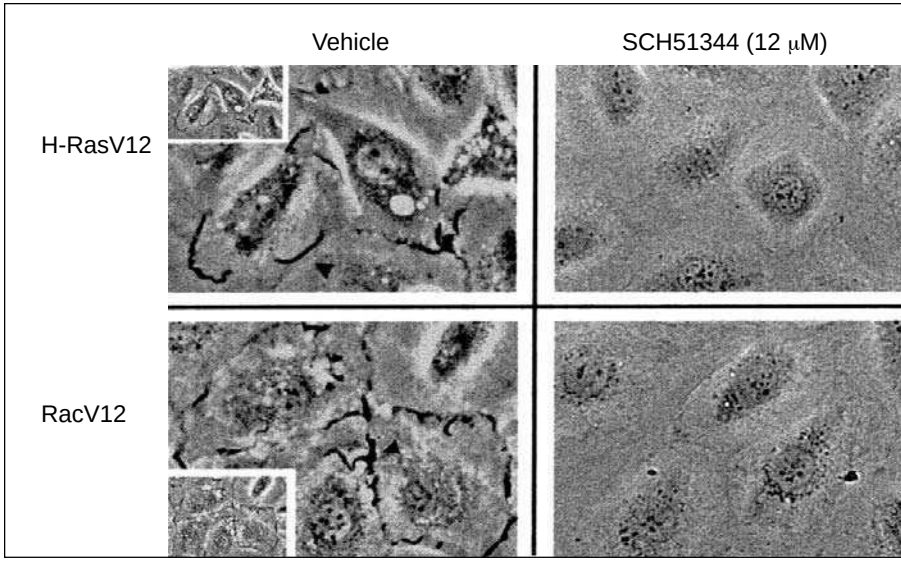


Fig. 20.4. Inhibition of H-RAS V12 and RAC V12 induced membrane ruffling by SCH51344. REF 52 cells were treated with 12.5 μM SCH51344 or DMSO (1:2000) for 24 hrs prior to microinjection with H-RAS V12, and RAC V12 (50 ng/μl)expression plasmids. Ruffling activity was scored by the appearance of transient phase bands actively traversing across the upper surface of the cells. The ruffling images were captured at 32x magnification 4 hrs after microinjection. Ruffles were enhanced by the auto-trace function of Corell Draw computer software. The insets show the unprocessed images. The cells were also fixed and immunostained with anti-T7 antibody (1:300) to monitor the expression of RAS and RAC V12 proteins. Reprinted with permission from Walsh AB et al. Oncogene 1997; 15:2553-2560.

White et al,[61] demonstrating that an effector domain mutant of H-RAS V12S35 was found to be severely impaired in its ability to induce foci in fibroblast cells. However, this mutant retained the ability to activate ERK and induce SRE-dependent transcription, but was defective for stimulation of membrane ruffling.[61,35] These results clearly show that stimulation of membrane ruffling pathway is critical for the transforming activity of RAS and SCH51344 represents a pharmacological agent that targets this pathway specifically at a point downstream from RAC.

RAS activation triggers two divergent signaling cascades that activate distinct MAPKs, ERK and Jun Kinase (JNK), with different substrate specificities and transcriptional functions.[62,63] Recent studies have demonstrated that the effects of RAS and RAC on JNK cascade and membrane ruffling formation are mediated by distinct effector pathways that diverge at the level of RAC (Fig. 20.5). While the role of ERK activation in RAS-mediated transformation is clearly established, the contribution of JNK pathway towards RAS transformation is not established. Our studies indicate that treatment of fibroblast cells with SCH51344 has very little effect on RAS-mediated activation of JNK (Fig. 20.5).[60a] These results suggest that inhibition of RAS-mediated activation of ERK and JNK pathways is not obligatory for blocking the transforming function of RAS. In addition, we have observed that SCH51344 is effective in inhibiting the anchorage-independent growth of Rat-2 fibroblast cells transformed by three forms of oncogenic RAS and RAC V12.[60a] These results indicate that SCH51344 inhibits a critical component of the membrane ruffling pathway

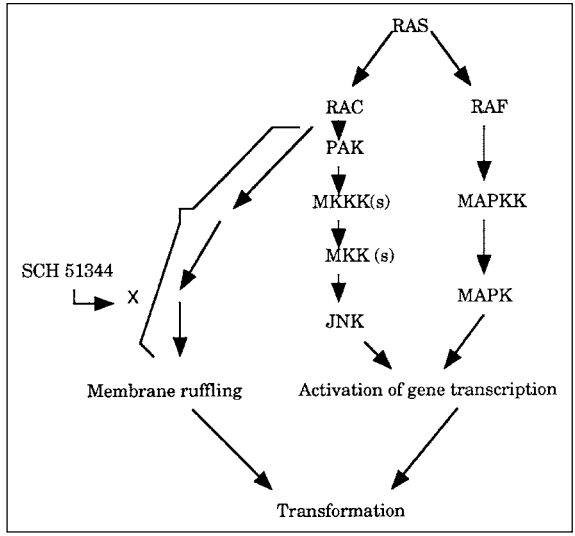


Fig. 20.5. Proposed signaling pathways mediated by RAS and inhibition of RAS and RAC mediated activation of membrane ruffling formation by SCH51344. See the text for details. Reprinted with permission from Walsh AB et al. Oncogene 1997; 15:2553-2560.

downstream from RAC and suggest that targeting the membrane ruffling pathway may be an effective approach to inhibit transformation by RAS.

The pathway by which RAS and RAC stimulate membrane ruffling pathway is not clearly established. Recently, a number of RAC1-interacting proteins, have been isolated using the yeast two-hybrid system.[66-68] POR1 binds directly to RAC1 in a GTP-dependent fashion and truncated versions of POR1 inhibit the induction of membrane ruffling by an activated mutant of RAC1, suggesting a potential role for POR1 in RAC-mediated cell morphology pathway.[66] The ser/thr kinase, $p160^{ROCK}$ which was originally identified as a putative RHO target has been recently shown to interact also with activated RAC L61.[64] Studies with mutants of RAC defective for membrane ruffling, suggest that this kinase might play a role in RAC-induced cytoskeletal changes.[64] However, recent studies using kinase defective, RHO-binding negative mutants clearly showed that $p160^{ROCK}$ selectively works downstream of RHO to induce focal adhesions and stress fibers in the cell.[69] The best characterized target protein for RAC is the CDC42/RAC-activated kinase (PAK) family of serine/threonine kinases.[70] PAK is involved in mediating the RAS and RAC-induced activation of JNK pathway (Fig. 20.5). So far there has been no definitive evidence to support or eliminate a role for PAK family kinases in the RAC-induced membrane ruffling, although PAK is involved in the RAC-induced disruption of actin stress fibers.[70a]

A potential link between RAC and increased actin polymerization involves phosphatidylinositol 4,5-biphosphate (PIP_2) generation. RAC affinity columns can bind PIP 5-kinase and RAC can stimulate PIP_2 production in permeabilized platelets by activating PI-4/PI-5 kinases.[71,72] In this system, RAC induces uncapping of actin filaments at the plus ends leading to actin polymerization through PIP_2 that inactivates F-actin cappers. However, in experiments using fibroblast cell extracts, RHO but not RAC was reported to stimulate PIP 5-kinase activity.[73] It is possible that PI-4 kinases or distinct isoforms of PIP 5-kinase may be responsive to RAC or RHO. It is clear that the pathway linking RAC to membrane ruffles needs to be worked out. The fact that SCH51344 seems to inhibit membrane ruffling pathway downstream of RAC suggests that this pathway is amenable to inhibition by small molecular weight compounds. Future studies aimed at identifying the target

protein that is inhibited by SCH51344 may provide a novel, therapeutically useful target for RAS-mediated malignancies.

Acknowledgments

I would like to thank Dr. Dafna Bar-Sagi and Amy Walsh for their contributions to the studies on membrane ruffling, ERK and JNK pathways and to Dr. Adrian Afonso and Joe Kelley for synthesizing SCH51344. I would also like to thank my colleagues and collaborators for their contributions to this work. A special thanks to Dr. Earl Ruley for his encouragement, guidance and many valuable suggestions during the course of this work.

References

1. Weber K, Lazarides E, Goldman RD et al. Localization and distribution of actin fibers in normal and transformed and revertant cells. Cold Spring Harbor Symp Quant Biol 1974; 39:363-369.
2. Pollack R, Osborn M, Weber K. Patterns of organization of actin and myosin in normal and transformed cultured cells. Proc Natl Acad Sci USA 1975; 72:994-998.
3. Pollard TD, Cooper JA. Actin and actin binding proteins: A critical evaluation of mechanisms and functions. Annu Rev Biochem 1986; 55:987-1035.
4. Pollack R, Chen S, Powers S et al. Transformation mechanisms at the cellular level. Adv Viral Oncol 1987; 4:3-27.
5. Zigmond SH. Signal transduction and actin filament organization. Curr Opin Cell Biol 1996; 8:66-73.
6. Chandra Kumar C, Chang C. Human smooth muscle myosin light chain-2 gene expression is repressed in Ras transformed fibroblast cells. Cell Growth & Diff 1992; 3:1-10.
7. Leavitt J, Gunning P, Kedes L et al. Smooth muscle α-actin is a sensitive marker for the transformed phenotype. Nature 1985; 316:840-842.
8. Vaderkerckhove J, Bauw G, Vancompernolle A et al. Comparative two-dimensional gel analysis and microsequencing identifies gelsolin as one of the most prominent down-regulated markers of transformed human fibroblast and epithelial cells. J Cell Biol 1990; 111; 95-102.
9. Raz A, Zoller M, Ben-Ze'ev. Cell configuration and adhesive properties of metastasizing and nonmetastasizing BSp73 rat adenocarcinoma cells. Exp Cell Res 1986; 162:127-141.
10. Bravo R, Celis JE. Human proteins sensitive to neoplastic transformation in cultured epithelial and fibroblast cells. Clin Chem 1982; 28:949-954.
11. Matsumura F, Lin JJC, Yamishiro-Matsumura S et al. Differential expression of tropomyosin forms in the microfilaments isolated from normal and transformed rat cultured cells. J Biol Chem 1983; 264:5299-5312.
12. Rodriguez-Fernandez JL, Geiger B et al. Suppression of tumorigenicity in transformed cells after transfection with vinculin cDNA. J Cell Biol 1992; 119:427-438.
13. Glueck U, Kwiatkowski DJ, Ben-Ze'ev A. Suppression of tumorigenicity in simian virus 40-transformed 3T3 cells transfected with α-actinin cDNA. Proc Natl Acad Sci USA 1993; 90:383-387.
14. Prasad, GL, Fuldner RA, Cooper HL. Expression of transduced tropomyosin I cDNA suppresses neoplastic growth of cells transformed by the Ras oncogene. Proc Natl Acad Sci USA 1993; 90:383-387.
15. Muellauer L, Fujita H, Shizaki A et al. Tumor-suppressive function of mutated gelsolin in Ras-transformed cells. Oncogene 1993; 8:2531-2536.
16. Hall A. Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu Rev Cell Biol 1994; 10:31-54.
17. Ridley AJ. Rho-related proteins: Actin cytoskeleton and cell cycle. Curr Opin Genet Dev 1995; 5:24-30.
18. Symons M. Rho family of GTPases: The cytoskeleton and beyond. Trends Biochem Sci 1996; 21:178-181.

19. Machesky LM, Hall A. Rho: A connection between membrane receptor signaling and the cytoskeleton. Trends Cell Biol 1996; 6:304-310.
20. Ridley AJ. Rho: Theme and variations. Curr Biol 1996; 6:1256-1264.
21. Ridley AJ. Membrane ruffling and signal transduction. BioEssays 1994; 16:321-327.
22. Ridley AJ, Hall A. The Rho GTPase regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992; 70:389-399.
23. Ridley AJ, Paterson HF, Johnston CL et al. Rac regulates growth factor-induced membrane ruffling. Cell 1992; 70:401-410.
24. Nobes CD, Hall A. Rho, Rac and Cdc 42GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia and filopodia. Cell 1995; 81:53-62.
25. Hart MJ, Eva A, Evans T et al. Catalysis of Guanine nucleotide exchange on the CDC42 by the Dbl oncogene product. Nature 1991; 354:311-314.
26. Adams JM, Houston H, Allen J et al. The hematopoietically expressed Vav proto-oncogene shares homology with Dbl GDP-GTP exchange factor, the Bcr gene and a yeast gene (CDC24) involved in cytoskeletal organization. Oncogene 1992; 7:611-618.
27. Symons M. The Rac and Rho pathways as a source of drug targets for Ras-mediated malignancies. Curr Opin Biotechnology 1995; 6:668-674.
28. Maruta H. Downstream of RAS and actin cytoskeleton. In: Maruta H, Burgess AW eds. Regulation of the RAS Signaling Network. Austin/Heidelberg: RG Landes/ Springer-Verlag, 1996:139-180.
29. Bos JL. Ras oncogenes in human cancer: A review. Cancer Res 1989; 49:4682-4689.
30. Barbacid M. Ras genes and their role in neoplacia. Eur J Clin Invest 1990; 20:225-235.
31. McCormick F. Activators and effectors of Ras. Curr Opin Genet Dev 1994; 4:71-76,.
32. Egan SE, Weinberg RA. The pathway to signal achievement. Nature 1993; 365:781-783.
33. Bar-Sagi D, Feramisco JR. Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibraoblast cells by ras proteins. Science 1986; 233:1061-1065.
34. Qiu RG, Chen J, Kirn D et al. An essential role for Rac in Ras transformation. Nature 1995; 374:457-459.
35. Joneson T, White M, Wigler M, Bar-Sagi D. Stimulation of membrane ruffling and MAP kinase activation by distinct effectors of RAS. Science 1996; 271:810-812.
36. Spaargaren M, Bischoff JR, McCormick F. Siganl transduction by Ras-like GTPases: A potential target for anticancer drugs. Gene Expression 1995; 4:345-356.
37. Khosravi-Far R, Solski PA, Clark GJ, Kinch MS, Der CJ. Activation of Rac and Rho and mitogen activated protein kinases are required for Ras transformation. Mol Cell Biol 1995; 15:6443-6453.
38. Gibbs, J. B. Ras C-terminal processing enzymes—new drug targets? Cell 1991:65:1-4.
39. Kohl NE, Wilson FR, Mosser SD et al. Protein farnesyl transferase inhibitors block the growth of Ras-dependent tumors in nude mice. Proc Natl Acad Sci USA 1994; 91:9141-9145.
40. Bishop WR, Petrin J, Bond R et al. Novel tricyclic inhibitors of farnesyl protein transferase: Biochemical characterization and inhibition of Ras modification in transfected Cos cells. J Biol Chem 1995; 270:30611-30618.
41. James GL, Goldstein JL, Brown MS et al. Benzodiezepine peptidominetics: potent inhibitors of Ras farnesylation in animal cells. Science 1993; 260:1937-1942.
42. Kohl NE, Wilson FR, Mosser SD et al. Protein farnesyl transferase inhibitors block the growth of *Ras*-dependent tumors in nude mice. Proc Natl Acad Sci USA 1994; 91:9141-9145.
42a. Zhao JF, Nakano H, Sharma S. Suppresion of RAS and MOS transformation by radicol. Oncogene 1995; 11:161-173.
42b. Futamura M, Monden Y, Okabe T et al. Trichostatin A inhibits both RAS-induced neurite outgrowth of PC 12 cells and morphological transformation of NIH 3T3 cells. Oncogene 1995; 11:1119-1123.
43. Cox LY, Moltz J, Troll W, Garte SJ. Antipain-induced suppression of oncogene expression in H-Ras transformed NIH 3T3 cells. Cancer Res 1991; 51:4810-4814.
44. Itoh O, Kuroiwa S, Atsumi S et al. Induction of guanosine analog oxanosine of reversion toward the normal phenotype of K-ras-transformed rat kidney cells. Cancer Res 1989; 49:996-1000.

45. Shindo-Okada N, Makabe O, Nagahara H et al. Permanent conversion of mouse and human cells transformed by activated ras and raf genes to apparently normal cells by treatment with antibiotic azatyrosine. Mol Carcinog 1989; 2:159-167.
46. Okamoto-Inoue M, Taniguchi S, Sadano H et al. Alteration in expression of smooth muscle α-actin associated with transformation of rat 3Y1 cells. J Cell Sci 1990; 96:631-637.
47. Garrels J, Gibson W. Identification and characterization of multiple forms of actin. Cell 1976; 9:793-805.
48. Vanderckhove J, Weber K. At least six different actins are expressed in higher mammals: An analysis based on amino acid sequence of the amino terminal tryptic peptide. J Mol Biol 1978; 126:783-802.
49. Owens GK. Regulation of diffrentiation of vascular smooth muscle cells. Physiol Rev 1995; 75:487-517.
50. Owens GK, Thompson MM. Developmental changes in isoactin expression in rat aortic smooth muscle cells in vivo. J Biol Chem 1986; 261:13373-13380.
51. Skalli OJ, Vandekerckhove J, Gabbiani G. Actin-isoform pattern as a marker of normal or pathological smooth muscle and fibroblast tissues. Diffrentiation 1987; 33:232-238.
52. DeNofrio D, Hoock TC, Herman IM. Functional sorting of actin isoforms in microvascular pericytes. J Cell Biol 1989; 109:191-202.
53. Herman IM. Actin isoforms. Curr Opin Cell Biol 1993; 5:48-55.
54. Ronnov-Jessen L, Peterson OW. A function for filamentous α-smooth muscle actin: Retardation of motility in fibroblasts. J Cell Biol 1996; 134:67-80.
55. Chandra Kumar C, Bushel P, Mohan SM et al. Regulation of smooth muscle α-actin promoter in Ras-transformed cells: Usefulness for setting up a reporter-gene based assay system for drug screening. Cancer Res 1992; 52:6877-6884.
56. Chandra Kumar C, Chang C. Human smooth muscle myosin light chain-2 gene expression is repressed in Ras transformed fibroblast cells. Cell Growth & Diff 1992; 3:1-10.
57. Bushel P, Kim JH, Chang W et al. Two serum response elements mediate transcriptional respression of human smooth muscle α-actin promoter in ras-transformed cells. Oncogene 1995; 10:1361-1370.
58. Kim JH, Johansen FE, Robertson N et al. Suppression of Ras transformation by serum response factor. J Biol Chem 1994; 269:13740-13743.
59. Lloyd A, Yancheva N, Wasylyk B. Transformation suppressor activity of a jun transcription factor lacking its activation domain. Nature 1991; 352:635-638.
60. Chandra Kumar C, Prorock-Rogers C, Kelley J et al. SCH51344 inhibits Ras transformation by a novel mechanism. Cancer Res 1995; 55:5106-5117.
60a. Walsh AB, Dhanasekaran M, Bar-Sagi D et al. SCH51344-induced reversal of RAS-transformation is accompanied by the specific inhibition of the RAS and RAC-dependent cell morphology pathway. Oncogene 1997; 15:2553-2560.
61. White MA, Nicolette C, Minden A et al. Multiple Ras functions can contribute to mammalian cell transformation. Cell 1995; 80:533-541.
62. Minden A, Lin A, McMahon M et al. Differential activation of ERK and JNK MAP kinases by Raf-1 and MEKK. Science 1994; 266:1719-1723.
63. Minden A, Lin A, Claret FX et al. Selective activation of the JNK signaling pathway and c-jun transcriptional activity by Rac and Cdc42 GTPases. Cell 1995; 81:1147-1157.
64. Lamarche N, Tapon N, Stowers L et al. Rac and cdc42 induce actin polymerization and G1 cell cycle progression independently of p65pak and the JNK/SAPK MAP kinase cascade. Cell 1996; 87:519-529.
65. Joneson T, McDonough M, Bar-Sagi D et al. RAC regulation of actin polymerization and proliferation by a pathway distinct from Jun kinase. Science 1996; 274:1374-1376.
66. Van Aelst L, Joneson T, Bar-Sagi D. Identification of a novel Rac1-interacting protein involved in membrane ruffling. EMBO J 1996; 15:3778-3786.
67. Ishizaki T, Maekawa M, Fujisawa K et al. The Rho GTPase binds and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J 1996; 15:1885-1893.

68. Burbelo PD, Drechsel D, Hall A. A conserved binding motif defines numerous candiadate target proteins for both Cdc42 and Rac GTPases. J Biol Chem 1995; 270:29071-29074.
69. Ishizaki T, Naito M, Fujisawa K et al. p160^{ROCK}, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. FEBS Lett 1997; 404:118-124.
70. Brown JL, Stowers L, Baer M et al. Human Ste 20 homologue hPAK1 links GTPases to the JNK MAP kinase pathway. Curr Biol 1996; 6:598-605.
70a. Manser E, Huang HY, Loo TH et al. Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. Mol Cell Biol 1997; 17:1129-1143.
71. Tolias KF, Cantley LC, Carpenter CL. Rho family GTPases bind to phosphoinositide kinases. J Biol Chem 1995; 270:17656-17659.
72. Hartwig JH, Bokoch GM, Carpenter CL et al. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell 1995; 82:643-653.
73. Chong LD, Traynor-kaplan A, Bokoch G et al. Rho GTPase regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. Cell 1994; 79:507-513.

CHAPTER 21

Azatyrosine: Mechanism of Action

Yoshiaki Monden, Nobuko Shindo-Okada and Susumu Nishimura

Introduction

Most of current anticancer drugs used clinically are based on the faster proliferation of cancer cells as compared with normal cells. However, normal cells such as myeloid cells and epithelial cells in the gastrointestinal tract also actively proliferate. Therefore, these drugs also affect such normal cells. It is difficult for these drugs to kill only cancer cells. A variety of new screening procedures targeting oncogenes specifically expressed in cancer cells were recently adopted for isolation of more specific and effective anticancer drugs. Our procedure for screening agents that specifically inhibit the growth of particular cancer cells was quite straightforward: Normal NIH3T3 cells and NIH3T3 cells transformed by the activated human c-Ha-*ras* gene (having a mutation at codon 61, resulting in replacement of glutamine by leucine) were used in parallel as test cell lines. Using this system to select agents that specifically inhibit growth of tumor cells with an activated c-Ha-*ras* gene, we have screened a variety of antibiotics and partially purified fractions of culture products of *Streptomyces* and thus isolated an antibiotic azatyrosine (L-β-(5-hydroxy-2-pyridyl)-alanine, Fig. 21.1), which specifically inhibited the growth of the transformed cells.

It was found that azatyrosine had an unique property to convert the transformed phenotype of the transformed cells to normal. However how azatyrosine functions and its major targets are largely unknown. If the mechanism of action of azatyrosine could be elucidated, it would contribute to the elucidation of novel mechanisms of cancer suppression. In this chapter we summarize the biological properties of azatyrosine reported earlier and our recent studies using c-*erb*B-2-transformed cells.

Inhibitory Effect of Azatyrosine on the *ras*-Mediated Signal Transduction

Azatyrosine inhibits the growth of c-Ha-*ras*-, c-*raf*-, or c-*erbB*-2-transformed NIH3T3 cells and induces the reversion of the transformed morphology to a normal phenotype.[1,2] It should be noted that NIH3T3 cells transformed by *hst*, *ret* or *src* are not reverted by azatyrosine.[1] It also induces the conversion of human pancreatic adenocarcinoma PSN-1 cells and human colon cancer HCT116 cells with a point mutation in c-K-*ras* and human prostate cancer TSU-Prl, DU-145 and PC-3 cells to revertant cells.[1,3] Moreover, azatyrosine inhibits not only transformed phenotypes but also inhibits Ras function in other aspects. Rat pheochromocytoma PC12 cells are chromaffin-like cells, but when these cells are treated with NGF (nerve growth factor), they differentiate into sympathetic neuron-like cells including neurite outgrowth and cessation of cell growth. In PC12 cells, Ras is considered to be a downstream factor of NGF. Microinjection of oncogenic Ras induces the

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

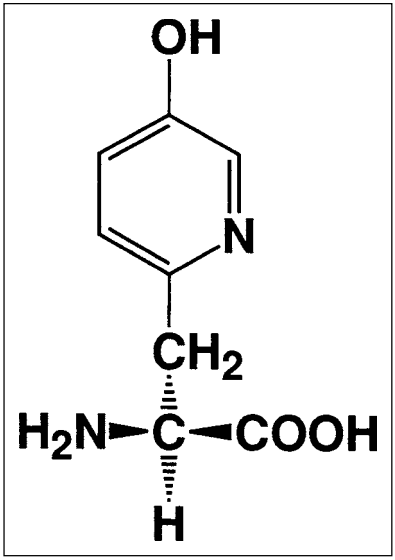


Fig. 21.1. Structure of azatyrosine.

differentiation of PC12 cells into the neuron-like cells in the absence of NGF. Azatyrosine also inhibits the oncogenic Ras-induced outgrowth of neurites from PC12 cells.[4] The oncogenic Ras is also known to induce cell maturation of *Xenopus laevis* oocytes when microinjected into the cells. Azatyrosine inhibits the maturation of oocytes induced by the oncogenic Ras.[5,6] Since azatyrosine inhibits signal transduction mediated by Ras protein in such cells, azatyrosine could inhibit the main pathway of *ras*-mediated signal transduction.

Another important feature of azatyrosine is that it inhibits dimethyl benzanthracene (DMBA)-induced formation of papillomas in skin and methylnitrosourea (MNU)-induced papillomas in the forestomach of transgenic mice that harbor the human protooncogene, c-Ha-*ras*, when azatyrosine was administered in mice by i.p. injection.[7] Many types of agents are found to exhibit an anti-cancer effect on cultured cancer cells, but few agents have efficacy on the animal models of cancer.

Effect of Azatyrosine on the Transformed Phenotypes of c-*erbB*-2-Transformed Cells

The c-*erbB*-2 is an oncogene of which abnormalities were found in many types of human cancer. Especially in breast and stomach cancers, gene amplification or overexpression of c-*erbB*-2 were found with high frequency.[8,9] These abnormalities of c-*erbB*-2 are considered to contribute to malignancy of clinical cancers.

A4 cells are a variant of NIH3T3 cells, transformed with oncogenic c-*erbB*-2.[10] Treatment of A4 cells with azatyrosine for 1 day did not affect cell growth. On day 2, however, the growth of A4 cells was inhibited in a dose-dependent manner. Treatment with azatyrosine at 1 mg/ml completely inhibited cell growth, but dead cells were not observed even 8 days after the addition of azatyrosine, suggesting that azatyrosine reduced the growth rate of cells without any effect on viability. Morphological analysis showed that azatyrosine at more than 500 μg/ml induced all of the cells to convert to normal morphology while azatyrosine at 250 μg/ml was partially effective.[2] Resembling the effect on cell growth, the morphological changes were also not observed on day 1. Thus, azatyrosine has the ability to reverse the transformed phenotype of c-*erbB*-2-transformed cells, as well as that of *ras*-transformed cells. However, it should be noted that the morphological change of c-*erbB*-2-transformed cells is not permanent since the reverted cells returned to the transformed phenotype when the cells were cultured in the absence of azatyrosine, unlike the case of activated c-Ha-*ras*-transformed NIH3T3 cells as previously reported.[1]

Anticancer drugs used in cancer chemotherapy can be divided into various classes on the basis of their mode of action. Many cytotoxic agents inhibit cell growth by interfering with the synthesis of DNA, RNA and/or protein and/or by inhibiting cell growth at a specific stage of the cell cycle. Many inducers of differentiation also induce the accumulation of cells at a specific stage of the cell cycle. We investigated the effects of azatyrosine on the

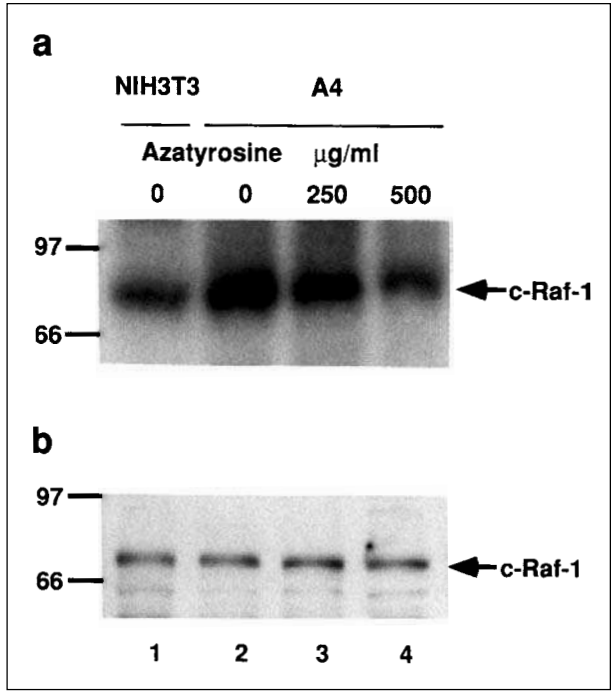


Fig. 21.2. Effects of azatyrosine on the phosphorylation and expression of c-Raf-1. NIH3T3 cells (lane 1) and A4 cells (lanes 2-4) were cultured with azatyrosine at the specified concentrations for 3 days. (a) After treatment with azatyrosine, the cells were labeled with [^{32}P]-phosphate and lysed. c-Raf-1 was immunoprecipitated with c-Raf-1-specific antibodies and analyzed by PAGE on an 8% polyacrylamide gel with subsequent autoradiography. (b) After treatment with azatyrosine, cells were lysed and c-Raf-1 was detected by Western blotting with the same antibodies. Positions of marker proteins with molecular masses in kDa are indicated on the left.

synthesis of DNA, RNA and protein in A4 cells. However, azatyrosine did not specifically interfere with DNA, RNA or protein synthesis in A4 cells.[11] To investigate the effect of azatyrosine on progression of the cell cycle in A4 cells, we measured the DNA content of A4 cells by flow-cytometric analysis after treatment with azatyrosine. However, treatment of A4 cells with azatyrosine did not affect the DNA histogram, suggesting that it does not interrupt the cell cycle at a specific stage.[11] These results indicated that the mechanism of action of azatyrosine is different from that of typical anticancer drugs.

Effects of Azatyrosine on Signal Transduction Triggered by Activated c-*erbB*-2

The c-*erbB*-2 oncogene encodes a receptor tyrosine kinase.[12,13] Activation of c-ErbB-2 kinase transmits cellular signaling from the plasma membrane to the nucleus via several factors. The resultant activation of transcriptional factors stimulate expression of particular genes which are implicated in proliferation of cells.[14,15]

In order to elucidate the mechanism of action of azatyrosine on cellular transformation, we attempted to identify the signal-transduction processes triggered by activated c-ErbB-2 that were inhibited by azatyrosine.

Oncogenic c-ErbB-2 has been shown to increase the relative level of the GTP-bound form of Ras.[16] Therefore, we investigated the effect of azatyrosine on activation of Ras induced by oncogenic c-ErbB-2.[16a] Ras protein was immunoprecipitated with Ras-specific antibodies from the cells labeled with [^{32}P]-phosphate. Ras-bound guanosine nucleotides were then separated by thin-layer chromatography. In A4 cells, the amount of Ras-bound GTP was approximately twice as that of normal NIH3T3 cells. Azatyrosine did not alter the level of GTP-bound Ras as well as the total amount of Ras, indicating that the activation of Ras (formation of the GTP bound-form of Ras) still occurred to the same extent in

azatyrosine-treated A4 cells. The results suggest that azatyrosine did not inhibit the Ras-activation pathway induced by oncogenic c-ErbB-2.

The c-*raf*-1 protooncogene encodes a cytoplasmic serine/threonine protein kinase, a downstream effector of Ras.[17-21] The activation of c-Raf-1 is induced by growth factors and oncogenes encoding Tyr kinases and is accompanied by its hyperphosphorylation.[22,23] Therefore, we next examined the effect of azatyrosine on the phosphorylation of c-Raf-1 in order to determine whether or not azatyrosine could inhibit this kinase cascade.[16a] As shown in Figure 21.2, c-Raf-1 was more heavily phosphorylated in A4 cells than in normal NIH3T3 cells, indicating the activation of c-Raf-1 protein by oncogenic c-ErbB-2. When A4 cells were treated with azatyrosine at 500 μg/ml, reduction of the extent of phosphorylation of c-Raf-1 (Fig. 21.2a, lane 4) without any effect on the amount of c-Raf-1 (Fig. 21.2b) was observed. Densitometric analysis of the autoradiogram in Figure 21.2a showed that the amount of phosphorylated c-Raf-1 in azatyrosine-treated cells (500 μg/ml) was reduced to approximately half of that of untreated cells. These results indicated that azatyrosine inhibited the activation of c-Raf-1 kinase by oncogenic c-ErbB-2.

Activated c-ErbB-2/Neu protein and activated c-Raf-1 protein have been reported to activate transcription factor AP1.[14,15] Activation of AP1, whereby transcription of genes that contain a TRE (12-*O*-tetradecanoylphorbol-13-acetate response element) is stimulated, plays a crucial role in cell transformation.[24,25] To investigate the effect of azatyrosine on stimulation of the AP1 of activity, NIH3T3 cells were cotransfected with the reporter plasmid pTREtkCAT and the oncogenic c-ErbB-2 expression plasmid pCOB2A7 in the presence or in the absence of azatyrosine.[16a] The pTREtkCAT plasmid contains TRE in the region upstream of the promoter of the gene for thymidine kinase (tk) and the gene for chloramphenicol acetyltransferase (CAT). The activation of AP1 was clearly detected; the CAT activity in the lysate from cells that had been cotransfected with pTREtkCAT and pCOB2A7 was higher than that of lysates prepared from cells that had been cotransfected with the enhancerless plasmid ptkCAT and pCOB2A7 (Fig. 21.3). When the cells were treated with azatyrosine at 800 μg/ml, stimulation of the AP1 activity was inhibited (approximately 40% of the control value; Fig. 21.3, lanes 3 and 5), but the background level of CAT activity from pTREtkCAT was barely affected (Fig. 21.3, lanes 2, 9 and 10). Therefore, it was concluded that azatyrosine inhibits the activation of AP1 by oncogenic c-ErbB-2. These results suggested that azatyrosine acts downstream of Ras in signal transduction from oncogenic c-ErbB-2 to nuclear factors.

Induction of Expression of Particular Genes by the Treatment with Azatyrosine

We showed previously that azatyrosine activates the transcription of several distinct genes such as *rrg* (*ras* recision gene), collagen type III, *rhoB,* fibronectin and Ca-binding protein Ca-31 genes.[27,28] The *rrg* was isolated as a gene which is associated with reversion of *ras*-transformed cells.[29] Recently it was reported to be a target of the antioncogenic transcription factor, IFN regulatory factor 1 (IRF-1).[30] Collagen type III and fibronectin are members of the extracellular matrix which are basement components of cell-adhesion. Rho proteins, members of small GTP-binding proteins, regulate the actin-cytoskeleton by assembling actin stress fibers.[31,32] We found that overexpression of RhoB in A4 cells could partially convert the transformed cells to normal morphology.[28] Lysyl oxidase encoded by *rrg* is considered to be responsible for the integrity of extracellular matrix such as collagen and elastin.[33] It was previously known that anchorage-dependent growth of normal cells requires the normal adhesive function of cells to extracellular matrix, but transformation of cells by activated oncogenes reduces the adhesive function of cells.[34,35] It is likely to be caused by alteration of actin-cytoskeleton or adhesion molecules such as integrin. Cytoskeleton

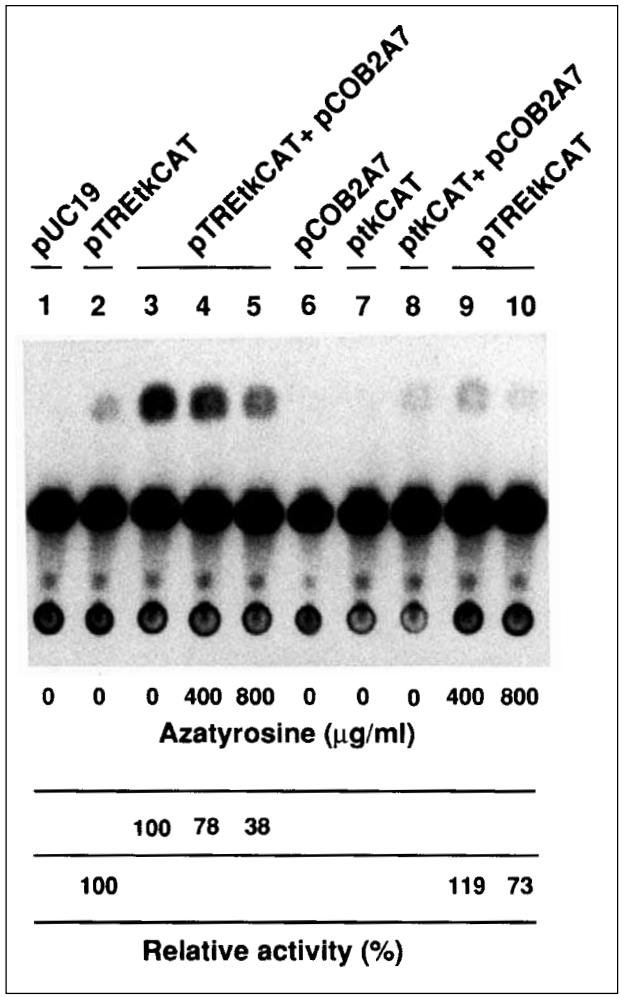


Fig. 21.3. Effects of azatyrosine on the activation of TRE. NIH3T3 cells were transfected with 6 μg of pUC19 (lane 1), 1 μg of pTREtkCAT and 5 μg of pUC19 (lane 2), 1 μg of pTREtkCAT and 5 μg of pCOB2A7 (lanes 3-5), 1 μg of pUC19 and 5 μg of pCOB2A7 (lane 6), 1 μg of ptkCAT and 5 μg of pUC19 (lane 7), 1 μg of ptkCAT and 5 μg of pUC19 (lane 8) or 1 μg of pTREtkCAT and 5 μg of pUC19 (lanes 9 and 10) in the presence and in the absence of azatyrosine at the specified concentrations. Cell lysates were prepared and analyzed for CAT activity. The activities, as percentages relative to the control (without azatyrosine), are indicated at the bottom.

and/or adhesion molecules induced by the treatment with azatyrosine might lead to the reversion of the transformed phenotype to the normal.

Incorporation of Azatyrosine into Proteins

Because of its structural similarity with tyrosine, it seemed possible that azatyrosine is incorporated into protein instead of tyrosine. To examine this possibility, we cultured A4 cells with [^{3}H]-azatyrosine and determined its incorporation into cellular proteins (Fig. 21.4).[11] [^{3}H]-Azatyrosine was in fact incorporated into the protein fraction (Fig. 21.4B). The amount of azatyrosine incorporated was approximately 8-fold lower than that of tyrosine, when the same concentrations of azatyrosine and tyrosine were added in the culture medium. To confirm the incorporation of azatyrosine into proteins, a lysate of [^{3}H]-azatyrosine-treated cells was fractionated by SDS-PAGE. Many radiolabeled proteins were detected in the lysate of [^{3}H]-azatyrosine-treated cells. To investigate whether azatyrosine is incorporated into proteins instead of tyrosine, we cultured A4 cells with [^{3}H]-tyrosine or [^{3}H]-azatyrosine in the presence of unlabeled tyrosine or unlabeled azatyrosine. Unlabeled

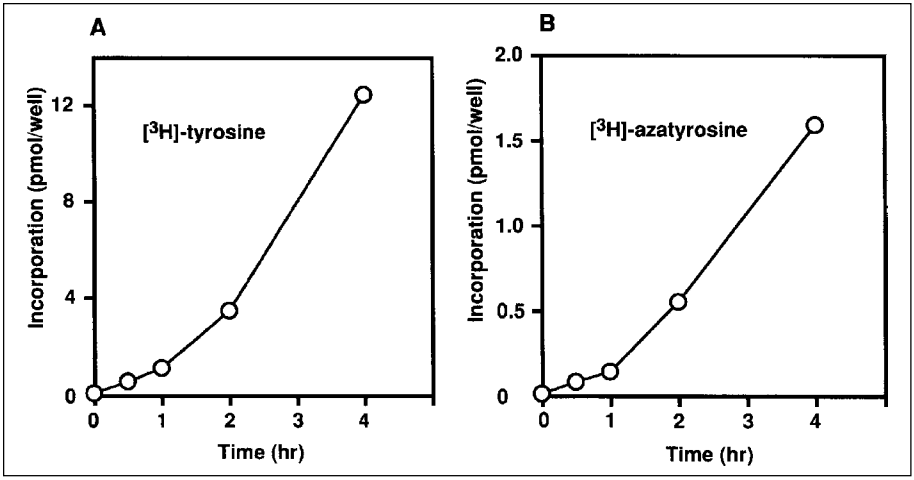


Fig. 21.4. Incorporation of [^{3}H]-azatyrosine into A4 cells. A4 cells were cultured with [^{3}H]-tyrosine (A) or [^{3}H]-azatyrosine (B) for the times indicated. Radioactivity of the TCA-insoluble fractions of cell lysates was measured.

tyrosine inhibited the incorporation of [^{3}H]-azatyrosine into proteins in a dose-dependent manner. Moreover, unlabeled azatyrosine similarly inhibited the incorporation of [^{3}H]-tyrosine. These results indicated that azatyrosine was incorporated into proteins instead of tyrosine.

To investigate whether the incorporation of azatyrosine instead of tyrosine is implicated in the ability of azatyrosine to convert the transformed to a normal phenotype, we examined the influence of a high concentration of tyrosine in the cultured medium on the effects of azatyrosine.[11] When A4 cells were cultured in the medium that originally contained 72 μg/ml tyrosine, azatyrosine converted the cells to a normal phenotype. By contrast, when A4 cells were cultured with azatyrosine together with 1 mg/ml of tyrosine, A4 cells still retained their transformed morphology. Thus, a high concentration of tyrosine interfered with the ability of azatyrosine to convert the transformed to a normal phenotype.

However, it should be noted that we do not understand as yet the mechanisms by which the incorporation of azatyrosine into proteins inhibits the signal transduction for proliferation downstream of Ras and induces expression of the genes.

Conclusion and Future View

We have shown that azatyrosine counteracts the transforming activity of activated Ras by acting downstream of Ras in signal transduction. Azatyrosine neither affected the phosphorylation of the introduced oncogenic c-ErbB-2 nor suppressed activation of Ras (amount of GTP-bound form of Ras) induced by oncogenic c-ErbB-2 in A4 cells. On the other hand, azatyrosine inhibited increases in phosphorylation of c-Raf-1 and c-Jun induced by oncogenic c-ErbB-2. In addition, azatyrosine inhibited activation of TPA response element in stimulation by oncogenic c-ErbB-2.

Azatyrosine was incorporated into proteins to partially replace tyrosine. The simultaneous presence of high concentration of tyrosine inhibited the conversion to a normal phenotype of the transformed cells by azatyrosine, with concomitant decrease of the azatyrosine incorporation into proteins.

We previously showed that the administration of azatyrosine into transgenic mice harboring human protooncogenic c-Ha-*ras* effectively inhibited papilloma formation initiated by chemical carcinogens.[7] However azatyrosine was found to be ineffective for the suppression of tumor growth which already proliferated in the transgenic mice, possibly due to insufficient concentrations of azatyrosine in the plasma (unpublished results). It is therefore likely that azatyrosine or its analogs may be useful as a chemopreventive agent rather than an anti-cancer agent.

The effective concentration of azatyrosine to convert the transformed cells to the normal is quite high (at least 500 μg/ml). Since the effect of azatyrosine is likely due to its incorporation into proteins, it seems to be difficult to develop more effective tyrosine analog, unless such a analog with the same biological potency can be more effectively aminoacylated by tyrosyl-tRNA synthetase and transferred to proteins through the tRNA, We have recently obtained a mutated *E. coli* tyrosyl tRNA synthetase which has lower Km for azatyrosine than tyrosine. *E. coli* cells transformed with such a mutated tyrosyl-tRNA synthetase gene was found to incorporate azatyrosine much more effectively than tyrosine (unpublished results). The similar approach can be made for mammalian tyrosyl-tRNA synthetase. If mammalian cells containing such a mutated tyrosyl-tRNA synthetase becomes available, such a cell line should be very useful for confirming the mode of action of azatyrosine to convert the transformed phenotype to the normal. The study of this line of approach is currently being undertaken in our laboratory. In any case, it is important to identify a crucial protein(s) which incorporates azatyrosine and is relevant to the conversion of the transformed phenotype to the normal. Identification of such a protein(s) will contribute to the understanding of cell proliferation and may elucidate a novel mechanism of cancer suppression.

References

1. Shindo-Okada N, Makabe O, Nagahara H et al. Permanent conversion of mouse and human cells transformed by activated *ras* or *raf* genes to apparently normal cells by treatment with the antibiotic azatyrosine. Mol Carcinog 1989; 2:159-167.
2. Monden Y, Nakamura T, Kojiri K et al. Azatyrosine inhibits the activation of c-Raf-1, c-Jun and AP1 but not the activation of Ras during signal transduction triggered by oncogenic c-ErbB-2. Oncol Rep 1996; 3:33-40.
3. Benoit RM, Eiseman J, Jacobs SC et al. Reversion of human prostate tumorigenic growth by azatyrosine. Urology 1995; 46:370-377.
4. Fujita-Yoshigaki J, Yokoyama S, Shindo-Okada N et al. Azatyrosine inhibits neurite outgrowth of PC12 cells induced by oncogenic Ras. Oncogene 1992; 7:2019-2024.
5. Chung DL, Brandt-Rauf PW, Weinstein IB et al. Evidence that the *ras* oncogene-encoded p21 protein induces oocyte maturation via activation of protein kinase C. Proc Natl Acad Sci USA 1992; 89:1993-1996.
6. Chung DL, Brandt-Rauf P, Murphy RB et al. A peptide from the GAP-binding domain of the *ras*-p21 protein as well as azatyrosine block *ras*-induced maturation of Xenopus oocytes. Biochem Biophys Res Commun 1991; 181:1378-1384.
7. Izawa M, Takayama S, Shindo-Okada N et al. Inhibition of chemical carcinogenesis in vivo by azatyrosine. Cancer Res 1992; 52:1628-1630.
8. Park J, Rhim JS, Park S et al. Amplification, overexpression, and rearrengement of the *erb*B-2 protooncogene in primary human stomach carcinomas. Cancer Res 1989; 49:6605-6609.
9. Bishop JM. Molecular themes in oncogenesis. Cell 1991; 64:235-248.
10. Shan R, Matsuda S, Ichino M et al. Detection of the ligand activity of the c-*erb*B-2 protein in calf serum. Jpn J Cancer Res 1992; 83:15-19.

11. Monden Y, Nakamura T, Kojiri K et al. Azatyrosine is incorporated into proteins instead of tyrosine residues, with the resulant conversion of transformed cells to cells with a normal phenotype. Oncol Rep 1996; 3:625-629.
12. Coussens L, Yang-Feng TL, Liao YC et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 1985; 230:1132-1139.
13. Yamamoto T, Ikawa S, Akiyama T et al. Similarity of protein encoded by the human c-*erb-B*-2 gene to epidermal growth factor receptor. Nature 1986; 319:230-234.
14. Sistonen L, Hölttä E, Lehväslaiho H et al. Activation of the *neu* tyrosine kinase induces the *fos/jun* transcription factor complex, the glucose transporter, and ornithine decarboxylase. J Cell Biol 1989; 109:1911-1919.
15. Fujimoto A, Kai S, Akiyama T et al. Transactivation of the TPA-responsive element by the oncogenic c-*erbB*-2 protein is partly mediated by protein kinase C. Biochem Biophys Res Commun 1991; 178:724-732.
16. Satoh T, Endo M, Nakafuku M et al. Accumulation of $p21^{ras}$•GTP in response to stimulation with epidermal growth factor and oncogene products with tyrosine kinase activity. Proc Natl Acad Sci USA 1990; 87:7926-7929.
16a. Monden Y, Shindo-Ikada N, Nishimura S. Mechanism of action of azatyrosine: recent progress. Jpn J Cancer Chemother 197; 24:1563-1570.
17. Kolch W, Heidecker G, Lloyd P et al. Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature 1991; 349:426-428.
18. Dickson B, Sprenger F, Morrison D et al. Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. Nature 1992; 360:600-603.
19. Moodie SA. Willumsen BM, Weber MJ et al. Complexes of Ras•GTP with Raf-1 and mitogen-activated protein kinase kinase. Science 1993; 260:1658-1661.
20. Warne PH, Viciana PR, Downward J. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature 1993; 364:352-355.
21. Zhang X, Settleman J, Kyriakis JM et al. Normal and oncogenic $p21^{ras}$ proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 1993; 364:308-313.
22. Morrison DK, Kaplan DR., Rapp U et al. Signal transduction from membrane to cytoplasm: Growth factors and membrane-bound oncogene products increase Raf-1 phosphorylation and associated protein kinase activity. Proc Natl Acad Sci USA 1988; 85:8855-8859.
23. Heidecker G, Kölch W, Morrison DK et al. The role of Raf-1 phosphorylation in signal transduction. Adv Cancer Res 1992; 58:53-73.
24. Wasylyk C, Imler JL, Wasylyk B. Transforming but not immortalizing oncogenes activate the transcription factor PEA1. EMBO J 1988; 7:2475-2483.
25. Schönthal A, Herrlich P, Rahmsdorf HJ et al. Requirement for *fos* gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. Cell 1988; 54:325-334.
26. Akiyama T, Matsuda S, Namba Y et al. The transforming potential of the c-*erbB*-2 protein is regulated by its autophosphorylation at the carboxyl-terminal domain. Mol Cell Biol 1991; 11:833-842.
27. Krzyzosiak WJ, Shindo-Okada N, Teshima H et al. Isolation of genes specifically expressed in flat revertant cells derived from activated *ras*-transformed NIH 3T3 cells by treatment with azatyrosine. Proc Natl Acad Sci USA 1992; 89:4879-4883.
28. Monden Y, Nakamura T, Kojiri K et al. Stimulation of the expression of *rho*B contributes to the ability of azatyrosine to convert c-*erb*B-2-transformed cells to a normal morphology. Oncol Rep 1996; 3:49-55.
29. Contente S, Kenyon K, Rimoldi D et al. Expression of gene *rrg* is associated with reversion of NIH3T3 transformed by LTR-c-H-*ras*. Science 1990; 249:796-798.
30. Tan RS, Taniguchi T, Harada H. Identification of the *Lysyl Oxidase* gene as a target of the antioncogenic transcription factor IRF-1, and its possible role in tumor suppression. Cancer Res 1996; 56:2417-2421.
31. Paterson HF, Self AJ, Garrett MD et al. Microinjection of recombinant $p21^{rho}$ induces rapid changes in cell morphology. J Cell Biol 1990; 111:1001-1007.

32. Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992; 70:389-399.
33. Kagan HM. Characterization and regulation of lysyl oxidase. In: Mecham RP, ed. Regulation of Matrix Accumulation. New York: Academic Press, 1986:321-398.
34. Weber K, Lazarides E, Goldman RD et al. Localization and distribution of actin fibers in normal, transformed and revertant cells. Cold Springs Harbor Symp Quant Biol 1974; 39:363-369.
35. Pollack R, Osborn M, Weber K. Patterns of organization of actin and myosin in normal and transformed cultured cells. Proc Natl Acad Sci USA 1975; 72:994-998.

Chapter 22

Histone Deacetylase Inhibitors: Possible Anti-Cancer Therapeutics

Minoru Yoshida and Sueharu Horinouchi

Introduction

The organization of chromatin is crucial for the regulation of gene expression. In particular, both the positioning and properties of nucleosomes influence promoter-specific transcription in response to extracellular or intracellular signals. The nucleosome core contains DNA of 146 bp tightly wrapped around a central histone octamer comprising two molecules of each of core histones. The four core histones (H2A, H2B, H3 and H4) are subject to a variety of enzyme-catalyzed post-translational modifications, thereby modulating the chromatin functions.[1] Of the modifications, acetylation has been the most extensively studied. The primary sites of histone acetylation are specific lysine residues in the positively charged N-terminal tails that protrude from the octamer, which are important for both histone-DNA and histone-nonhistone protein interactions. The neutralization of the positive charge by acetylation has long been proposed to lead to loosening histone-DNA contacts, which facilitates the accessibility of a variety of factors to DNA.[2-4] Acetylation also appears to play a critical role in various histone interactions with specific nonhistone regulatory proteins.[5] The acetyl groups on histone molecules continuously turn over and the net level of acetylation is controlled by equilibrium between the two specific enzyme activities, histone acetyltransferase (HAT) and deacetylase (HDAC). The genes encoding these enzymes have not been cloned until recently and the mechanism of regulation of the enzyme activities is still largely unknown. Early studies showed that *n*-butyrate, a naturally occurring short chain fatty acid, inhibited histone deacetylation and induced a variety of biological phenotypes. However, the relationship between inhibition of histone deacetylation and *n*-butyrate-induced biological phenotypes should be interpreted with caution, since its effective concentration exceeds millimolar levels and other enzymes are possibly affected.[6] Recently, two microbial metabolites, trichostatin A and trapoxin (Fig. 22.1), were found as potent inhibitors of HDAC.[7,8] They induce hyperacetylation of core histones at very low concentrations, which is accompanied by characteristic blockage of the cell cycle as well as various cellular phenotypic changes. These agents have now been revealed to be useful in analyzing the possible roles of histone acetylation at the cellular level.[9]

Most recently, a human histone deacetylase (HDAC1) was isolated[10] as a protein that binds trapoxin.[8] HDAC1 was significantly similar to the yeast transcriptional regulator, Rpd3.[10] On the other hand, four different human histone acetyltransferases (hGCN5, P/CAF, p300/CBP and $TAF_{II}250$) have been identified to date.[11-15] It was shown that the adenoviral oncoprotein E1A disturbed the normal cellular interaction of P/CAF with p300/CBP, also

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

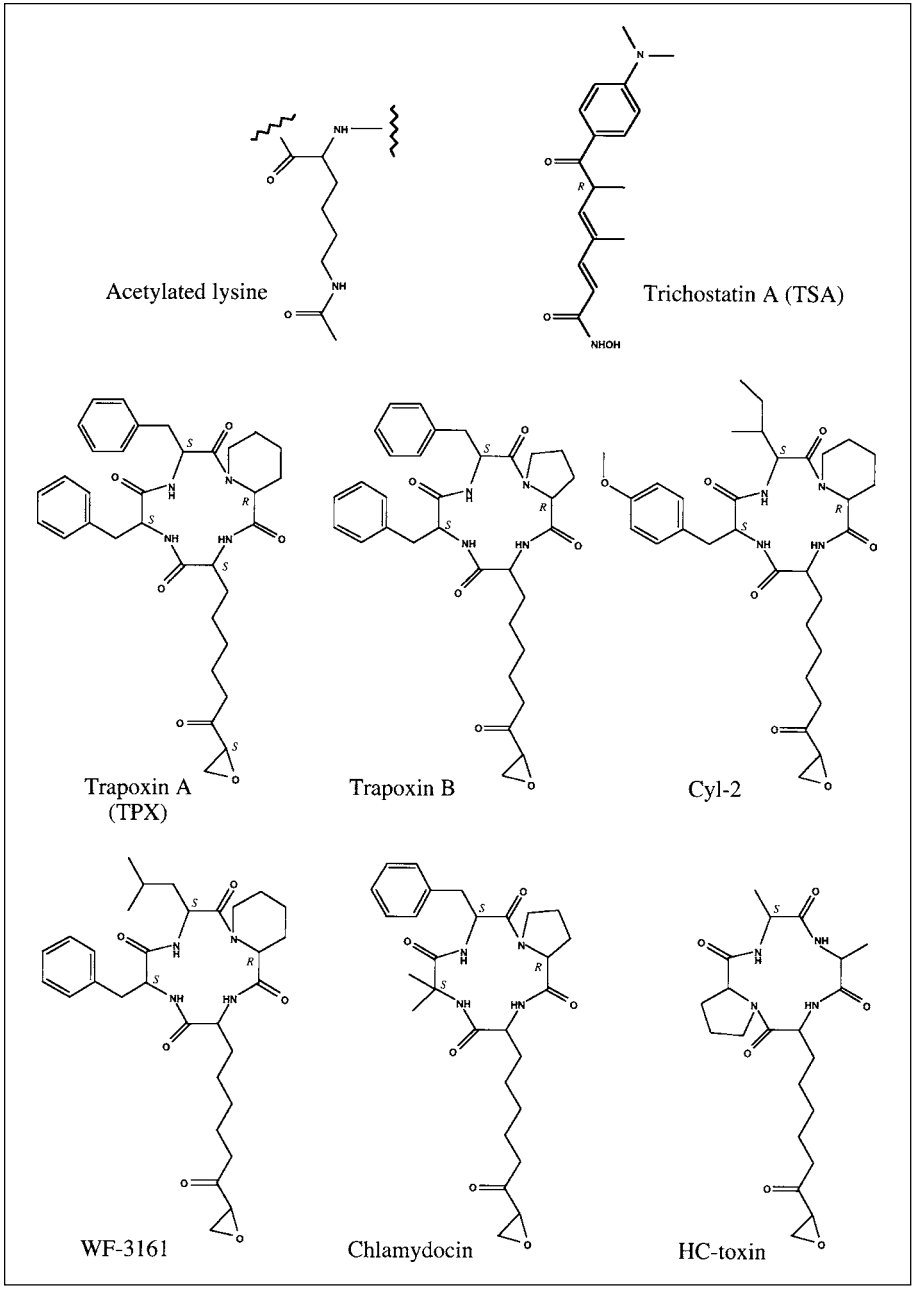


Fig. 22.1. Chemical structures of TSA, TPX and other related microbial metabolites. These compounds mimic the acetylated lysine residue.

known as transcriptional coactivators.[16] Furthermore, overexpression of P/CAF in cultured cells inhibited cell cycle progression and counteracted the activity of E1A.[12] These findings suggest that control of the chromatin function by histone acetylation is one of the critical targets of oncoproteins and that its deregulation may lead to cellular transformation. Thus, inhibitors of histone acetylation or deacetylation may serve as not only biochemical tools for basic research but also promising lead compounds for cancer chemotherapy. Here we review the biological activities of these inhibitors and discuss on their application.

Trichostatin and Trapoxin: Specific Inhibitors of Histone Deacetylase

Trichostatin A (TSA) and its glucopyranosyl derivative (trichostatin C) were originally isolated from *Streptomyces hygroscopicus* as antifungal antibiotics active against *Trichophyton*[17,18] (Figs. 22.1 and 22.3). About ten years later, very potent activities of trichostatins in inducing differentiation of Friend murine erythroleukemia (MEL) cells and inhibiting cell proliferation of mammalian cells were found by two groups.[19-21] Exposure of the MEL cells to the nanomolar concentrations of TSA for 4-5 days produced hemoglobin-positive cells detected by benzidine-staining with a high efficiency. The skeletal structure of trichostatins possesses a chiral center at the 6 position. Stereoselective synthesis of both enantiomer of TSA by Mori and Koseki[22] confirmed that the natural TSA was *R* configuration. Its antipode (*S*)-TSA was biologically inactive and showed no agonistic or antagonistic effect on (*R*)-TSA. An extremely low effective concentration and strict structural specificity of (*R*)-TSA suggest the presence of a specific target molecule to which (*R*)-TSA binds.[23]

A clue to understanding the target of TSA was incidentally obtained from the analysis of the modification of histones. Acid urea Triton (AUT) gel electrophoresis which can separate core histone molecules with different extents of acetylation revealed that the histones in the cells treated with TSA were acetylated to unusually high extents. Pulse-chase experiments revealed that histone hyperacetylation induced by TSA was not due to increased acetylation but decreased deacetylation of histones. In vitro experiments using partially purified histone deacetylase from mouse mammary tumor cells (FM3A) showed that TSA was a potent inhibitor with the Ki value of 3.4 nM, which almost corresponded to the in vivo effective concentrations of TSA. Furthermore, we derived a mutant cell line named TR303 resistant to only TSA from FM3A and found that the histone deacetylase from the mutant cells possessed the markedly increased Ki value of 31 nM, indicating that the enzyme itself had changed to being resistant to TSA. This genetic evidence has clearly indicated that histone deacetylase is the intracellular primary target of TSA.[7] Sodium *n*-butyrate had been reported to induce histone hyperacetylation as well as erythroid differentiation of MEL cells and arrest of the cell cycle of fibroblast cells at G1 and G2 phases. The effect of butyrate as a noncompetitive inhibitor on the histone deacetylase was shown with a partially purified enzyme.[24] However, *n*-butyrate might cause subsidiary effects on other enzymes, cytoskeleton, and cell membrane, since millimolar concentrations were required for its action. For instance, Munks and Turner[25] showed that butyrate and alcohols but not TSA caused a major reduction in heat-shock protein synthesis when added to the growth medium of *Drosophila* cultured cells prior to initiation of stress such as the increased temperature. TSA at nanomolar concentrations causes an increase in the acetylation of all histones that are naturally acetylated in vivo, as butyrate does at millimolar concentrations. The very potent activity of TSA with high specificity allows its use as a well-defined biochemical probe for histone acetylation instead of butyrate.[9]

Trapoxins A and B, novel cyclic tetrapeptides (Fig. 22.1), were isolated from fungal metabolites as agents inducing morphological reversion of v-*sis*-transformed NIH3T3 cells by Itazaki et al[26] in 1990. Since TSA was also reported to possess a similar activity inducing morphological reversion of v-*sis*-transformed cells, we examined the similarity between the

molecular action of these agents. Trapoxin A (TPX) was found to cause accumulation of highly acetylated core histones in a variety of mammalian cells. In vitro experiments using partially purified histone deacetylase showed that a low concentration of TPX irreversibly inhibited deacetylation of acetylated histone molecules, in contrast to reversible inhibition by TSA. The kinetic analysis indicates that TPX is classified as a "slow-binding inhibitor". TPX contains an unusual amino acid, 2-amino-8-oxo-9,10-epoxy-decanoic acid (AOE), which may act as a lysine substrate mimic (Fig. 22.1). Since only the epoxy ketone group in the AOE residue is a chemically reactive moiety, this moiety seemed likely to play a role in forming a covalent bond between TPX and the enzyme molecule. This idea was supported by chemical reduction of the epoxide group. The reduced TPX lost the inhibitory activity, suggesting that TPX inactivates the histone deacetylase by binding the enzyme via its epoxide group. TR303, a TSA-resistant cell line derived from FM3A, showed cross-resistance to TPX. Thus, TPX is a new additional member of specific inhibitors of histone deacetylase.[8] Several cyclic tetrapeptide antibiotics structurally related to TPX, chlamydocin,[27] HC-toxin,[28] Cyl-2,[29] and WF-3161[30] have been isolated from cultures of fungal strains. All these cyclic tetrapeptides have a similar reactive residue, in which the epoxy ketone was shown to be essential for inhibiting histone deacetylase in TPX (Fig. 22.1). HC-toxin and chlamydocin were actually found to inhibit histone deacetylase.[31] It seems probable that several microorganisms evolutionally acquired the ability to inhibit the plant or fungal histone deacetylases in order to survive in their natural environment. The use of TPX in addition to TSA may be extremely useful in investigating the role of histone acetylation in a variety of biological systems.

Molecular Cloning of Histone Acetylating or Deacetylating Enzymes

Transcriptionally active or competent genes are preferentially localized in the acetylated chromatin domains.[2,3] For example, early studies using Hg-agarose affinity chromatography revealed that DNA sequences of transcriptionally active genes were copurified with highly acetylated histones.[32,33] Consistently, immunolabeling of metaphase chromosomes with antisera to acetylated H4 indicated that R-bands, the regions enriched in coding DNA, were the most strongly labeled whereas heterochromatin, such as the inactive X chromosome in female mammals or those regions of the autosomes adjacent to the centromeres, were unlabeled.[34] Genetic analysis using mutant strains of yeast *Saccharomyces cerevisiae* in which individual lysines in the N-terminal region of histone H4 were substituted by other amino acids demonstrated that substitution of 3 or 4 of the lysine residues inhibited the induced expression of *GAL1*, *PHO5*, and *CUP1* genes and lysine-16 was essential for silencing of the mating-type genes.[35-37] All these observations support the intimate correlation between transcriptional activity and increased histone H4 acetylation. Although it has long been postulated that hyperacetylation of histones facilitates the accessibility of RNA polymerase and transcription factors to DNA, the mechanism of regulation of the enzyme activities has been mostly unknown, since the genes encoding these enzymes had not been cloned until recently.

The first cloning of a gene encoding histone acetyltransferase (HAT) was done by Sternglanz and his colleagues using a yeast mutant defective in histone acetylation.[38] However, the acetyltransferase named HAT1 was a cytoplasmic deposition-related acetyltransferase that adds acetyl groups to newly formed histones. The nuclear HAT (type A) and its gene were isolated by Brownell et al[11] from the ciliated protozoan *Tetrahymena* in 1996. A search of databases for genes whose sequences resemble those of HAT A yielded an unexpected result that HAT A turned out to be very similar to a yeast protein called GCN5. GCN5 was known as an adaptor protein originally defined by genetic analysis as being required for the full activity of a subset of transcriptional activators such as GCN4. In fact,

GCN5 was soon proved to act as a HAT acetylating histones H3 and H4. GCN5 is one component of an adaptor complex including ADA2 and ADA3, and its HAT activity was found to be essential for the in vivo transcriptional activation by GCN4.[39] Since ADA2 and mammalian p300/CBP share significant sequence similarity and GCN4 is believed to be a c-Jun counterpart, the mammalian GCN5 homolog was assumed to interact with p300/CBP, coactivators for several transcription factors including c-Jun. Nakatani and his colleagues[12] isolated P/CAF as a p300/CBP-associated factor having HAT activity by looking for the mammalian counterpart of GCN5. Actually, P/CAF showed significant homology with the yeast GCN5 protein. Furthermore, p300 and CBP themselves were found to possess HAT activity, which can acetylate all the core histone molecules.[13,15] In addition, TAFII230/250, a large subunit of the transcription factor complex TFIID, was also a HAT.[14] These findings imply that histone-acetylating enzymes are complexed with other proteins and recruited by sequence-specific transcription factors to the promoter and/or enhancer regions, which is directly involved in transcriptional "on" signals (Fig. 22.2).

Conversely, it has been believed that core histone deacetylation leads to transcriptional repression. This hypothesis was found to be partly consistent with the observation that a mammalian histone deacetylase, HDAC1, is related to the yeast RPD3 protein. HDAC1 was isolated by Schreiber and his colleagues[10] as a protein that binds the irreversible inhibitor, TPX.[8] HDAC1 was significantly similar to the yeast transcriptional regulator, RPD3, which is required for full repression as well as full activation of gene expression. Two yeast HDAC complexes were also isolated, one of which contained RPD3 and the other contained HDA1, also related to RPD3.[40] Search of the yeast genome database revealed at least three other homologs, HOS1, HOS2 and HOS3, are present in yeast, although their enzymatic activity was yet unidentified. These results suggest that HDAC genes constitute a gene family like HAT genes. Mammalian HDAC also forms large protein complexes. The affinity-purified HDAC1 was complexed with RbAp48,[10] whereas HDAC2, also known as mRPD3, was identified as a binding protein for YY1, a sequence-specific DNA-binding protein that acts as both a repressor and an activator (Yin and Yang).[41] More recently, HDAC complexes were found to be associated with several sequence-specific DNA binding repressors, such as Mad:Max, Ume6 and nuclear receptors without ligands. The interaction of HDAC with these sequence-specific transcriptional repressors was mediated by Sin3 and N-CoR/SMRT.[42-46] Inhibition of HDAC activity by TSA or TPX alleviated the transcriptional repression directed by the DNA-bound repressors. Thus, HDAC can be recruited by the sequence-specific transcriptional repressors to promoters, which causes deacetylation of core histones, leading to gene-specific or chromatin region-specific transcriptional repression (Fig. 22.2).

Histone Acetylation and Gene Expression

The simple notion that histone acetylation and deacetylation is necessary for transcriptional activation and repression, respectively, cannot explain several important experiments. Immunolabeling of *Drosophila* polytene chromosomes with specific antibodies to H4 acetylated at different sites demonstrated that the transcriptionally hyperactive X chromosome related to dosage compensation in male larvae was greatly enriched in H4 acetylated at lysine-16 (H4Ac16), but not in H4 acetylated at other sites.[47] In addition, while transcriptionally active regions on the autosomes labeled weakly with antibodies to H4Ac16, the male X chromosome was marked by relatively high levels of H4Ac16 in mitotic cells, where transcriptional activity is minimal. These findings suggest that H4Ac16 is not a consequence of transcriptional activity but a genetic mark for a chromosome that is to be hyperactive.[4,47] In addition, a differential display analysis of cells treated with TSA exhibited only 8 out of 340 genes were changed upon drug treatment, suggesting that most of

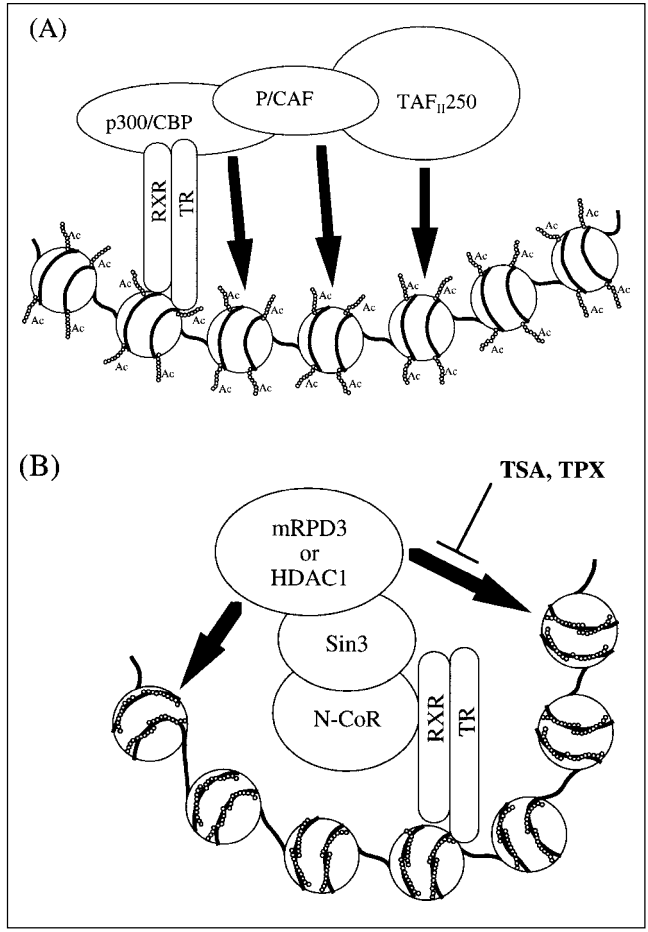


Fig. 22.2. Protein complexes that acetylate or deacetylate nucleosomal histones. (A) The histone acetyltransferase complex was recruited by sequence-specific transcription factors such as liganded nuclear receptors (RXR, TR) to gene promoters. (B) The histone deacetylase complex containing corepressors was recruited by sequence-specific transcriptional repressors such as unliganded nuclear receptors (RXR, TR) to gene promoters.

endogenous genes are unaffected but a small number of genes are specifically induced despite bulk histone hyperacetylation.[48] Recently, the role of Rpd3 in position effect variegation and telomeric silencing was genetically studied and the results were opposite to the proposed model.[49] Position effect variegation and telomeric silencing are thought to be a consequence of localized transcriptional repression that occurs in the vicinity of heterochromatin and negatively regulated by histone acetylation. However, the deletion of *RPD3* in yeast caused a significant decrease in transcriptional activity near telomeres, although the level of histone acetylation increased. Enhanced position effect variegation was also observed in *Drosophila rpd3* mutants, indicating that transcriptional repression was enhanced by the increase in histone acetylation. These observations indicate that the relationship between histone acetylation and transcriptional silencing is much more complex than we thought previously.

Histone Acetylation and Cancer

An initial clue to understanding the intimate relation between cancer and cell cycle control was obtained from the observations that the viral oncogene products such as E1A

and large T antigen are directly associated with pRb, the most important substrate for cyclin-dependent kinases (CDK). There has been a similar situation in the research on histone acetylation and cancer. The transcriptional coactivators, p300/CBP, are well known targets for an adenoviral oncoprotein E1A. Recent studies clearly showed that p300/CBP possessed HAT activity, as mentioned above. Another histone acetyltransferase P/CAF was also found to be associated with p300/CBP. Both P/CAF and E1A bind to the same or to very closely spaced sites on p300/CBP and compete in vitro, and the endogenous P/CAF-p300 complex was disrupted by E1A in vivo. Furthermore, overexpression of P/CAF in cultured cells inhibited cell cycle progression and counteracted the activity of E1A.[12] Thus, P/CAF, a HAT related to GCN5, might act as a tumor suppressor.

Molecular analyses of human diseases have also suggested that changes in acetylation may play a role in the uncontrolled cell growth of cancer. The Rubinstein-Taybi syndrome (RTS) is a well-defined syndrome with facial abnormalities, broad thumbs, broad big toes and mental retardation as the main clinical features. In addition, the high incidence of neoplasms as well as the formation of keloids, a hyperproliferative response of fibroblasts to dermal injury, in RTS indicates the relevance of the susceptible gene of this disease to malignancy. Many patients with RTS have been shown to have breakpoints in or deletions of chromosome 16p13.3. Petrij et al[50] identified the region of the breakpoints, where the CBP gene was found to be mutated. Because the patients are heterozygous for the mutations, the loss of one functional copy of the CBP gene underlies the abnormalities in RTS. One can assume that the decrease in the gene dosage of CBP may cause insufficient histone acetylation in some specific chromatin regions and disturb correct gene expression, thereby promoting tumor incidence. A certain type of acute myeloid leukemia provided another hint for the link between histone acetylation and cancer. The recurrent translocation t(8;16)(p11;p13) is a cytogenic hallmark for the M4/M5 subtype of acute myeloid leukemia. Identification of the breakpoint-associated genes revealed that MOZ, a novel gene encoding a putative acetyltransferase with zinc fingers, was fused to CBP, resulting in the combination of the putative acetyltransferase domain of MOZ with a largely intact CBP.[51] SAS2, a yeast homolog of MOZ, was identified as a gene involved in silencing at the *HM* loci in the absence of SIR1.[52] As described above, unacetylated acidic histone tails have a significant role in silencing. For example, overproduction of SIR2 leads to decreased acetylation of core histones, either directly or perhaps by inhibition of a HAT.[53] SIR1 might act in the recruitment or assembly of other silencing proteins such as SIR2 into inactive chromatin. The *sas2* and *sir1* double mutant was defective in mating due to uncontrolled expression of *HM* loci. Furthermore, the *sas2* mutant showed loss of silencing of the telomere-proximal URA3 gene.[52] SAS2 and MOZ share extensive similarity and are homologous to known acetyltransferases. Since the *sas2* phenotypes were closely similar to those of *nat1* and *ard1* mutants defective in N-terminal acetyltransferase but not to those of hat1 mutants defective in HAT, it seems likely that SAS2/MOS may have N-terminal protein acetylation activities on substrates other than histones. If so, it seems possible to assume that the MOZ-CBP chimera enhances silencing of the chromatin regions directed by the CBP portion and acts as a dominant-negative regulator of chromatin structure and transcriptional regulation, antagonizing the CBP action. Conversely, it is also possible that the extensive histone acetylation by recruiting the MOZ acetyltransferase to the chromatin domain causes unusually high expression of growth-associated genes, if MOZ can acetylate histones. Identification of the t(8;16) breakpoint genes also provides insight into other variant translocations such as t(8;22)(p11;q13) that causes a similar monocytic leukemia. Presumably, MOZ is involved in most of these recurrent translocations, whereas CBP can be replaced with analogous genes. p300 is functionally similar to CBP and is also targeted by E1A. The p300 gene maps to

22q13, which strongly suggests that t(8;22)(p11;q13) results in a MOZ-p300 fusion protein that may act similarly to MOZ-CBP.

Potential Antitumor Activity of Histone Deacetylase Inhibitors

Several lines of evidence suggest that decreased histone acetylation in at least some specific chromatin domains is involved in tumorigenesis. This hypothesis is partly consistent with the effects of histone deacetylase inhibitors. The increase in histone acetylation in chromatin by TSA or TPX induces arrest of the cell cycle in G1 and/or G2 in a variety of normal and transformed cell lines. Since TR303 cells possessing a TSA-resistant HDAC are resistant to TSA and TPX, cell cycle arrest may be due to histone hyperacetylation induced by these agents.[7] However, it is unclear how the induced hyperacetylation leads to such an arrest. One explanation is that perturbation of transcriptional regulation by histone hyperacetylation interferes with correct gene expression or activation of the cell cycle regulators such as cyclins and Cdks.[54] Alternatively, the G1 or G2 progression may be blocked by the checkpoint control mechanism which detects the aberrant chromatin hyperacetylation as one of the chromatin structural abnormalities like DNA damage.[55] We have observed that the level of p21WAF1, a potent CDK-inhibitory protein normally produced under the control of p53,[56] was highly enhanced by TSA treatment irrespective of the p53 state (unpublished results). The p21WAF1 induction is one of the possible reasons for the TSA-induced cell cycle arrest (Fig. 22.3).

Sodium *n*-butyrate, a weak inhibitor of histone deacetylase, has hitherto been well known to induce biochemical or morphological differentiation of several tumor cell lines. Differentiation of MEL,[57] neuroblastoma,[58] and teratocarcinoma cells[59] are effectively induced by the exposure to butyrate at millimolar concentrations. The morphology of HeLa cells is changed to that with neurite-like processes,[60] and several oncogene-transformed fibroblast cell lines are morphologically reverted to an apparently normal state in which the actin stress fibers are restored.[61,62] Since butyrate is formed naturally in the large intestine as a bacterial fermentation product of fiber carbohydrate,[63] it has been speculated that increased intestinal butyrate levels may account for the decreased colon cancer incidence in individuals with high-fiber diets.[64-66] These observations suggest a link between histone acetylation and differentiation in vivo and in vitro. Recent studies using TSA have indicated that similar phenotypic changes of MEL, F9, HeLa, and other transformed cells are induced by TSA at the very low concentrations, and the effects are confined only to the (*R*)-isomer of TSA.[23,67,68] TSA was also reported as an inducer of morphological reversion of several transformed cells, such as v-*sis*- and v-*ras*-transformed NIH3T3 fibroblasts and human carcinoma cell lines.[68-70] These findings demonstrate that hyperacetylation of chromatin by TSA or TPX induces the transformed cells to revert to the apparently normal state showing differentiated phenotypes.

Some proteins whose expression was stimulated by butyrate have been confirmed to be enhanced by TSA. Histone $H1^0$, a member of the differentiation-associated histone H1 family, is expressed in vertebrates when cells are committed to a large number of differentiation programs[71,72] or exposed to a high concentration of butyrate.[73] Giardot et al[74] have demonstrated a striking correlation between the extent of histone acetylation and the induced expression of $H1^0$ by TSA. The $H1^0$ promoter is highly sensitive to the state of bulk chromatin acetylation and the increase in the proportion of mono-acetylated H4 is sufficient to induce a higher level of expression. High $H1^0$ gene expression is observed in the wild-type FM3A cells treated with 5 ng/ml TSA and in their derived TSA-resistant cells (TR303) without TSA treatment, in which the level of the mono-acetylated histone H4 is already high even in the absence of TSA. In contrast, such activation was not observed with the H1 or H4 gene promoter, despite sharing regulatory elements with $H1^0$. Similar specific induction of

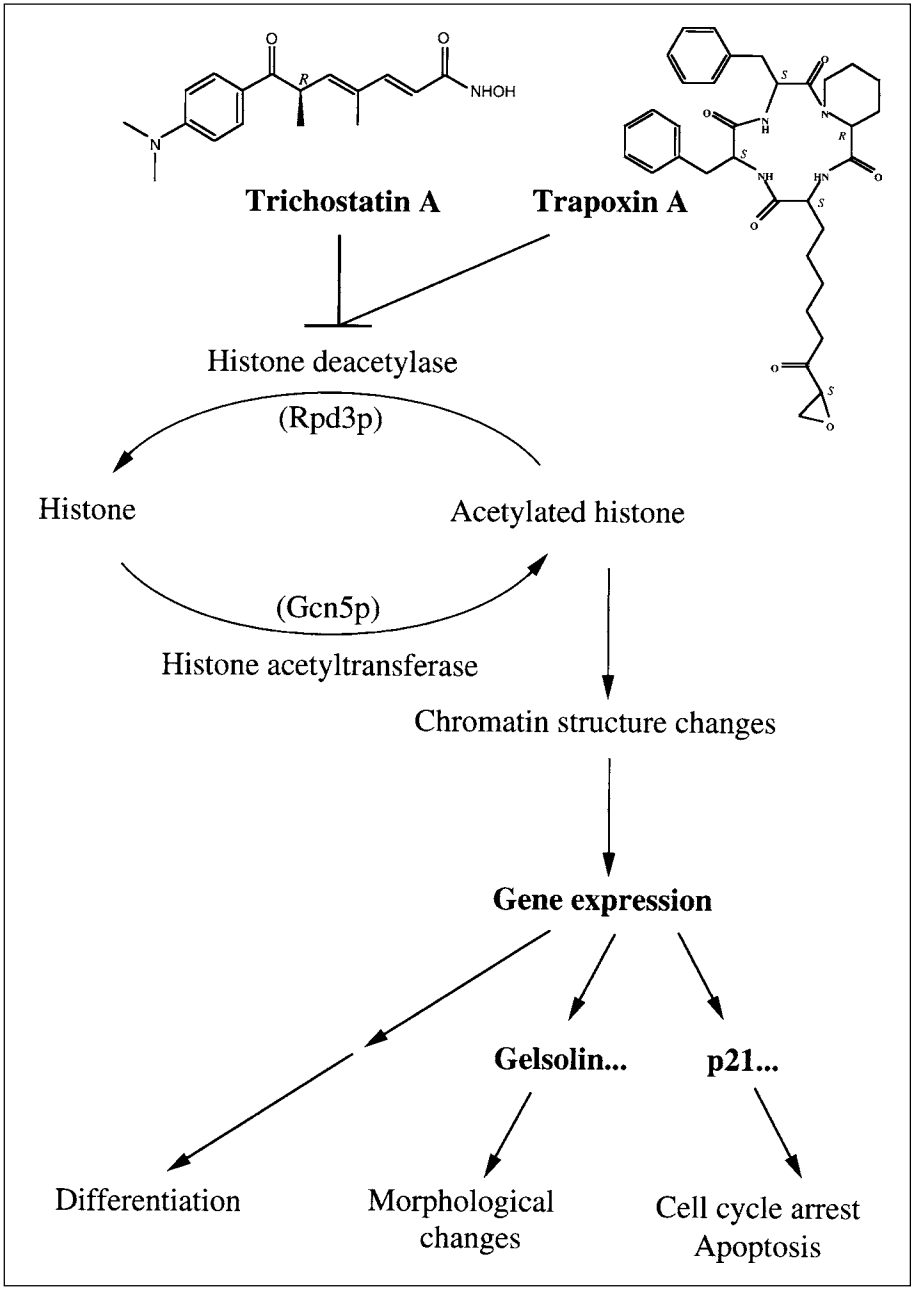


Fig. 22.3. Schematic representation of the biological activity of TSA and TPX.

$H1^0$ gene expression by TSA or butyrate has been observed in *Xenopus* embryogenesis, but no activation has occurred in *Xenopus* oocytes in which the acetylation level is insensitive to TSA or butyrate.[75] Thus, it seems likely that $H1^0$ gene expression is developmentally regulated through histone acetylation control.

Recently, we identified gelsolin, a Ca^{2+}-dependent actin filament severing and capping protein,[76] as one of the induced proteins in the TSA-treated human carcinoma cell lines.[68] On the other hand, apparent increases in the other actin-binding proteins were not observed during TSA treatment. Gelsolin, a protein which is highly conserved in vertebrates, controls the length of actin filaments in vitro and the cell shape and motility in vivo by a variety of mechanisms.[77] Gelsolin expression also appears to be involved in cell differentiation and neoplasia; gelsolin is produced in an undetectable amount in mouse embryonic and immature myeloid cells but increasingly produced during cell differentiation.[78,79] The downregulation of gelsolin expression is observed in a variety of transformed cells and human invasive tumor cells.[80,81] It has been reported that a drastic increase in the gelsolin level is also observed during flat reversion of v-*ras*-transformed fibroblasts by butyrate treatment.[82] Furthermore, it was recently reported that overexpression of gelsolin causes tumor cells to appear flatter, and the chromosomal alteration of 9q found in human bladder carcinoma correlates with diminished expression of the gelsolin gene that is mapped onto chromosome 9q33.[83] Moreover, a mutation of gelsolin at His321 or overproduction of wild-type gelsolin has been shown to induce morphological reversion and marked reduction of in vitro colony-forming activity as well as in vivo tumor incidence.[84] All of these observations strongly suggest a suppressive potential of gelsolin against cancer. The induction of gelsolin gene expression may be involved in morphological reversion depending on de novo protein synthesis of several tumor and transformed cell lines by TSA or butyrate.

To determine whether the drug-induced gelsolin expression is associated with the morphological changes of tumor cells, we injected the anti-human gelsolin antibody into T24 human carcinoma cells prior to drug treatment and observed the effects on the cellular morphology. T24 contains an activated Ras mutation. The antibody used was shown to inhibit the actin severing activity of gelsolin.[85] The TSA treatment dramatically caused the cells to become flat, as is determined by phalloidin staining, and actin stress fibers became prominent. The cells injected with the control IgG were also flat with stress fibers being restored upon TSA treatment. However, approximately 80% of the cells injected with the anti-gelsolin antibody exhibited a morphology similar to that of untreated cells, even when they were treated with TSA. Similar results were obtained with radicicol, a Src kinase inhibitor inducing morphological reversion of several transformed cells with enhanced gelsolin expression.[86] These results show that elevated expression of gelsolin is associated, at least in part, with the suppression of transformation and the restoration of actin stress fibers in human carcinoma cells by TSA (Fig. 22.3).

Clinical Possibility of Histone Deacetylase Inhibitors

Cancer Chemotherapy

The potent activity of TSA or TPX to induce cell cycle arrest and subsequent apoptotic cell death suggests its potential usefulness in cancer chemotherapy. However, TSA and TPX *per se* were not sufficiently effective in the experimental tumor models, probably due to their instability. Recently, a promising antitumor agent was found to be a new HDAC inhibitor. FR901228 (Fig. 22.4) was isolated from *Chromobacterium violaceum* as an agent inducing morphological reversion of H-*ras*-transformed NIH3T3 cells.[87-89] FR901228 also strongly inhibited proliferation of tumor cells in vitro by arresting cell cycle transition at G1 and G2. In addition, FR901228 was found to greatly induce transcription of the SV40 pro-

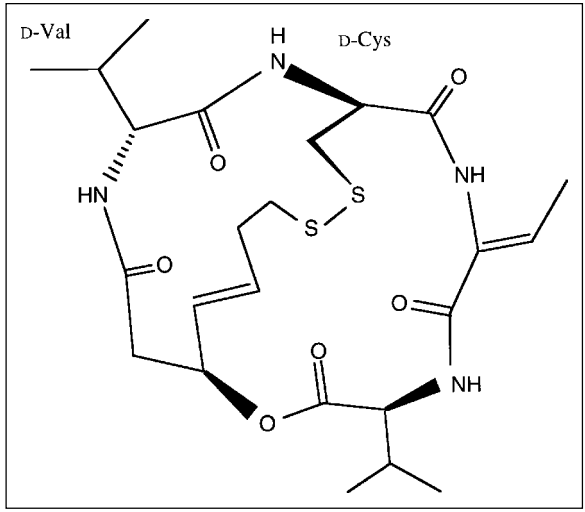


Fig. 22.4. Chemical structure of FR901228, a new histone deacetylase inhibitor with potent antitumor activity.

moter-driven CAT reporter gene. Since TSA also activates the SV40 promoter,[90] we examined whether FR901228 inhibits HDAC. FR901228 apparently induced the accumulation of highly acetylated histones, characterized by decreases in the most rapid migration bands of each histone species and appearance of additional slower ones like a ladder on the AUT gel. In fact, FR901228 inhibited HDAC partially purified from cells in a dose-dependent manner.[91] Thus, FR901228, a cyclic depsipeptide lacking both AOE and a hydroxamate group, is a novel inhibitor of HDAC, structurally distinct from known inhibitors, TSA, TPX, and *n*-butyrate. One of the possible mechanisms of HDAC inhibition by FR901228 is that the SH group produced by reducing the disulfide bond in the cell may serve as a reactive moiety such as a hydroxamate of TSA or an epoxy ketone of TPX. In contrast to TSA or TPX, FR901228 greatly suppressed the growth of transplanted mouse and human tumors in mice.[89] Since the antitumor activity of FR901228 against xenografted human solid tumors was comparable to those of present antitumor drugs such as cisplatinum (CDDP) and mitomycin C, the antitumor potential of FR901228 is further evaluated at the National Cancer Institute (Bethesda, MD). FR901228 is now under clinical trials for cancer therapy. Identification of FR901228 as an HDAC inhibitor with potent antitumor activity implies that HDAC is an attractive target for cancer therapy and that the potential activity of TSA, TPX derivatives, and short-chain fatty acids may be improved by chemical modification.

Differentiation Therapy

The ability of histone deacetylase inhibitors to induce differentiation or morphological normalization indicates the possibility of their clinical use for patients of diseases involving defective cellular differentiation. The embryonic, fetal and adult globin genes in the human β-globin cluster are regulated in a stage-specific manner with the fetal-to-adult switch occurring soon after birth.[92] The pharmacologic elevation of fetal γ-globin gene expression is being explored as a treatment for patients suffering from β-globin deficiencies. One inducer of fetal hemoglobin, hydroxyurea, has shown clinical efficacy for treatment of sickle cell anemia,[93] making it the first real treatment option for some patients. A separate group of compounds currently under investigation in the clinic are short-chain fatty acids, including *n*-butyrate. Since *n*-butyrate treatment increases levels of fetal hemoglobin in experimental models and in humans,[94] other histone deacetylase inhibitors are also expected to be

effective, if the butyrate effect on γ-globin gene expression is due to its inhibition of HDAC. Recently, McCaffrey et al[95] examined whether TSA, TPX and HC-toxin have similar ability to induce fetal hemoglobin expression in erythroid cells. In both primary erythroid cell cultures and in human erythroleukemia K562 cells, these compounds were found to induce hemoglobin at the nanomolar concentrations. Despite their potency, the clinical use of all of these agents may be limited by their cytotoxicity, which occurs at concentrations only slightly higher than those required to detect hemoglobin induction. It is possible that specific changes in histone acetylation are required for transcriptional regulation of the γ-globin gene, while excessive or inappropriate acetylation leads to aberrant changes in chromatin structure that can cause cells to arrest and undergo apoptosis. Therefore, it seems necessary for differentiation therapy to induce domain-specific histone hyperacetylation which allows selective upregulation of gene expression.

Gene Therapy

Retroviral and adeno-associated viral vectors are two widely used systems for stably transferring genes into mammalian cells. Despite the highly efficient gene transfer by these vectors, maintenance of high-level and long-term expression of the transgenes is generally difficult. Inhibition or suppression of expression of the integrated transgenes was frequently observed during culture. This transcriptional repression is probably due to alteration of the chromatin structure similar to silencing. Recently, it was reported that the silenced, virally transduced genes were reactivated upon treatment with TSA and *n*-butyrate.[96] Since 5-azacytidine did not reactivate the transgenes, histone deacetylation but not DNA methylation may have a role in silencing virally transduced genes. In addition, these inhibitors were shown to amplify transgene expression by more than 100-fold in cells infected with E1-defective adenoviruses. This amplification by TSA was not observed in TR303 cells in which the HDAC is resistant to TSA, clearly indicating that increased histone acetylation is responsible for the transgene amplification.[97] These results imply that efficient viral gene transfer followed by treatment of HDAC inhibitors to relieve transcriptional suppression provides a powerful combination for treatment of various genetic and infectious diseases.

Conclusions and Perspectives

As a result of the impressive speed with which the HAT and HDAC genes have been identified in the past few years, we now know their identities with transcriptional coactivators and corepressors, respectively. Specific HDAC inhibitors TSA/TPX did play an important role in this history, and perhaps will continue to do so. However, accumulating evidence has suggested that each lysine residue on different core histone molecules has an independent regulatory function in chromatin structure and gene expression.[98,99] Therefore, the overall inhibition of HDAC by the present inhibitors may not help to analyze the different functional roles for acetylation of different histones and of different lysine residues on the same histone. It is apparently important to discover or design different HDAC inhibitors which specifically accumulate a specific acetylated lysine residue for dissecting the biological functions of histone acetylation. In addition, we have not yet seen what happens if the cellular HAT activity is inhibited, although a HAT has been shown to be involved in RTS and leukemia. The inhibitors of HAT would also be extremely useful in understanding the relationship between histone acetylation and cancer. These new inhibitors, in addition to FR901228, a new HDAC inhibitor with potent antitumor activity that are currently under clinical investigation, will serve as novel drug candidates for therapy of human disease including cancer.

References

1. Bradbury EM. Reversible histone modifications and the chromosome cell cycle. BioEssays 1992; 14:9-16.
2. Csordas A. On the biological role of histone acetylation. Biochem J 1990; 265:23-38.
3. Turner BM. Histone acetylation and control of gene expression. J Cell Sci 1991; 99:13-20.
4. Turner BM. Decoding the nucleosome. Cell 1993; 75:5-8.
5. Kingston RE, Bunker CA, Imbalzano AN. Repression and activation by multiprotein complexes that alter chromatin structure. Genes Dev 1996; 10:905-920.
6. Kruh J. Effects of sodium butyrate, a new pharmacological agent, on cells in culture. Mol Cell Biochem 1982; 42:65-82.
7. Yoshida M, Kijima M, Akita M et al. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem 1990; 265:17174-17179.
8. Kijima M, Yoshida M, Sugita K et al. Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase. J Biol Chem 1993; 268:22429-22435.
9. Yoshida M, Horinouchi S, Beppu T. Trichostatin A and trapoxin: Novel chemical probes for the role of histone acetylation in chromatin structure and function. BioEssays 1995; 17:423-430.
10. Taunton J, Hassig CA, Schreiber SL. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science 1996; 272:408-411.
11. Brownell JE, Zhou J, Ranalli T et al. Tetrahymena histone acetyltransferase A: A homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell 1996; 84:843-851.
12. Yang XJ, Ogryzko VV, Nishikawa J et al. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature 1996; 382:319-324.
13. Ogryzko VV, Schitz RL, Russanova V et al. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 1996; 87:953-959.
14. Mizzen CA, Yang X-J, Kokubo T et al. The TAFII250 subunit of TFIID has histone acetyltransferase activity. Cell 1996; 87:1261-1270.
15. Bannister AJ, Kouzarides T. The CBP co-activator is a histone acetyltransferase. Nature 1996; 384:641-643.
16. Janknecht R, Hunter T. A growing coactivator network. Nature 1996; 383:22-23.
17. Tsuji N, Kobayashi M, Nagashima K et al. A new antifungal antibiotic, trichostatin. J Antibiot 1976; 29:1-6.
18. Tsuji N, Kobayashi M. Trichostatin C, a glucopyranosyl hydroxamate. J Antibio 1978; 31:939-944.
19. Yoshida M, Iwamoto Y, Uozumi T et al. Trichostatin C, a new inducer of differentiation of Friend leukemic cells. Agric Biol Chem 1985; 49:563-565.
20. Yoshida M, Nomura S, Beppu T. Effects of trichostatins on differentiation of murine erythroleukemia cells. Cancer Res 1987; 47:3688-3691.
21. Morioka H, Ishihara M, Takezawa M et al. (+)-Trichostatin A, a potent differentiation inducer of Friend leukemia cells. Agric Biol Chem 1988; 52:251-253.
22. Mori K, Koseki K. Synthesis of trichostatin A, a potent differentiation inducer of Friend leukemic cells, and its antipode. Tetrahedron 1988; 44:6013-6020.
23. Yoshida M, Hoshikawa Y, Koseki K et al. Structural specificity for biological activity of trichostatin A, a specific inhibitor of mammalian cell cycle with potent differentiation-inducing activity in Friend leukemia cells. J Antibiotics 1990; 43:1101-1106.
24. Cousens LS, Gallwitz D, Alberts BM. Different accessibilities in chromatin to histone acetylase. J Biol Chem 1979; 254:1716-1723.
25. Munks RJL, Turner BM. Suppression of heat-shock protein synthesis by short-chain fatty acids and alcohols. Biochim Biophys Acta 1994; 1223:23-28.
26. Itazaki H, Nagashima K, Sugita K et al. Isolation and structural elucidation of new cyclotetrapeptides, trapoxins A and B, having detransformation activities as antitumor agents. J Antibiot 1990; 43:1524-1532.
27. Closse A, Hugenin R. Isolation and structure elucidation of chlamydocin. Helv Chim Acta 1974; 57:533-545.

28. Liesch JM, Sweeley CC, Staffeld GD et al. Structure of HC-toxin, a cyclic tetrapeptide from *Helminthosporium carbonum*. Tetrahedron 1982; 38:45-48.
29. Hirota A, Suzuki A, Aizawa K et al. Structure of Cyl-2, a novel cyclotetrapeptide from *Cylindrocladium scoparium*. Agric Biol Chem 1973; 37:955-956.
30. Umehara K, Nakahara K, Kiyoto S et al. Studies on WF-3161, a new antitumor antibiotic. J Antibiot 1983; 36:478-483.
31. Brosch G, Ransom R, Lechner T et al. Inhibition of maize histone deacetylases by HC toxin, the host- selective toxin of Cochliobolus carbonum. Plant Cell 1995; 7:1941-1950.
32. Chen TA, Allfrey VG. Rapid and reversible changes in nucleosome structure accompany the activation, repression, and superinduction of murine fibroblast protooncogenes c-*fos* and c-*myc*. Proc Natl Acad Sci USA 1987; 84:5252-5256.
33. Allegra P, Sterner R, Clayton DF et al. Affinity chromatographic purification of nucleosomes containing transcriptionally active DNA sequences. J Mol Biol 1987; 196:379-388.
34. Jeppesen P, Turner BM. The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. Cell 1993; 74:281-9.
35. Durrin LK, Mann RK, Kayne PS et al. Yeast histone H4 N-terminal sequence is required for promoter activation in vivo. Cell 1991; 65:1023-1031.
36. Johnson JM, Kayne PS, Kahn ES et al. Genetic evidence for an interaction between SIR3 and histone H4 in the repression of the silent mating loci in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 1990; 87:6286-6290.
37. Megee PC, Morgan BA, Mittman BA et al. Genetic analysis of histone H4: essential role of lysines subject to reversible acetylation. Science 1990; 247:841-845.
38. Kleff S, Andrulis ED, Anderson CW et al. Identification of a gene encoding a yeast histone H4 acetyltransferase. J Biol Chem 1995; 270:24674-24677.
39. Candau R, Zhou JX, Allis CD et al. Histone acetyltransferase activity and interaction with ADA2 are critical for GCN5 function in vivo. EMBO J 1997; 16:555-565.
40. Rundlett SE, Carmen AA, Kobayashi R et al. HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc Natl Acad Sci USA 1996; 93:14503-14508.
41. Yang W, Inouye C, Zeng Y et al. Transcriptional repression by YY1 is mediated by interaction with the mammalian homolog of the yeast global regulator RPD3. Proc Natl Acad Sci USA 1996; 93:12845-12850.
42. Ayer DE, Lawrence QA, Eisenman RN. Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell 1997; 80:767-776.
43. Kadosh D, Struhl K. Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. Cell 1997; 89:365-371.
44. Hassig CA, Fleischer TC, Billin AN et al. Histone deacetylase activity is required for full transcriptional repression by mSin3A. Cell 1997; 89:341-347.
45. Nagy L, Kao HY, Chakravarti D et al. Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. Cell 1997; 89:373-380.
46. Laherty CD, Yang WM, Sun JM et al. Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. Cell 1997; 89:349-356.
47. Turner BM, Birley AJ, Lavender J. Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in Drosophila polytene nuclei. Cell 1992; 69:375-384.
48. Van Lint C, Emiliani S, Verdin E. The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. Gene Expr 1996; 5:245-253.
49. De Rubertis F, Kadosh D, Henchoz S et al. The histone deacetylase RPD3 conteracts genomic silencing in *Drosophila* and yeast. Nature 1997; 384:589-591.
50. Petrij F, Giles RH, Dauwerse HG et al. Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. Nature 1995; 376:348-351.
51. Borrow J, Stanton VP, Andresen JM et al. The translocation t(8;16)(p11;p13) of acute myeloid leukemia fuses a putative acetyltransferase to the CREB-binding protein. Nature Genet 1996; 14:33-41.

52. Reifsnyder C, Lowell J, Clarke A et al. Yeast *SAS* silencing genes and human genes associated with AML and HIV-1 Tat interactions are homologous with acetyltransferases. Nature Genet 1996; 14:42-49.
53. Braunstein M, Rose AB, Holmes SG et al. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. Genes Dev 1993; 7:592-604.
54. Sherr CJ. Mammalian G1 cyclins. Cell 1993; 73:1059-1065.
55. Hartwell L. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell 1992; 71:543-546.
56. El-Deiry WS, Tokino T, Velculescu VE et al. *WAF1*, a potential mediator of p53 tumor suppression. Cell 1993; 75:817-825.
57. Leder A, Leder P. Butyric acid, a potent inducer of erythroid differentiation in cultured erythroleukemic cells. Cell 1975; 5:319-322.
58. Prasad K, Sinha PK. Effect of sodium butyrate on mammalian cells in culture: a review. In Vitro 1976; 12:125-132.
59. Nishimune Y, Kume A, Ogiso Y et al. Induction of teratocarcinoma cell differentiation: effect of the inhibitors of DNA synthesis. Exp Cell Res 1983; 146:439-444.
60. Ginsburg E, Salomon D, Sreevalsan T et al. Growth inhibition and morphological changes caused by lipophilic acids in mammalian cells. Proc Natl Acad Sci USA 1973; 70:2457-2461.
61. Leavitt J, Barrett JC, Crawford BD et al. Butyric acid suppression of the in vitro neoplastic state of Syrian hamster cells. Nature 1978; 271:262-265.
62. Ryan MP, Higgins PJ. Cytoarchitecture of Kirsten sarcoma virus-transformed rat kidney fibroblasts: butyrate-induced reorganization within the actin microfilament network. J Cell Physiol 1988; 137:25-34.
63. Remesy C, Demigne C. Partition and absorption of volatile fatty acids in the alimentary canal of the rat. Ann Rech Vet 1976; 7:39-55.
64. Freeman HJ. Effects of differing concentrations of sodium butyrate on rat intestinal neoplasia. Gastroenterology 1986; 91:596-602.
65. Gamet L, Daviaud D, Denis-Pouxviel C et al. Effects of short-chain fatty acids on growth and differentiation of the human colon-cancer cell line HT29. Int J Cancer 1992; 52:286-289.
66. Jass JR. Diet, butyric acid and differentiation of gastrointestinal tract tumors. Med Hypotheses 1985; 18:113-118.
67. Hoshikawa Y, Kijima M, Yoshida M et al. Expression of differentiation-related markers in teratocarcinoma cells via histone hyperacetylation by trichostatin A. Agric Biol Chem 1991; 55:1491-1495.
68. Hoshikawa Y, Kwon HJ, Yoshida M et al. Trichostatin A induces morphological changes and gelsolin expression by inhibiting histone deacetylase in human carcinoma cell lines. Exp Cell Res 1994; 214:189-197.
69. Futamura M, Monden Y, Okabe T et al. Trichostatin A inhibits both ras-induced neurite outgrowth of PC12 cells and morphological transformation of NIH3T3 cells. Oncogene 1995; 10:1119-1123.
70. Sugita K, Koizumi K, Yoshida H. Morphological reversion of *sis*-transformed NIH3T3 cells by trichostatin A. Cancer Res 1992; 52:168-172.
71. Alonso A, Breuer B, Bouterfa H et al. Early increase in histone $H1^0$ mRNA during differentiation of F9 cells to parietal endoderm. EMBO J 1988; 7:3003-3008.
72. Rousseau D, Khochbin S, Gorka C et al. Regulation of histone $H1^0$ accumulation during induced differentiation of murine erythroleukemia cells. J Mol Biol 1991; 217:85-92.
73. Kress H, Tönjes R, Doenecke D. Butyrate induced accumulation of a 2.3 kb polyadenylated $H1^0$ histone mRNA in HeLa cells. Nucleic Acid Res 1986; 14:7189-7197.
74. Girardot V, Rabilloud T, Yoshida M et al. Relationship between core histone acetylation and histone $H1^0$ gene activity. Eur J Biochem 1994; 224:885-892.
75. Almouzni G, Khochbin S, Dimitrov S et al. Histone acetylation influences both gene expression and development of *Xenopus laevis*. Dev Biol 1994; 165:654-669.
76. Stossel TP, Chaponnier C, Ezzell RM et al. Nonmuscle actin-binding proteins. Annu Rev Cell Biol 1985; 1:353-402.

77. Cunningham CC, Stossel TP, Kwiatkowski DJ. Enhanced motility in NIH3T3 fibroblasts that overexpress gelsolin. Science 1991; 251:1233-1236.
78. Kwiatkowski DJ. Predominant induction in gelsolin and actin-binding protein during myeloid differentiation. J Biol Chem 1988; 263:13857-13862.
79. Dieffenbach CW, SenGupta DN, Krause D et al. Cloning of murine gelsolin and its regulation during differentiation of embryonal carcinoma cells. J Biol Chem 1989; 264: 13281-13288.
80. Chaponnier C, Gabbiani G. Gelsolin modulation in epithelial and stromal cells of mammary carcinoma. Am J Pathol 1989; 134:597-603.
81. Vandekerckhove J, Bauw G, Vancompernolle K et al. Comparative two-dimensional gel analysis and microsequencing identifies gelsolin as one of the most prominent down-regulated markers of transformed human fibroblast and epithelial cells. J Cell Biol 1990; 111:95-102.
82. Higgins PJ, Ryan MP. p52(PAI-1) and actin expression in butyrate-induced flat revertants of v-*ras*-transformed rat kidney cells. Biochem J 1991; 279:883-890.
83. Tanaka M, Müllauer L, Ogiso Y et al. Gelsolin: A candidate for suppressor of human bladder cancer. Cancer Res 1995; 55:3228-3232.
84. Müllauer L, Fujita H, Ishizaki A et al. Tumor-suppressive function of mutated gelsolin in *ras*-transformed cells. Oncogene 1993; 8:2531-2536.
85. Arora PD, McCulloch CAG. Dependence of fibroblast migration on actin severing activity of gelsolin. J Biol Chem 1996; 271:20516-20523.
86. Kwon HJ, Yoshida M, Nagaoka R et al. Suppression of morphological transformation by radicicol is accompanied by enhanced gelsolin expression. Oncogene 1997; (in press).
87. Ueda M, Nakajima H, Hori Y et al. FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties, and antitumor activity. J Antibiot 1994; 47:301-310.
88. Ueda H, Manda T, Matsumoto S et al. FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968. II. Antitumor activities on experimental tumors in mice. J Antibiot 1994; 47:311-314.
89. Ueda H, Manda T, Matsumoto S et al. FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968. III. Antitumor activities on experimental tumors in mice. J Antibiot 1994; 47:315-323.
90. Schlake T, Klehr WD, Yoshida M et al. Gene expression within a chromatin domain: The role of core histone hyperacetylation. Biochemistry 1994; 33:4197-4206.
91. Nakajima H, Kim YB, Terano H et al. FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. Exp Cell Res 1998; 241:126-133.
92. Stamatoyannopoulos G, Nienhuis AW. Hemoglobin switching. In: Stamatoyannopoulos G, Nienhuis AW, Majerus PW, Varmus H, eds. The Molecular Basis of Blood Disease. Philadelphia, PA: Saunders, 1994:107-156.
93. Charache S, Terrin ML, Moore RD et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. N Engl J Med 1995; 332:1317-1326.
94. Perrine SP, Rudolph A, Faller DV et al. Butyrate infusions in the ovine fetus delay the biologic clock for globin gene switching. Proc Natl Acad Sci USA 1988; 85:8540-8544.
95. McCaffrey PG, Newsome DA, Fibach E et al. Induction of γ-globin by histone deacetylase inhibitors. Blood 1997; 90:2075-2083.
96. Chen WY, Bailey EC, McCune SL et al. Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase. Proc Natl Acad Sci USA 1997; 94:5798-5803.
97. Dion LD, Goldsmith KT, Tang D-c et al. Amplification of recombinant adenoviral transgene products occurs by inhibition of histone deacetylase. Virology 1997; 231:201-209.
98. Turner BM. Histone H4, the cell cycle and a question of integrity. BioEssays 1995; 17:1013-1015.
99. Turner BM, O'Neill LP. Histone acetylation in chromatin and chromosomes. Semin Cell Biol 1995; 6:229-236.

CHAPTER 23

Bacterial Toxins Controlling Rho-GTPases

Klaus Aktories

Introduction

Modification of GTP-binding proteins is a well-known mechanism by which bacterial toxins attack eukaryotic cells. This applies to diphtheria toxin and *Pseudomonas* exotoxin A, which ADP-ribosylate elongation factor 2, and is also true for cholera toxin, the related *Escherichia coli* heat labile toxins and pertussis toxin. These toxins are important virulence factors and valuable tools in cell biology. Research from the last few years also showed that small GTPases are specific targets for bacterial toxins. In particular Rho GTPases, which are involved in the organization of the actin cytoskeleton and act as molecular switches in various signaling pathways, are modified by various bacterial toxins. These toxins inactivate Rho proteins by ADP-ribosylation (*Clostridium botulinum* C3 exoenzyme) or by glucosylation ("large" clostridial cytotoxins). Moreover, it has been shown recently that Rho proteins are also activated by bacterial toxins (e.g., CNF). In this chapter, these various bacterial protein toxins which act on Rho GTPases are briefly reviewed. Several more comprehensive reviews on C3-like exoenzymes[1-6] and on large clostridial cytotoxins have been published recently.[7-11]

Rho Family GTPases

At least 11 proteins belong to the family of mammalian Rho GTPases (a detailed description of the role and functions of Rho family proteins and their regulation is given refs. 12-20). Best studied examples are RhoA, B, C, Rac 1, 2 and the two isoforms of Cdc42 (Cdc42Hs, G25K). Other members of the GTPase family are Rho D, E, G and TC10. Like other monomeric G proteins, Rho proteins are regulated by a GTPase cycle which is controlled by at least three groups of Rho-interacting proteins. Whereas the inactive, GDP-bound form of Rho proteins is stabilized and retained in the cytosol by a group of GDP dissociation inhibitors (GDIs),[21] activation of Rho proteins is induced by GDP dissociation stimulators (GDSs).[22] Finally, inactivation of the active GTP-bound Rho proteins is dramatically accelerated by GTPase-activating proteins (GAPs).[12]

All three subgroups of Rho family proteins, Rho, Rac and Cdc42 are key players in the receptor-mediated regulation of the actin cytoskeleton. Rho induces formation of stress fibers and adhesion complexes, Rac is involved in lamellipodia formation[23] and Cdc42 induces microspikes.[24] Moreover, it has been suggested that the GTPases govern actin polymerization in a hierarchical manner. Thus, Cdc42 activates Rac which is then capable of activating Rho.[24,25] Beside their roles in the organization of the actin cytoskeleton, Rho

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

proteins act as molecular switches in various signal transduction processes including control of cell aggregation,[26] integrin signaling,[27,28] control of phosphatidylinositol-3-kinase,[29] phosphatidylinositol-4-phosphate-5-kinase,[30] phospholipase D.[31-33] Rho family proteins are implicated in endocytosis,[34,35] in control of transcription,[36] cell cycle progression,[37] apoptosis[38] and cell transformation.[39] Furthermore, Rac is essential for control NADPH oxidase in monocytes and neutrophils.[40,41]

Rho-ADP-Ribosylating Exoenzymes

During the last few years various bacterial ADP-ribosylating exoenzymes have been described that modify Rho proteins. The prototype of this toxin family is *Clostridium botulinum* C3 ADP-ribosyltransferase (Fig. 23.1). C3 was serendipitously detected during screening for a higher producer strain of *Clostridium botulinum* C2 toxin, a toxin that ADP-ribosylates actin.[42,43] Consequently, the novel transferase was termed C3 to distinguish the ADP-ribosyltransferases from *Clostridium botulinum* C2 toxin and from *Clostridium botulinum* neurotoxin C1.[44,45] C3 is produced by various strains of *Clostridium botulinum* types C and D. However, the exoenzymes from these strains are not entirely identical and show some heterogeneity in their structure and activity representing a group of isoenzymes. Popoff and co-workers cloned and sequenced a gene for C3 from strains D1873 and C468 coding for a 251-amino acid protein with a molecular mass of 27,823. The mature transferase has 211 amino acids and a mass of 23,546.[46] Narumiya and co-workers cloned the gene of C3 from strain C003-9 that encodes a protein of 244 amino acids with a mass of 27,362. The mature protein has 204 amino acids (M_r 23,119).[47] The two isoforms differ by about 40% in their amino acid sequences. Beside the transferases produced by *Clostridium botulinum*, several other bacterial exoenzymes have been described that ADP-ribosylate Rho selectively. These are *Clostridium limosum* transferase,[48] a transferase from *Bacillus cereus*[49] and a transferase from *Staphylococcus aureus* that was called EDIN (epidermal differentiation inhibitor).[50,51] Whereas the exoenzyme from *Clostridium limosum* is closely related to C3 with about 70% identity on the amino acid level, the mature EDIN transferase of 212 amino acids shares only about 35% amino acid identity with C3. All C3-like transferases are basic proteins (PI >9) with masses of 25,000-28,000.

Most toxins acting inside eukaryotic cells possess at least three functional domains which are involved in the binding to the target cell, translocation of the toxin to the cytosol and modification of the intracellular target. It appears that C3-like transferases do not possess binding and/or translocation domains or subunits. Most likely, these exoenzymes are taken up by an nonspecific mechanism (pinocytosis). The cell accessibility of C3-like transferases is rather poor. Therefore, these transferases are introduced into cells by osmotic shock, electropermeabilization, microinjection or by prolonged incubation (>1 day) in the presence of high enzyme concentrations (>10 μg/ml).[52-55] For unknown reason the cell accessibility of the various C3 isoforms differ and/or some cell types may be much more sensitive towards C3.

C3 and the related C3-like transferases ADP-ribosylate RhoA, B and C with high specificity.[56-58] Rac is a very poor substrate for C3 and is modified to a minor extent (5-10%) only in the presence of detergent, and Cdc42 is not at all ADP-ribosylated.[48] ADP-ribosylation of Rho by all these C3-like transferases occurs at asparagine-41 of the GTPase.[59] This amino acid residue is located in or near the effector region of the GTPase. ADP-ribosylated Rho is biologically inactive. However, the precise functional basis for this inactivation is not known. Most likely, inhibition is not merely the consequence of sterical hindrance between modified Rho and effectors. For example, ADP-ribosylated Rho is still able to interact with protein kinase N (P. Sehr et al, unpublished observations). It is feasible that ADP-ribosylation

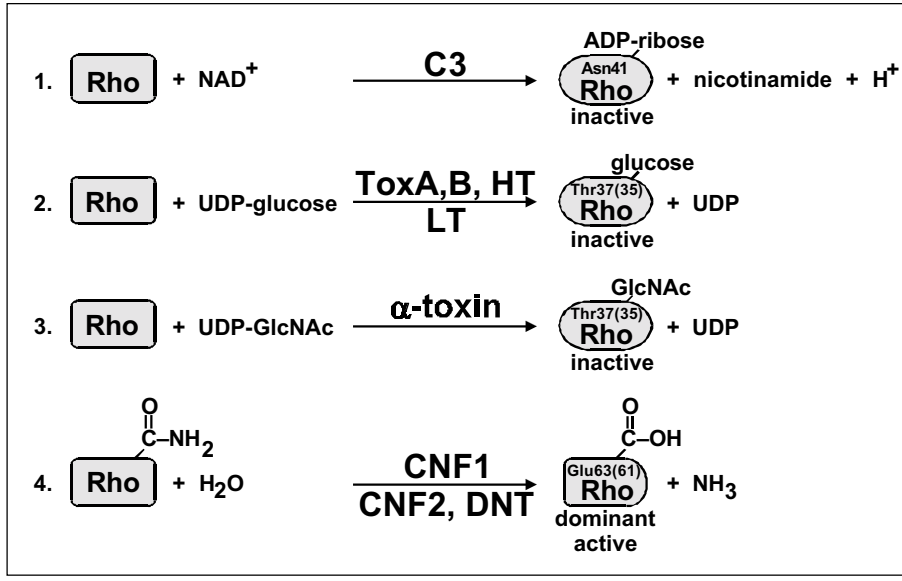


Fig. 23.1. Modification of Rho family proteins by bacterial toxins. (1) *Clostridium botulinum* exoenzyme C3 and C3-like transferases ADP-ribosylate RhoA, B and C at asparagine-41 by using NAD^+ as a cosubstrate. (2) *Clostridium difficile* Toxin A and B and the hemorrhagic toxin from *Clostridium sordellii* glucosylate Rho, Rac and Cdc42 at threonine-37 (Rho) or threonine-35 (Rac and Cdc42) by using UDP-glucose as a cosubstrate. The lethal toxin from *Clostridium sordellii* glucosylate Rac and Cdc42 (dependent on the toxin isoforms) and also Ras family proteins (Ras, Rap, Ral). (3) The α-toxin of *Clostridium novyi* catalyzes the O-GlucN-acetylation of Rho subfamily members. (4) The cytotoxic-necrotizing factors CNF1 and 2 from *Escherichia coli* and the dermonecrotic toxin DNT from *Bordetella ssp*. deamidate glutamine-63 of Rho (glutamine-61 of Cdc42).

blocks the activation of the effectors by Rho but not the physical interaction or, alternatively, a sequestration of Rho GDSs/GEFs occurs.

The effects of C3 are best studied in fibroblasts (3T3 cells).[55] In these cells, C3 induces rounding up and destruction of the microfilament network especially of stress fibers.[60] Because C3 is highly specific for Rho, the transferase was recognized as an important cell biological tool to study the role of Rho not only in regulation of the actin cytoskeleton but also in other signal processes (Fig. 23.2).[1,61]

"Large" Clostridial Cytotoxins

Recently, it has been shown that Rho family proteins are glucosylated by the family of large clostridial cytotoxins. Members of this toxin family are *Clostridium difficile* toxin A and B, the lethal and the hemorrhagic toxins of *Clostridium sordellii*, and the α-toxin of *Clostridium novyi*.

Clostridium Difficile *Toxins A and B*

Clostridium difficile toxins A and B are important pathogenic factors of the antibiotic-associated diarrhea and colitis.[62] It has been suggested that toxin-producing strains of *Clostridium difficile* are implicated in about 20% of cases with antibiotic-associated diarrhea

and are responsible for most, if not all, cases of the pseudomembranous colitis, which is a severe complication of antibiotic therapy.[63]

Clostridium difficile toxins A and B are large single-chain peptide toxins of about 308 and 270 kDa, respectively.[64] Both toxins are about 50% identical on the amino acid level and exhibit a similar primary structure which is shared by all large clostridial toxins.[64,65] The C-terminal part of the toxins is comprised of oligopeptide repeats, which are suggested to be important for cell surface binding. The N-terminal part of the toxin harbors the toxin activity and, in between, is a rather small hydrophobic part located that might be involved in membrane translocation.[64] The biological activities of both toxins differ in animal models. Toxin A is enterotoxic and causes tissue damage and permeability changes with fluid accumulation in rabbit ileal and colonic loops which are followed or accompanied by a neutrophilic inflammatory response, epithelial cell necrosis and ulceration with hemorrhagic edema.[66,67] Under same conditions, toxin B shows no overt enterotoxicity.[66,67] However, when toxin B was given with small amounts of toxin A, animals died suggesting that both toxins act synergistically.[68] Notably, it was shown recently that toxin B is potently enterotoxic in human intestine preparations.[69] Most likely, these discrepancies are caused by binding of toxins to different cell receptors. Both toxins are cytotoxic in cell culture. However, toxin B is at least 1000-fold more potent than toxin A. Therefore, toxin B was termed cytotoxin and toxin A enterotoxin.[70,71] Given intravenously, both toxins are lethal at the same dose (minimal toxic dose 50 ng in mice, intraperitoneal injection).[11]

It has been known for some time that *Clostridium difficile* toxins selectively affect the actin cytoskeleton and much less the microtubule system.[72] The cell morphological changes induced by the toxins are characterized by destruction of the cytoskeleton and rounding up of cells with dramatic retraction of the cell body.[72,73] Often network-like or neurite-like processes remain. At an early stage, these morphological changes are quite similar to changes induced by C3-like transferases. These observations led to the suggestion that Rho proteins are also the target of *Clostridium difficile* toxins. This hypothesis was further strengthened by the findings that pretreatment of Chinese hamster ovary (CHO) cells by *Clostridium difficile* toxin B inhibits subsequent ADP-ribosylation of Rho by C3. Moreover, the decrease in the ability of Rho to serve as a substrate for ADP-ribosylation was shown to precede *Clostridium difficile* toxin-induced morphological changes, suggesting a cause and effect relationship between these events.[74] Inhibition of the C3-catalyzed ADP-ribosylation of Rho proteins by toxin A and B was also observed in a cell-free system, but this effect was strictly dependent on the presence of a cytosolic factor (see below). Gel electrophoresis studies demonstrated that Rho proteins treated with *Clostridium difficile* toxin migrated more slowly on nondenaturing gels compared to control Rho.[74-77] Altogether, these data suggested that *Clostridium difficile* toxins induce a covalent modification of Rho that depends on a low M_r molecule as a cosubstrate.[78,79] By means of electrospray-mass-spectrometry it was demonstrated that toxin B[78] as well as toxin A[79] increase the molecular mass of recombinant Rho exactly by 162 Da, consistent with the incorporation of hexose into this small G protein (Fig. 23.1). Further studies identified UDP-glucose as the essential cosubstrate involved in the modification of Rho proteins by toxins A and B. In contrast to the C3-like transferases that ADP-ribosylate only RhoA, B and C, the *Clostridium difficile* toxins glucosylate all members of the Rho subfamily, i.e., Rho, Rac and Cdc42. However, other monomeric proteins, including Ras, Rab, Arf or Ran or heterotrimeric G proteins, are not modified by these toxins. One mol of glucose per mol of GTPase is incorporated by the *Clostridium difficile* toxins indicating a monoglucosylation reaction. In addition to the glucosyltransferase activity, the *Clostridium difficile* toxins also possess UDP-glucose hydrolase activity, i.e., in the absence of a protein substrate, they cleave UDP-glucose into UDP and glucose. However, the hydrolase activity is 10-to 100-fold lower than the glucosyltransferase activity. This

is similar to the ratio of NAD glycohydrolase activity to ADP-ribosyltransferase activity that has been reported for the ADP-ribosylating toxins, e.g., C3 transferases.[49]

Using massspectrometric techniques and site-directed mutagenesis the threonine-37 residue of Rho was identified as the amino acid acceptor for glucosylation by toxins A and B.[78,79] This threonine residue is highly conserved in the effector domain of all monomeric G proteins.[80,81] Threonine-37 is located in the effector region of Rho where coupling with the effector protein takes place. Thus, glucosylation of Rho GTPases at threonine-37/35 inhibits the interaction with their effectors (Fig. 23.2).

Threonine-37 in Rho (equivalent to Thr-35 in Ras, Rac and Cdc42) is involved in the binding of the nucleotide (GDP/GTP) through coordination of the magnesium cation. This region adjacent to this crucial threonine residue undergoes major conformational changes upon activation (switch-I region).[82,83] In the active GTP-bound form, the threonine residue coordinates the Mg^{2+} ion and might participate in the binding of the γ-phosphate of the nucleotide. In the inactive GDP-bound form, the side chain of the threonine residue is directed to the solvent and no longer available for interaction with the cation. Therefore, the GDP-bound form is a superior substrate for glucosylation compared to the GTP-bound form. This explains why the GTPγS-bound form of Rho is not glucosylated by the toxins,[78] whereas the toxins are able to incorporate about 1 mol of glucose per mol GTPase into the GDP-bound form.[79]

Other "Large" Clostridial Cytotoxins

The hemorrhagic and lethal toxins from *Clostridium sordellii* and the α-toxin from *Clostridium novyi* are further members of the family of large clostridial toxins. These toxins are implicated to play pathogenic roles in gas gangrene syndromes. Moreover, *Clostridium sordellii* toxins are thought to be responsible for induction of diarrhea and enterotoxemia in sheep and cattle. The toxins are structurally and functionally related to *Clostridium difficile* toxins. Studies on the enzymatic activities of these toxins showed that they are also glucosyltransferases that modify small GTPases. Whereas the hemorrhagic toxin from *Clostridium sordellii* shares the cosubstrate and substrate specificity with *Clostridium difficile* toxins, α-toxin from *Clostridium novyi* and the lethal toxin from *Clostridium sordellii* exhibit interesting differences in substrate and cosubstrate specificity when compared with toxin A and B. *Clostridium novyi* α-toxin uses UDP-N-acetylglucosamine (UDP-GlcNAc) as cosubstrate but not UDP-glucose (Fig. 23.1).[84] Because α-toxin treatment of Rho inhibited subsequent ADP-ribosylation by *Clostridium difficile* toxins, it is suggested that the G protein is modified at the same acceptor amino acid as found for *Clostridium difficile* toxins. In line with this notion is the finding that the Thr37Ala mutation of Rho is not substrate for α-toxin. The protein substrates (all Rho family proteins) are also identical. Using galactosyltransferase which modifies only GlcNAc-bearing proteins, it was shown that O-GlcN-acylation by α-toxin occurs also in intact cells.[84] In this respect, it is worth noting that endogenous, reversible mono-O-GlcN-acylation of eukaryotic cytosolic proteins has been described recently.[85,86]

Lethal toxin from *Clostridium sordellii* that is 90% similar to *Clostridium difficile* toxin B also uses UDP-glucose as a cosubstrate; however, this toxin differs in its protein substrate specificity.[87,88] *Clostridium sordellii* lethal toxin glucosylates Rac but not Rho. The ability to modify Cdc42 varies between toxins from various strains. Most interestingly, the lethal toxin glucosylates also Ras family proteins including Ras, Rap and Ral. To test whether Ras proteins are substrates in intact cells, the influence of *Clostridium sordellii* lethal toxin on the Ras signal pathway was studied. Ras, which is activated via growth factor receptors (receptor tyrosine kinases), interacts and activates RAF kinase which subsequently activates the MAP kinase cascade by phosphorylation.[89,90] When serum-starved cells were treated with

Clostridium sordellii lethal toxin, addition of epidermal growth factor did not increase MAP kinase (ERK) activity indicating inhibition of Ras and blockage of the signaling pathway downstream of Ras.[87] It is known for some time that the morphology of the cytotoxic effects of lethal toxin differ from those induced by *Clostridium difficile* toxins A and B.[91] Thus, most likely the different protein substrate specificity of *Clostridium sordellii* lethal toxin is responsible for the different morphological changes induced by this toxin compared to *Clostridium difficile* toxins.

Toxins Activating Rho Proteins

Cytotoxic Necrotizing Factors

The cytotoxic necrotizing factors CNF1 and CNF2 which are produced by various pathogenic *Escherichia coli* strains cause multinucleation in culture cells[92-94] and induce necrosis in rabbit skin when injected intradermally.[95,96] CNF is thought to be important for *Escherichia coli* pathogenicity and is found in up to 20% of *Escherichia coli* strains isolated from diarrhea and up to 50% of strains isolated from extraintestinal infections.[97] These protein toxins are 115 kDa proteins of 85% identity and 99% similarity.[98] At the carboxy-terminal end which is suggested to harbor the enzyme activity, the toxins show significant homology with the dermonecrotic toxin (DNT) from *Bordetella bronchiseptica*.[99] Moreover, the amino-terminus exhibits about 27% identity (80% conserved residues) with the amino acid sequence of *Pasteurella multocida* toxin.[99] Because it was observed that CNFs induce massive reorganization of the actin cytoskeleton by actin polymerization and increase the F-actin content of cells, it was suggested that the toxins affect Rho proteins, a hypothesis that was corroborated by the findings that the toxins induce changes in the migration of Rho on SDS-PAGE.[95,96] Very recently, the molecular mechanism by which CNF affects Rho has been elucidated. Schmidt and co-workers observed that treatment of RhoA in the presence of CNF1 causes inhibition of the intrinsic GTPase activity and blocks the stimulation of GTP hydrolysis by p50 Rho-GAP. Mass spectrometric analysis showed that CNF induced the increase in mass of the Rho peptide covering amino acids Gln52 through Arg68 exactly by 1 Da. Sequencing of this peptide revealed that a glutamine residue in position 63 was changed to glutamic acid. Thus, CNF1 causes a deamidation of Gln63 of RhoA resulting in a mutant Gln63Glu RhoA protein (Fig. 23.1).[100,101] Gln63 of Rho is essential for GTPase activity of the G protein. Therefore, deamidation of this amino acid residue inhibits basal and GAP-stimulated GTPase activity and, thereby, turns Rho into a dominant active protein (Fig. 23.2). Data available suggest also that other Rho family proteins (e.g., Cdc42) are substrates for deamidation by CNF1.[100] Thus activation of various Rho family proteins may be involved in formation of the CNF1-induced phenotype, which cannot be explained only by activation of RhoA.

Dermonecrotic Toxin

Various *Bordetella* species produce dermonecrotic toxins (DNTs) that show similar biological and immunological properties.[99,102,103] DNT is a heat-labile protein toxin of 154 kDa that causes dermonecrotic lesions when injected intradermally and is lethal after i.v. injection. DNT is thought to be a virulence factor for porcine atrophic rhinitis. The genes for DNT from *Bordetella bronchiseptica* and *Bordetella pertussis* are 99% identical and show significant similarity to the cytotoxic necrotizing factors (CNFs) from *Escherichia coli*.[103] The toxin stimulates DNA synthesis but inhibits cell division leading to multinucleated cells. DNT stimulates actin filament assembly and formation of focal adhesions as reported for CNFs. In fact, DNT treatment causes changes in the migration of Rho on SDS-PAGE and

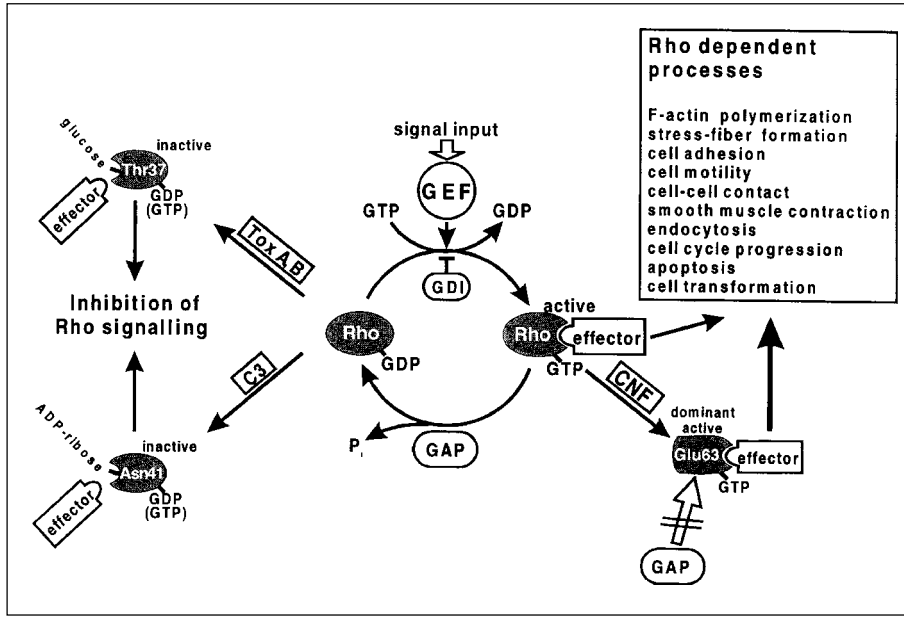


Fig. 23.2. GDP exchange factors (GEF) activate Rho-GTPases. Activation is blocked by GDP dissociation inhibitors (GDI). GTPase activating proteins (GAP) terminate the active state of Rho family proteins by stimulation of GTP hydrolysis. Rho are molecular switches in diverse signal processes (box). *Clostridium botulinum* ADP-ribosyltransferase C3 and the large clostridial cytotoxins (ToxA, B) inactivate Rho GTPases. The cytotoxic-necrotizing factors CNF from *Escherichia coli* deamidate Rho at glutamine-63 thereby activating the Rho.

recent studies indicate that DNT also modifies Rho by deamidation (Horiguchi et al, personal communication).

Conclusions

Rho GTPases are molecular switches in diverse signal processes. Therefore, these key regulators are perfect targets for bacterial toxins that inactivate proteins by ADP-ribosylation or monoglucosylation. Moreover, recent studies have shown that toxins also act on Rho family proteins by persistently activating these molecular switches. The bacterial toxins are not only important as virulence factors. They are also widely used as cell biological tools. Thus, the availability of C3 was most important in the rapid elucidation of the functional role of Rho. In comparison with C3-like transferases, the cell accessibility of large clostridial cytotoxins is much better, a fact that facilitates usage as a biological tool. However, this advantage is counterbalanced by the broader protein substrate specificity.

Rho family modifying toxins may have potential relevance as therapeutic agents because the GTPases they modify are essential for transformation of cells[39,104,105] and/or for control of invasiveness of tumor cells.[106-108] Moreover, the findings that the lethal toxin of *Clostridium sordellii* inactivates oncogenic Ras mutants found in a large number of tumors may have implications for future therapeutic usage of the toxin to inhibit cell transformation, metastasis and invasiveness of tumor cells.

References

1. Aktories K, Mohr C, Koch G. *Clostridium botulinum* C3 ADP-ribosyltransferase. Curr Top Microbiol Immunol 1992; 175:115-31.
2. Aktories K, Braun U, Habermann B et al. Botulinum ADP-ribosyltransferase C3. In: Moss J, Vaughan M, eds. ADP-Ribosylating Toxins and G Proteins. Washington: American Society for Microbiology, 1990:97-115.
3. Aktories K. Clostridial ADP-ribosylating toxins: Effects on ATP and GTP-binding proteins. Mol Cell Biochem 1994; 138:167-76.
4. Aktories K, Koch G. Modification of actin and of Rho proteins by clostridial ADP-ribosylating toxins. In: Iglewski B, Moss J, Tu AT, Vaughan M, eds. Microbial Toxins and Virulence Factors in Desease. New York, Basal: Marcel Dekker, 1995:491-520.
5. Aktories K, Koch G. *Clostridium botulinum* ADP-ribosyltransferase C3. In: Aktories K, ed. Bacterial Toxins. Weinheim: Chapman & Hall, 1997:61-9.
6. Aktories K, Just I. In vitro ADP-ribosylation of Rho by bacterial ADP-ribosyltransferases. Methods Enzymol 1995; 256:184-95.
7. Von Eichel-Streiber C, Boquet P, Sauerborn M et al. Large clostridial cytotoxins—a family of glycosyltransferases modifying small GTP-binding proteins. Trends Microbiol 1996; 4:375-82.
8. Aktories K, Just I. Monoglucosylation of low-molecular-mass GTP-binding Rho proteins by clostridial cytotoxins. Trends Cell Biol 1995; 5:441-3.
9. Moncrief JS, Lyerly DM, Wilkins TD. Molecular biology of the C*lostridium difficile* toxins. In: Rood JI, McClane BA, Songer JG, Titball RW, eds. The Clostridia: Molecular Biology and Pathogenesis. San Diego: Academic Press, 1997:369-92.
10. Lyerly DM, Krivan HC, Wilkins TD. *Clostridium difficile*: Its disease and toxins. Clin Microbiol Rev 1988; 1:1-18.
11. Lyerly DM, Wilkins TD. *Clostridium difficile*. In: Blaser MJ, Smith PD, Ravdin JI et al, eds. Infections of the Gastrointestinal Tract. New York: Raven Press, 1995:867-91.
12. Nobes C, Hall A. Regulation and function of the Rho subfamily of small GTPases. Curr Opin Genet Dev 1994; 4:77-81.
13. Machesky LM, Hall A. Rho: A connection between membrane receptor signalling and the cytoskeleton. Trends in Cell Biology 1996; 6(8):304-10.
14. Narumiya S. The small GTPase Rho: Cellular functions and signal transduction. J Biochem (Tokyo) 1996; 120(2):215-28.
15. Ridley AJ. Rho: Theme and variations. Curr Biol 1996; 6:1256-64.
16. Denhardt DT. Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: The potential for multiplex signalling. Biochem J 1996; 318(3):729-47.
17. Takai A, Sasaki T, Tanaka K et al. Rho as a regulator of the cytoskeleton. Trends Biochem Sci 1995; 20:227-31.
18. Symons M. Rho family GTPases: The cytoskeleton and beyond. Trends Biochem Sci 1996; 21(5):178-81.
19. Ridley AJ. Rho-related proteins: Actin cytoskeleton and cell cycle. Curr Opin Genet Dev 1995; 5:24-30.
20. Price LS, Norman JC, Ridley AJ et al. The small GTPases Rac and Rho as regulators of secretion in mast cells. Curr Biol 1995; 5:68-73.
21. Fukumoto Y, Kaibuchi K, Hori Y et al. Molecular cloning and characterization of a novel type of regulatory protein (GDI) for the *rho* proteins, *ras* p21-like small GTP-binding proteins. Oncogene 1990; 5:1321-8.
22. Feig LA. Guanine-nucleotide exchange factors: A family of positive regulators of Ras and related GTPases. Curr Opin Cell Biol 1994; 6:204-11.
23. Ridley AJ, Paterson HF, Johnston CL et al. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 1992; 70:401-10.

24. Kozma R, Ahmed S, Best A et al. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol Cell Biol 1995; 15:1942-52.
25. Nobes CD, Hall A. Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell 1995; 81:53-62.
26. Tominaga T, Sugie K, Hirata M et al. Inhibition of PMA-induced, LFA-1-dependent Lymphocyte aggregation by ADP-ribosylation of the small molecular weight GTP binding protein, *rho*. J Cell Biol 1993; 120:1529-37.
27. Laudanna C, Campbell JJ, Butcher EC. Role of Rho in chemoattractant-activated leukocyte adhesion through integrins. Science 1996; 271:981-3.
28. Udagawa T, McIntyre BW. ADP-ribosylation of the G protein Rho inhibits integrin regulation of tumor cell growth. J Biol Chem 1996; 271:12542-8.
29. Zhang J, King WG, Dillon S et al. Activation of platelet phosphatidylinositide 3-kinase requires the small GTP-binding protein Rho. J Biol Chem 1993; 268:22251-4.
30. Chong LD, Traynor-Kaplan A, Bokoch GM et al. The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. Cell 1994; 79:507-13.
31. Malcolm KC, Ross AH, Qiu R-G et al. Activation of rat liver phospholipase D by the small GTP-binding protein RhoA. J Biol Chem 1994; 269:25951-4.
32. Kuribara H, Tago K, Yokozeki T et al. Synergistic activation of rat brain phospholipase D by ADP-ribosylation factor and *rho*A p21, and its inhibition by *Clostridium botulinum* C3 exoenzyme. J Biol Chem 1995; 270:25667-71.
33. Siddiqi AR, Smith JL, Ross AH et al. Regulation of phospholipase D in HL60 cells. J Biol Chem 1995; 270:8466-73.
34. Schmalzing G, Richter HP, Hansen A et al. Involvement of the GTP binding protein Rho in constitutive endocytosis in *Xenopus laevis* oocytes. J Cell Biol 1995;130:1319-32.
35. Lamaze C, Chuang TH, Terlecky LJ et al. Regulation of receptor-mediated endocytosis by Rho and Rac. Nature 1996; 382(6587):177-9.
36. Minden A, Lin A, Claret F-X et al. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell 1995; 81:1147-57.
37. Olson MF, Ashworth A, Hall A. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G_1. Science 1995; 269:1270-2.
38. Moorman JP, Bobak DA, Hahn CS. Inactivation of the small GTP binding protein Rho induces multinucleate cell formation and apoptosis in murine T lymphoma EL4. J Immunol 1996; 156:4146-53.
39. Khosravi-Far R, Solski PA, Clark GJ et al. Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol Cell Biol 1995; 15:6443-53.
40. Abo A, Pick E, Hall A et al. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac. Nature 1991; 353:668-70.
41. Bokoch GM. Regulation of the phagocyte respiratory burst by small GTP-binding proteins. Trends Cell Biol 1995; 5:109-13.
42. Aktories K, Weller U, Chhatwal GS. *Clostridium botulinum* type C produces a novel ADP-ribosyltransferase distinct from botulinum C2 toxin. FEBS Lett 1987; 212:109-13.
43. Aktories K, Rösener S, Blaschke U et al. Botulinum ADP-ribosyltransferase C3. Purification of the enzyme and characterization of the ADP-ribosylation reaction in platelet membranes. Eur J Biochem 1988; 172:445-50.
44. Adam-Vizi V, Rösener S, Aktories K et al. Botulinum toxin-induced ADP-ribosylation and inhibition of exocytosis are unrelated events. FEBS Lett 1988; 238:277-80.
45. Rösener S, Chhatwal GS, Aktories K. Botulinum ADP-ribosyltransferase C3 but not botulinum neurotoxins C1 and D ADP-ribosylates low molecular mass GTP-binding proteins. FEBS Lett 1987; 224:38-42.
46. Popoff MR, Hauser D, Boquet P et al. Characterization of the C3 gene of *Clostridium botulinum* types C and D and its expression in *Escherichia coli*. Infect Immun 1991; 59:3673-9.

47. Nemoto Y, Namba T, Kozaki S et al. *Clostridium botulinum* C3 ADP-ribosyltransferase gene. J Biol Chem 1991; 266:19312-9.
48. Just I, Mohr C, Schallehn G et al. Purification and characterization of an ADP-ribosyltransferase produced by *Clostridium limosum*. J Biol Chem 1992; 267:10274-80.
49. Just I, Selzer J, Jung M et al. Rho-ADP-ribosylating exoenzyme from *Bacillus cereus*-purification, characterization and identification of the NAD-binding site. Biochemistry 1995; 34:334-40.
50. Sugai M, Enomoto T, Hashimoto K et al. A novel epidermal cell differentiation inhibitor (EDIN): Purification and characterization from *Staphylococcus aureus*. Biochem Biophys Res Commun 1990; 173:92-8.
51. Inoue S, Sugai M, Murooka Y et al. Molecular cloning and sequencing of the epidermal cell differentiation inhibitor gene from *Staphylococcus aureus*. Biochem Biophys Res Commun 1991; 174:459-64.
52. Rubin EJ, Gill DM, Boquet P et al. Functional modification of a 21-Kilodalton G protein when ADP-ribosylated by exoenzyme C3 of *Clostridium botulinum*. Mol Cell Biol 1988; 8:418-26.
53. Stasia M-J, Jouan A, Bourmeyster N et al. ADP-ribosylation of a small size GTP-binding protein in bovine neutrophils by the C3 exoenzyme of *Clostridium botulinum* and effect on the cell motility. Biochem Biophys Res Commun 1991; 180:615-22.
54. Wiegers W, Just I, Müller H et al. Alteration of the cytoskeleton of mammalian cells cultured in vitro by *Clostridium botulinum* C2 toxin and C3 ADP-ribosyltransferase. Eur J Cell Biol 1991; 54:237-45.
55. Paterson HF, Self AJ, Garrett MD et al. Microinjection of recombinant $p21^{rho}$ induces rapid changes in cell morphology. J Cell Biol 1990; 111:1001-7.
56. Aktories K, Braun U, Rösener S et al. The rho gene product expressed in *E. coli* is a substrate of botulinum ADP-ribosyltransferase C3. Biochem Biophys Res Commun 1989; 158:209-13.
57. Chardin P, Boquet P, Madaule P et al. The mammalian G protein rho C is ADP- ribosylated by *Clostridium botulinum* exoenzyme C3 and affects actin microfilament in Vero cells. EMBO J 1989; 8:1087-92.
58. Braun U, Habermann B, Just I et al. Purification of the 22 kDa protein substrate of botulinum ADP-ribosyltransferase C3 from porcine brain cytosol and its characterization as a GTP-binding protein highly homologous to the rho gene product. FEBS Lett 1989; 243:70-6.
59. Sekine A, Fujiwara M, Narumiya S. Asparagine residue in the rho gene product is the modification site for botulinum ADP-ribosyltransferase. J Biol Chem 1989; 264:8602-5.
60. Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992; 70:389-99.
61. Narumiya S, Morii N. *rho* gene products, botulinum C3 exoenzyme and cell adhesion. Cell Signal 1993; 5:9-19.
62. Kelly CP, Pothoulakis C, LaMont JT. *Clostridium difficile* colitis. New England J Med 1994; 330:257-62.
63. Bongaerts GPA, Lyerly DM. Role of toxins A and B in the pathogenesis of *Clostridium difficile* disease. Microb Pathog 1994; 17:1-12.
64. Eichel-Streiber C, Laufenberg-Feldmann R, Sartingen S et al. Comparative sequence analysis of the Clostridium difficile toxins A and B. Mol Gen Genet 1992; 233:260-8.
65. Dove CH, Wang SZ, Price SB et al. Molecular characterization of the *Clostridium difficile* toxin A gene. Infect Immun 1990; 58:480-8.
66. Triadafilopoulos G, Pothoulakis C, O'Brien MJ et al. Differential effects of *Clostridium difficile* toxins A and B on rabbit ileum. Gastroenterology 1987; 93:273-9.
67. Lyerly DM, Lockwood DE, Richardson SH et al. Biological activities of toxins A and B of *Clostridium difficile*. Infect Immun 1982; 35:1147-50.
68. Lyerly DM, Saum KE, MacDonald DK et al. Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect Immun 1985; 47:349-52.
69. Riegler M, Sedivy R, Pothoulakis C et al. *Clostridium difficile* toxin B is more potent than toxin A in damaging human colonic epithelium in vitro. J Clin Invest 1995; 95:2004-11.

70. Rothman SW, Brown JE, Diecidue A et al. Differential cytotoxic effects of toxin A and B isolated from *Clostridium difficile*. Infect Immun 1984; 46:324-31.
71. Eichel-Streiber C, Warfolomeow I, Knautz D et al. Morphological changes in adherent cells induced by *Clostridium difficile* toxins. Biochem Soc Trans 1991; 19:1154-60.
72. Ottlinger ME, Lin S. *Clostridium difficile* toxin B induces reorganization of actin, vinculin, and talin in cultures cells. Exp Cell Res 1988; 174:215-29.
73. Fiorentini C, Malorni W, Paradisi S et al. Interaction of *Clostridium difficile* toxin A with cultured cells: cytoskeletal changes and nuclear polarization. Infect Immun 1990; 58:2329-36.
74. Just I, Fritz G, Aktories K et al. *Clostridium difficile* toxin B acts on the GTP-binding protein Rho. J Biol Chem 1994; 269:10706-12.
75. Just I, Richter H-P, Prepens U et al. Probing the action of *Clostridium difficile* toxin B in *Xenopus laevis* oocytes. J Cell Science 1994; 107:1653-9.
76. Just I, Selzer J, Von Eichel-Streiber C et al. The low molecular mass GTP-binding protein Rho is affected by toxin A from *Clostridium difficile*. J Clin Invest 1995; 95:1026-31.
77. Dillon ST, Rubin EJ, Yakubovich M et al. Involvement of Ras-related Rho proteins in the mechanisms of action of *Clostridium difficile* toxin A and toxin B. Infect Immun 1995; 63:1421-6.
78. Just I, Selzer J, Wilm M et al. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. Nature 1995; 375:500-3.
79. Just I, Wilm M, Selzer J et al. The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. J Biol Chem 1995; 270:13932-6.
80. Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: Conserved structure and molecular mechanism. Nature 1991; 349:117-27.
81. Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: A conserved switch for diverse cell functions. Nature 1990; 348:125-32.
82. Kim S-H, Privé GG, Milburn MV. Conformational switch and structural basis for oncogenic mutations of Ras proteins. In: Dickey BF, Birnbaumer L, eds. GTPases in Biology I. Berlin, Heidelberg: Springer-Verlag, 1993:177-94.
83. Wittinghofer A, Pai EF, Goody RS. Structural and mechanistic aspects of the GTPase reaction of H-ras p21. In: Dickey F, Birnbaumer L, eds. GTPases in Biology I. Berlin, Heidelberg: Springer-Verlag, 1993:195-211.
84. Selzer J, Hofmann F, Rex G et al. *Clostridium novyi* a-toxin-catalyzed incorporation of GlcNAc into Rho subfamily proteins. J Biol Chem 1996; 271:25173-7.
85. Hart GW. Dynamic O-GlcNAcytation of nuclear and cytoskeletal proteins. Ann Rev Biochem 1997; 66:315-335.
86. Kreppel LK, Blomberg MA, Hart GW. Dynamic glycosylation of nuclear and cytosolic proteins. J Biol Chem 1997; 272:9308-15.
87. Just I, Selzer J, Hofmann F et al. Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalyzed glucosylation. J Biol Chem 1996; 271:10149-53.
88. Popoff MR, Chaves OE, Lemichez E et al. Ras, Rap, and Rac small GTP-binding proteins are targets for Clostridium sordellii lethal toxin glucosylation. J Biol Chem 1996; 271:10217-24.
89. McCormick F. Activators and effectors of *ras* p21 proteins. Curr Opin Genet Dev 1994; 4:71-6.
90. Satoh T, Nakafuku M, Kaziro Y. Function of Ras as a molecular switch in signal transduction. J Biol Chem 1992; 267:24149-52.
91. Bette P, Oksche A, Mauler F et al. A comparative biochemical, pharmacological and immunological study of clostridium novyi a-toxin, *C. difficile* toxin B and *C. sordellii* lethal toxin. Toxicon 1991; 29:877-87.
92. Caprioli A, Falbo V, Roda LG et al. Partial purification and characterization of an *escherichia coli* toxic factor that induces morphological cell alterations. Infect Immun 1983; 39:1300-6.
93. de Rycke J, González EA, Blanco J et al. Evidence for two types of cytotoxic necrotizing factor in human and animal clinical isolates of *Escherichia coli*. J Clin Microbiol 1990; 28:694-9.

94. Falzano L, Fiorentini C, Donelli G et al. Induction of phagocytic behaviour in human epithelial cells by *Escherichia coli* cytotoxic necrotizing factor type 1. Molecular Microbiology 1993; 9:1247-54.
95. Fiorentini C, Donelli G, Matarrese P et al. *Escherichia coli* cytotoxic necrotizing factor 1: Evidence for induction of actin assembly by constitutive activation of the p21 Rho GTPase. Infect Immun 1995; 63:3936-44.
96. Oswald E, Sugai M, Labigne A et al. Cytotoxic necrotizing factor type 2 produced by virulent *Escherichia coli* modifies the small GTP-binding proteins Rho involved in assembly of actin stress fibers. Proc Natl Acad Sci USA 1994; 91:3814-8.
97. Caprioli A, Falbo V, Ruggeri FM et al. Cytotoxic necrotizing factor production by hemolytic strains of *escherichia coli* causing extraintestinal infections. J Clin Microbiol 1987; 25:146-9.
98. Falbo V, Pace T, Picci L et al. Isolation and nucleotide sequence of the gene encoding cytotoxic necrotizing factor 1 of *Escherichia coli.* Infect Immun 1993; 61:4909-14.
99. Walker KE, Weiss AA. Characterization of the dermonecrotic toxin in members of the genus *Bordetella*.. Infection and Immunity 1994; 62:3817-28.
100. Schmidt G, Sehr P, Wilm M, Selzer J, Mann M, and Aktories K. Deamidation of Gln63 of Rho induced by *Escherichia coli* cytotoxic necrotizing factor 1. Nature 1997; 387725-9.
101. Flatau G, Lemichez E, Gauthier M et al. Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. Nature 1997; 387:729-33.
102. Horiguchi Y, Senda T, Sugimoto N et al. *Bordetella bronchiseptica* dermonecrotizing toxin stimulates assembly of actin stress fibers and focal adhesions by modifying the small GTP-binding protein rho. J Cell Sci 1995; 108:3243-51.
103. Pullinger GD, Adams TE, Mullan PB et al. Cloning, expression, and molecular characterization of the dermonecrotic toxin gene of *Bordetella* spp. Infection and Immunity 1996; 64(10):4163-71.
104. Qiu R-G, Chen J, Kirn D et al. An essential role for Rac in Ras transformation. Nature 1995; 374:457-9.
105. Qiu RG, Chen J, McCormick F et al. A role for Rho in Ras transformation. Proc Natl Acad Sci USA 1995; 92:11781-5.
106. Imamura F, Shinkai K, Mukai M et al. rho-mediated protein tyrosine phosphorylation in lysophosphatidic-acid-induced tumor-cell invasion. Int J Cancer 1996; 65(5):627-32.
107. Michiels F, Habets GGM, Stam JC et al. A role for rac in Tiam1-induced membrane ruffling and invasion. Nature 1995; 375:338-40.
108. Collard JG. Signaling pathways regulated by Rho-like proteins: A possible role in tumor transformation and metastasis (Review). Int J Oncol 1996; 8:131-8.

Chapter 24

Antisense Oligonucleotides Against *Ras*

Brett P. Monia

Introduction

Novel experimental approaches for determining the biological functions of cellular gene products has always been, and remains, one of the most intriguing and controversial subjects in molecular biology. Although significant progress has been achieved in the identification of new genes that are associated with specific diseases, progress has generally been slow in determining the function of these genes in biological processes.

This is largely due to the difficulties associated with identifying relevant methods for determining gene function that offer acceptable specificity and do so in a time- and cost-effective manner. Furthermore, when success has been achieved in demonstrating an essential role for a particular gene product in a disease process, the emergence of novel therapies that target the gene product and effectively treat the disease have been slow. This is largely due to the fact that most molecular or pharmacological approaches that are useful for determining gene function in preclinical models are not suitable as pharmaceutical agents for humans.

The need for novel methods to determine the function of specific gene products is probably best exemplified in the field of signal transduction research. Virtually all diseases can be traced in some way to a defect in cell signaling. The number of regulatory proteins that have been shown or are believed to participate in the transduction of signals from the external environment into and through cells, resulting in cellular responses, are growing at an unprecedented rate. Virtually every signal transduction protein that has been identified to date is just one member of a much larger multigene family that contains multiple isoforms that possess similar but distinct cell signaling functions. The complexities of signal transduction pathways are even further complicated by the fairly recent discovery that individual signaling pathways, once thought of as having unique and exclusive functions in cells, often overlap and "cross-talk" with other pathways, resulting in modulation or redundancy of cell signaling. Thus, due to the rate of discovery of new gene products involved in signal transduction, and the fact that cell signaling proteins are commonly structurally homologous at the amino acid level, the need for novel approaches to rapidly and selectively discriminate between highly related gene family members and inhibit the activity of those gene products to assess biological function is great.

In this overview, I will attempt to summarize the advantages and disadvantages of using antisense oligonucleotide technology as a tool to determine the function of signal transduction proteins and as a novel class of pharmacological agents for treating human disease. Examples in which antisense approaches have been successfully utilized to address cell signaling mechanisms will be addressed. However, the review will focus primarily on the progress

G Proteins, Cytoskeleton and Cancer, edited by Hiroshi Maruta and Kazuhiro Kohama.

that has been made using antisense oligonucleotides to target specific members of the *ras* multigene family.

Antisense Approaches to Study Signal Transduction Pathways

Antisense oligonucleotides represent a new paradigm for the discovery of potent and specific drugs with fewer undesired side effects. The antisense concept derives from an understanding of nucleic acid structure and function and depends on Watson-Crick hybridization mechanisms.[1] Unlike traditional pharmacological approaches that attempt to identify inhibitors of gene product function by targeting proteins, antisense oligonucleotides are designed to specifically modulate the information transfer of a particular gene into protein by hybridizing with and disrupting the function of pre-mRNA and mRNA, thereby preventing the mRNA from being translated (Fig. 24.1).

A number of mechanisms have been demonstrated by which antisense oligonucleotides exert their inhibitory effects on mRNA function. These include inhibition of splicing, inhibition of protein translation, and most commonly, destruction of steady-state mRNA levels through the utilization of RNase H enzymes in cells.[2-7] The exact mechanism by which a particular antisense oligonucleotide acts is usually dependent on the chemical composition of the oligonucleotide and the hybridization site within the target mRNA.[2-7] Since antisense oligonucleotides display extremely high affinity and selectivity for their RNA targets, these compounds have the potential to be far better drugs than classical, small molecular weight chemicals that bind to proteins with relatively low affinity and selectivity. Since virtually every step within cell signaling cascades is governed, not by a single protein with a unique structure, but instead, by a family of highly homologous proteins encoded by multigene families, the specificity that antisense offers to selectively inhibit the expression of highly-related regulatory proteins is an extremely valuable approach to determine the detailed mechanisms of signal transduction processes. Despite the fact that this technology is relatively new, it has already been successfully exploited to address cell signaling mechanisms involving a number of gene families within a variety of signal transduction pathways. Some of these studies are listed in Table 24.1 for reference.

Advantages of Antisense Approaches

Specificity and simplicity are two of the major advantages of the antisense approach for the discovery of novel inhibitors of protein expression. Since antisense oligonucleotides act by targeting virtually any region (including nontranslated sequences) within a pre-mRNA or mRNA, and due to the degeneracy of the genetic code, it is relatively easy to design an antisense compound that specifically inhibits a member of a multigene family.[2-7,29] Furthermore, the synthesis of virtually any class or type of protein can be inhibited through an antisense approach. This includes proteins that are fairly easy to approach using traditional methods (e.g., enzymes and receptors) as well as proteins that are very difficult to obtain inhibitors against (e.g., adaptor proteins, structural proteins). Traditional approaches that design inhibitors to bind to enzyme active sites (e.g., low molecular weight chemicals) or mimic natural ligands for receptors (e.g., dominant negative mutants, peptide antagonists) often fail to demonstrate the desired level of specificity due to the similarity of protein structure amongst different family members, and sometimes nonfamily members. Thus, pharmacological targeting of specific members of multigene families is a logical approach for the utilization of antisense technology. Moreover, with little more than a partial gene sequence, the practitioner of antisense drug discovery can rapidly design, synthesize, and test a series of compounds in cell culture and determine if the target gene is specifically inhibited. A compound thus identified can then be tested in the relevant biological assays to determine the functional consequences of inhibiting the target gene product. The length of

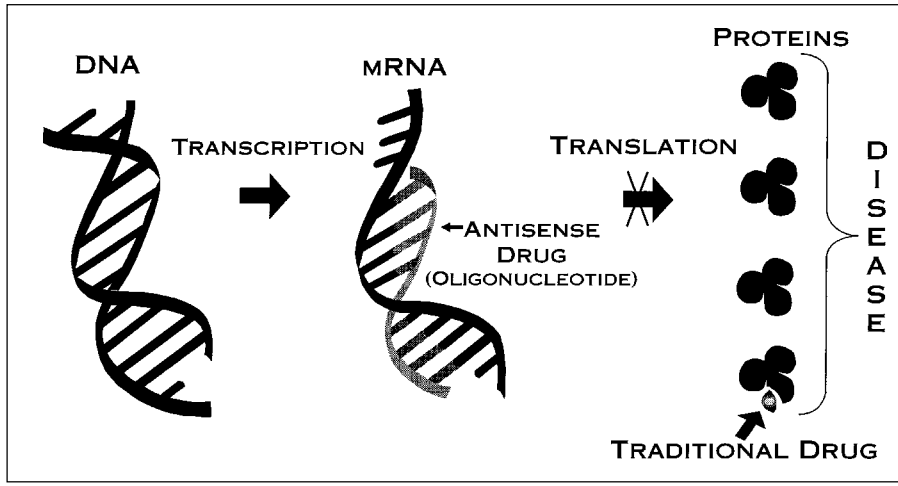


Fig. 24.1. Mechanism of action of antisense oligonucleotides. Antisense oligonucleotides selectively inhibit the synthesis of proteins by hybridizing with a target RNA transcript.

time and the resources required to identify a lead compound by the antisense paradigm is much less than by any other drug discovery method.

The third major advantage of antisense technology is the ability to directly utilize the antisense compound that was selected from cell culture studies in animal models and, ultimately, in humans. Although significant progress has been achieved in the identification of gene products implicated as casual factors in the onset or maintenance of human diseases, the emergence of novel therapies that specifically reverse the effects of these gene products has generally been slow. However, several publications have recently documented that antisense oligonucleotides identified in cellular based assays as inhibitors of gene expression are also effective in animal models of cancer and other diseases in a manner that is highly consistent with an in vivo antisense mechanism of action.[2,9-38] These results indicate that antisense technology has considerable potential both as a tool for the functional validation of specific molecular targets as well as a novel therapeutic approach for the treatment of human disease in the clinic.

Disadvantages of Antisense Approaches

All molecular and pharmacological approaches for determining the function of gene products in biological processes possess certain properties that may limit or prevent their utility as molecular probes under certain conditions. Despite the fact that antisense technology offers a variety of beneficial properties that make them very attractive as tools for determining gene function relative to more traditional approaches, they do possess certain limitations. The first, and probably greatest, limitation to the antisense approach is inherent to its mechanism of action. Since antisense inhibitors target RNA and not protein, biological consequences resulting from inhibition of a particular gene product is dependent on the normal decay rate (i.e., half-life) of the encoded protein product. In most cases, this is not a significant concern in that the majority of proteins in cells possess a normal half-life on the order of a few minutes (e.g., cell cycle proteins) to a day (e.g., *ras* proteins). However, some proteins are very long-lived and may require up to several days to sufficiently reduce steady-state levels of a particular protein product. An example of this is protein kinase C-α.[39]

Table 24.1. Examples in which antisense technology has been employed to determine protein function in signal transduction pathways

Antisense Target	Biological System	Reference
Ha-*ras*, Ki-*ras*	Cell proliferation	Chen et al (1996)[8]
Ha-*ras*, Ki-*ras*	CEA upregulation	Yan et al (1997)[9]
Ha-*ras*, Ki-*ras*	Maturation of $ß_1$ integins	Yan et al (1997)[10]
C-*raf*	PMA stimulation of ERK activity	Schulte et al (1996)[11]
A-*raf*, C-*raf*	Cell proliferation	Cioffi et al (1997)[12] Schumacher et al (1997)[13]
MAP Kinase Phosphatase-1	Angiotensin-II regulation of ERK activity	Duff et al (1995)[14]
PKC-zeta	Angiotensin-II stimulation of ERK activity	Liao et al (1997)[15]
PKC-epsilon	Mechano-sensitive stimulation of ERK activity	Traub et al (1997)[16]
PKC-α	Regulation of ICAM-1 expression	Dean et al (1994)[17]
Bcl-2	Tumor cell apoptosis	Ziegler et al (1997)[18]
$p125^{FAK}$	Tumor cell apoptosis	Xu et al (1996)[19]
c-fos/c-jun	Matrix metalloproteinase induction	McKay and Dean (1997)[20]
$G_{ao,ai}$	Regulation of PLC activity	Chen et al (1995)[21], Quick et al (1994)[22], Kasahara and Sugiyama (1994)[23]
G_{ai2}	Regulation of Ca^{2+} mobilization	Tang et al (1995)[24]
G_{a13}	Regulation of adenylate cyclase activity	deMazancourt et al (1994)[25]
G_{a11}	Regulation of K^+ channel activity	ffrench-Mullen et al (1994)[26]
PLA_2	Prostaglandin biosynthesis	Roshak et al (1994)[27]
ERK1,2	Stimulation of S6 Kinase activity, proliferation	Robinson et al (1996)[28]

Obviously, being forced to wait for such extended periods may have certain practical limitations and may impact the biological responses observed as a result of inhibiting gene expression. Thus, the most attractive antisense targets for determining gene function are those that are either inducible or encode proteins with short to moderate half-lives.

One additional limitation to antisense technology that is inherent to its mechanism of action is the potential inability to inhibit gene function for proteins that are regulated post-translationally. Obviously, if a cell regulates the steady-state levels of a particular protein product by adjusting its protein half-life relative to mRNA production, approaches that inhibit RNA production may be unable to effectively reduce steady-state protein levels. Although this is a purely theoretical concept at present, it demonstrates the importance of considering the possible mechanisms by which cells may compensate post-translationally upon inhibition of mRNA function.

In addition to the inherent limitations of the antisense approach that are described above, one must also realize that certain other limitations may exist when using this approach to determine gene function. The first generation of antisense analogs to be broadly examined for their properties as drugs are the phosphorothioates, where one of the nonbridging phosphoryl oxygens of DNA is substituted with a sulfur. This relatively simple modification results in dramatic improvements in nuclease stability and in the in vitro and in vivo pharmacokinetics.[33,40] However, phosphorothioates have been reported to possess certain nonspecific effects on protein function under certain conditions.[3,6,41,42] To circumvent these problems, newer oligonucleotide modifications have been identified that reduce or eliminate the nonspecific activities associated with phosphorothioates.[42] Typically, this is accomplished using chemistries that leave the natural phospodiester DNA backbone intact while modifying the 2' sugar position, rendering a molecule with increased affinity for RNA and sufficient stability against nucleases. Thus, when possible, it is prudent to utilize newer oligonucleotide chemistries that display a level of specificity that is even greater than that of phosphorothioates.

Finally, one must realize that the selection of the optimal antisense sequence for inhibiting a particular mRNA requires a fairly empirical approach in which a series of oligonucleotides are evaluated for inhibition of target gene expression.[3,6,29] This restriction is due to the inability to predict the optimal hybridization sites within a pre-mRNA sequence which, in turn, is probably due to our inability to accurately predict RNA structure in cells. Thus, the practical aspects of synthesizing and testing a series of oligonucleotides should be considered prior to embarking on an antisense approach against a particular molecular target.

Antisense Approaches to Study G-Protein Function

G-proteins represent a highly diverse group of proteins that are critical for the regulation and flow of information from external stimuli to final destinations within the cell. G-proteins function at critical checkpoints along complex signaling cascades, receiving information from multiple sources and transmitting the information to a variety of effectors. The proper functioning of cells and tissues relies on the fidelity of these signaling cascades. It is widely recognized that many diseases result from a malfunctioning of G-protein regulated cell signaling pathways, including diseases of inflammation, the cardiovascular system and cancer. Therefore, experimental approaches that can determine the roles that individual G-proteins play in normal and diseased conditions are in high demand and novel therapeutic approaches against G-proteins will be extremely valuable for the treatment of a wide range of human diseases.

Although large in number and diverse in nature, G-proteins can be categorized into distinct groupings based on structural and functional similarities.[43,44] At the most superficial level, G-proteins can be classified into two groups. High molecular weight or

heterotrimeric G-proteins, consisting of three subunits termed α, β and γ, and low molecular weight, monomeric G-proteins. Based on functional and structural similarities, G-proteins can be further subdivided into four broad categories, (a) translation factors, (b) heterotrimeric G-proteins involved in transmembrane receptor signaling, (c) *ras* and *ras*-like proteins, and (d) tubulins. Each of these in turn can be further subdivided based on structure and function.

Antisense oligonucleotide approaches have been utilized for determining the function of different G-protein isoforms under normal and diseased conditions for heterotrimeric G-proteins and for *ras* G-proteins. Antisense approaches against heterotrimeric G-proteins have been used to successfully inhibit the expression of multiple isoforms of all three heterotrimeric subunits (α, β, γ), and these studies have led to important insights into the function of these isoforms in a wide variety of cell signaling processes including ion channel gating, phospholipase activation, and regulation of intracellular kinase activity. This subject has been the topic of a very recent and extensive review, and therefore, will not be addressed further here (For a review see ref. 45). This review will summarize the progress that has been made in the application of antisense oligonucleotide technology for the study of the *ras* multigene family and as a novel therapeutic approach for targeting the *ras* oncogene for the treatment of human cancer.

Antisense as a Novel Anticancer Approach Against *Ras*

The discovery of viral oncogenes in the mid 1960s was a major breakthrough in understanding the molecular origins of cancer and led directly to the identification of the first human oncogene, *ras*, in 1982 (For a review see ref. 46). Since then, *ras* has been the focus of intense research that has resulted in the discovery of multiple *ras* isoforms and the elucidation of the mechanisms by which *ras* proteins function in normal cells and promote malignancy in cancer. However, despite intense research on *ras* since 1982, very few anticancer drugs that are known to act by inhibiting *ras* function have entered the clinic for the treatment of human cancer. Furthermore, very little information has been generated on the cellular functions of different *ras* isoforms in cells. The latter point is due mostly to the difficulties associated with generating isoform-specific inhibitors using traditional approaches.

Three different *ras* genes (Ki-*ras*, Ha-*ras*, and N-*ras*) have been identified and characterized in mammalian tissues. *Ras* genes can acquire transforming potential through a number of mechanisms, the best characterized being the acquisition of single base point mutations in their coding regions that result in amino acid substitutions in critical GTP regulatory domains of the protein. These mutations abrogate the normal function of *ras*, thereby converting a normally regulated cell protein to one that is constitutively active. Such deregulation of normal *ras* protein function is believed to contribute to the transforming activity of *ras* gene products. For reviews see refs. 46-49.

Discovery of Ras Antisense Inhibitors

We have focused our attention initially on the discovery of antisense inhibitors against the human Ha-*ras* and Ki-*ras* isoforms. To identify antisense oligonucleotides capable of inhibiting expression of these isoforms, a series of phosphorothioate oligodeoxynucleotides were designed and tested for inhibition of the appropriate *ras* isotype.[33] In both cases, oligonucleotides 15 to 20 bases in length were targeted to mRNA sequences comprising the 5'-untranslated regions, coding regions (including codons 12 and 61), and the 3' untranslated regions. Two cell lines were chosen for these studies: the T24 bladder carcinoma cell line, which expresses a mutation-bearing Ha-*ras* mRNA (codon-12, GGC → GTC), and the SW480 colon carcinoma cell line, which expresses a mutant Ki-*ras* mRNA (codon-12,

GGT→GTT).[50,51] Cells were treated with oligonucleotides at a concentration of 200 nM in the presence of cationic lipid to enhance cell uptake efficiency in vitro.[52] Inhibition of Ha-*ras* and Ki-*ras* mRNA expression was observed for only a subset of the oligonucleotides that were tested (Fig. 24.2). The degree of inhibition of the two different *ras* gene products varied depending on the mRNA target site and the particular *ras* message. For example, the 5'-untranslated region, including the AUG site of Ha-*ras* mRNA, was very sensitive to inhibition with antisense oligonucleotides, whereas oligonucleotides targeted to the 3'-untranslated region of this message were without effect. In contrast, oligonucleotides targeted to the AUG site of Ki-*ras* mRNA were poor inhibitors of Ki-*ras* expression whereas the 5'-untranslated region was very sensitive to antisense activity. Interestingly, for both target mRNAs, oligonucleotides designed to hybridize with codons 12 or 61 were effective in inhibiting expression of the respective mRNA targets, suggesting that mutant-specific inhibition of *ras* mRNA expression is feasible. In subsequent studies, we have taken a similar approach for the discovery of antisense inhibitors against the human N-*ras* isoform (L. Cowsert, unpublished results). In these experiments, we have found that the most effective antisense inhibitors against N-*ras* were targeted to the 3'untranslated region of the N-*ras* mRNA.

In addition to measuring the effects of antisense oligonucleotides on *ras* mRNA levels, we have also demonstrated that these compounds are effective inhibitors of *ras* protein synthesis and reduce steady-state levels of *ras* protein in cells.[8,57] As expected based on the predicted half-life of *ras* proteins in cells,[44] a 50% reduction of steady-state *ras* protein levels following initiation of oligonucleotide treatment requires a period of time between 12 and 20 hours. These results are remarkably consistent from cell type to cell type.

Specificity of Ras *Antisense Inhibition*

The structures of the three *ras* isotypes (Ha-*ras*, Ki-*ras*, and N-*ras*) at the protein level are virtually identical throughout the protein, except for a short region at the carboxy terminus.[44] Thus, protein targeting drugs which can selectively target different *ras* isozymes without affecting the function of other G-proteins have not been described. However, because of the redundancy of the genetic code and the presence of noncoding (untranslated) sequences, highly related proteins are often encoded by highly diverged mRNA sequences. Therefore, it should be possible to design antisense inhibitors to block expression of one particular isotype with minimal consequence to related isotypes.

To demonstrate isotype-specific inhibition of *ras* gene expression using antisense technology, oligonucleotides that were specifically designed to hybridize with either the Ha-*ras* mRNA or the Ki-*ras* mRNA were evaluated for isotype-specificity. ISIS 2503, an active 20 base phosphorothioate targeted to the Ha-*ras* mRNA AUG region, is complementary to the AUG region of the Ki-*ras* message in only 9 of 20 bases and, therefore, would not be expected to bind efficiently to Ki-*ras* mRNA.[53] Similarly, ISIS 6957, an active 20-base phosphorothioate targeted to the 5'-untranslated region of Ki-*ras* mRNA is complementary to the Ha-*ras* mRNA in only 4 of 20 bases, and therefore should not affect Ha-*ras* mRNA expression.[33] Cells treated with each of these oligonucleotides were analyzed for Ha-*ras* and Ki-*ras* mRNA expression by Northern analysis. ISIS 2503 reduced Ha-*ras* mRNA to virtually undetectable levels without affecting Ki-*ras* mRNA levels, whereas ISIS 6957 inhibited Ki-*ras* mRNA expression without affecting Ha-*ras* mRNA levels (Fig. 24.3). We have also demonstrated isotype-specific reduction of Ha-*ras* and Ki-*ras* protein levels for these oligonucleotides (Fig. 24.3).

Ras genes often acquire their tumor-promoting properties by single base point mutations in their coding regions.[44] Since the function of normal *ras* isotypes may be important for normal cell survival, inhibition of expression of the mutated *ras* gene in tumors may be

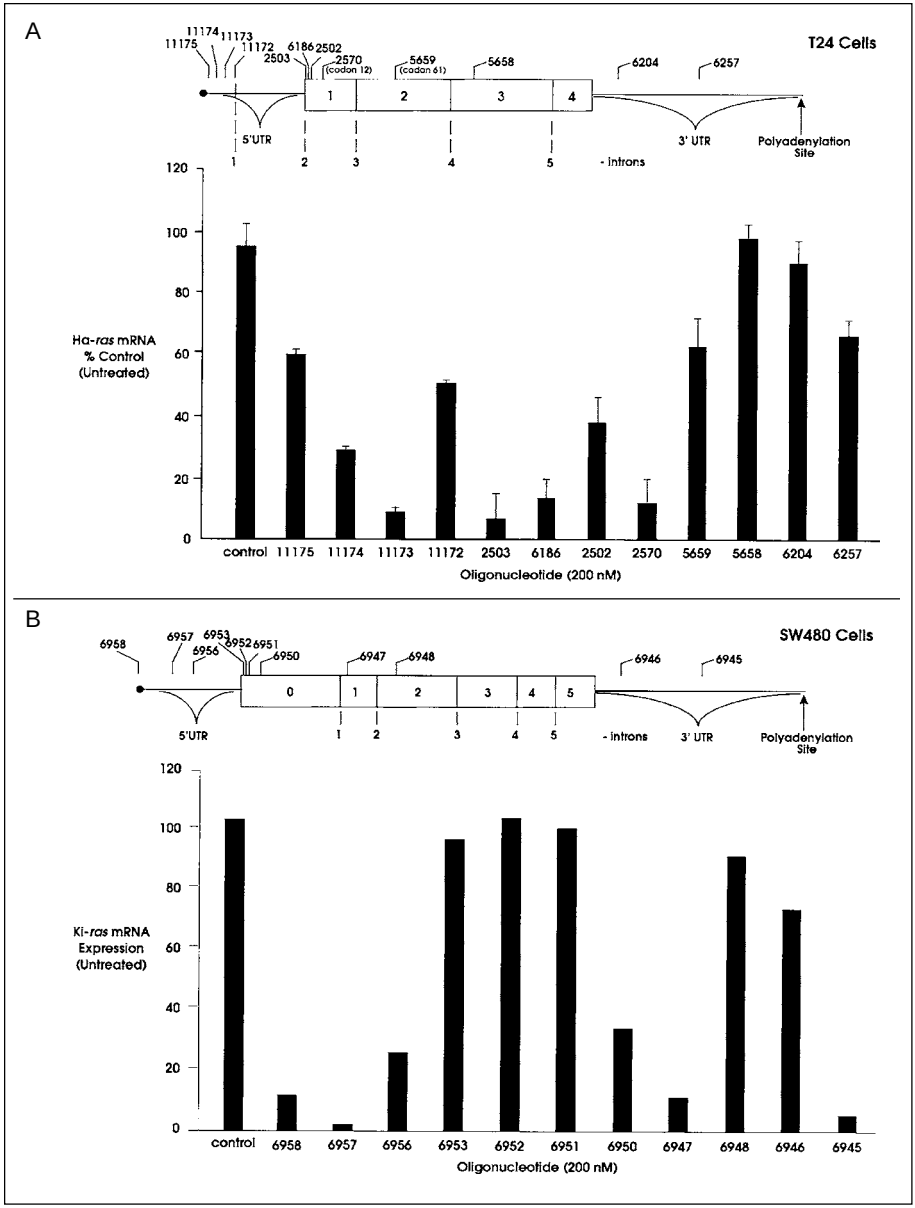


Fig. 24.2. Reduction of Ha-*ras* and Ki-*ras* mRNA levels in tumor cells following treatment with the indicated phosphorothioate antisense oligonucleotides. A, T24 bladder carcinoma cells were treated with oligonucleotides (200 nm) and Ha-*ras* mRNA levels were determined 24 hours later by northern blot analysis. B, SW480 colon carcinoma cells were treated with oligonucleotides (200 nm) and Ki-*ras* mRNA levels were determined 24 hours later by northern blot analysis. Relative positioning of the predicted hybridization sites of the oligonucleotides within Ha- and Ki-*ras* mRNAs is indicated schematically. For both targets, mRNA levels are expressed as a percentage of the levels of the target mRNA in untreated control cells. Target mRNA levels were analyzed, quantified and normalized as previously described.

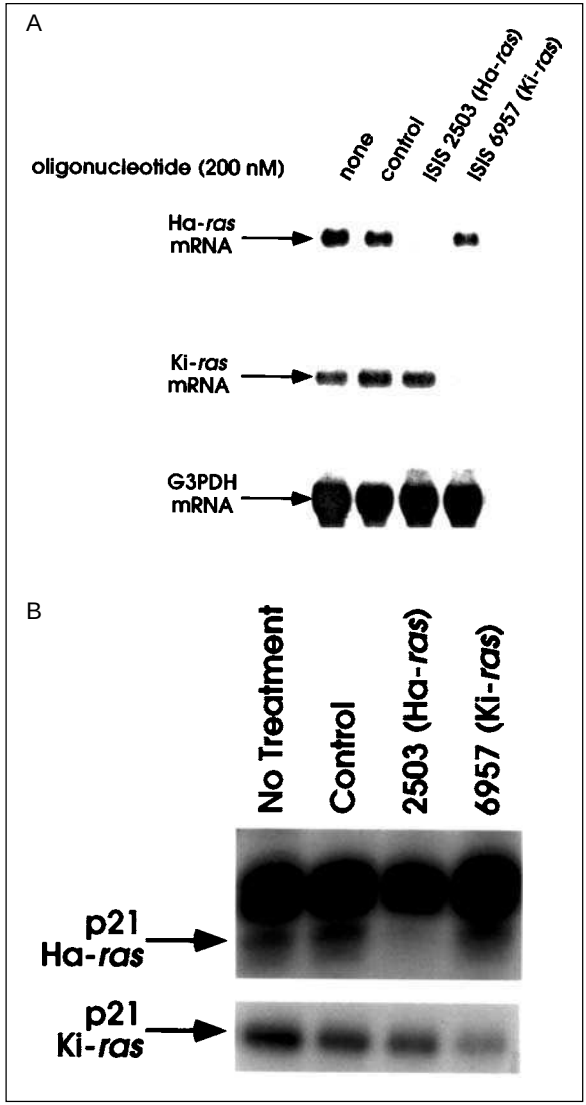


Fig. 24.3. Isoform-specific inhibition of *ras* mRNA and protein expression in tumor cell lines. T24 cells were treated with the indicated Ha-*ras*-specific (ISIS 2503) or Ki-*ras*-specific (ISIS 6957) antisense oligonucleotide (200 nM) and cell lysates were analyzed for Ha-*ras* and Ki-*ras* mRNA levels 30 hours post-treatment. A, Northern blot analysis of Ha-*ras* and Ki-*ras* mRNA levels. B, Western blot analysis of Ha-*ras* and Ki-*ras* protein levels using isoform-specific antisera.

preferred without affecting expression of the nonmutated *ras* isotypes. Helene and co-workers have demonstrated inhibition of a mutant form of Ha-*ras* using a 9-base phosphodiester antisense oligonucleotide linked to an acridine intercalating agent.[54] Chang and co-workers have also demonstrated selective targeting of a mutant Ha-*ras* message in which a mutation at codon 61 was targeted and methylphosphonate oligodeoxynucleotides were employed.[55] Studies from our laboratory have demonstrated similar antisense specificity targeting the Ha-*ras* EJ bladder carcinoma point mutation (GGC→GTC) at codon 12 using phosphorothioate oligodeoxynucleotides.[53] In our studies, we demonstrated that mutation-specific inhibition can be achieved with phosphorothioate oligodeoxynucleotides, but that oligonucleotide affinity and concentration were critical to maintaining the selectivity. Oligonucleotides targeted to codon 12 ranging in length between 5 and 25 bases targeted to

Ha-*ras* codon 12 were tested for overall activity and point mutation selectivity. Oligonucleotides < 15 bases in length were inactive, whereas all oligonucleotides greater in length displayed good activity with potency correlating directly with oligonucleotide chain length (affinity). However, selective inhibition of mutant Ha-*ras* expression did not increase with oligonucleotide chain length, but required a specific length between 15 and 19 bases. The maximum selectivity observed for inhibition of mutant Ha-*ras* expression relative to normal Ha-*ras* was achieved with a 17-mer oligonucleotide (ISIS 2570).[53]

Point mutation-specific targeting of Ki-*ras* oncogenes has also been demonstrated using phosphorothioate antisense oligonucleotides.[33] In this study, 15 bases was shown to be the optimal length for selectively targeting a codon 12 mutation (GGT→GTT) within the Ki-*ras* gene of SW480 colon carcinoma cells. Treatment of cells with the 15 base antisense oligonucleotide had no effect on Ki-*ras* mRNA levels in cells expressing nonmutated Ki-*ras* whereas Ki-*ras* mRNA expression was undetectable in SW480 cells. Furthermore, no inhibition of Ha-*ras* gene expression was observed in SW480 cells following treatment with the Ki-*ras* 15 base oligonucleotide. These types of studies demonstrate that point mutation-specific and isoform-specific inhibition of both Ha-*ras* and Ki-*ras* mRNA expression in tissue culture is possible through the use of properly designed antisense oligonucleotides.

Cellular Responses Resulting from Inhibition of Ras *Gene Expression Using Antisense Oligonucleotides*

Based on the generally accepted function of the MAP kinase signaling pathway in the transduction of extracellular signals that promote cellular proliferation and survival, the expected outcome of inhibiting *ras* expression using antisense oligonucleotides is modulation of downstream kinase and transcriptional activity, and attenuation of cellular proliferation and/or promotion of cell death, provided that inhibiting a single isotype of the *ras* multigene family is sufficient to promote these types of responses. We and others have investigated these types of effects in various nontransformed and tumor cell lines and have found that inhibition of a single *ras* isoform in cells is sufficient to abrogate downstream cell signaling pathways and impede cellular proliferation.[8,33,54-56] Moreover, we have generated results supporting the conclusion that different *ras* isoforms possess unique functions in cells that often appear to be cell-type specific.

We have measured the ability of *ras* antisense inhibitors to block stimulation of ERK activity in response to specific stimuli, and to modulate activation of specific transcription units. As shown in Figure 24.4, ERK stimulation by PDGF, angiotensin II, and TGFβ can be blocked either partially or completely in vascular smooth muscle cells following treatment with an isoform-specific antisense inhibitor against Ha-*ras*. However, Ha-*ras* inhibition does not affect the ability of phorbol ester to stimulate ERK activity in these cells. Interestingly, antisense inhibition of Ki-*ras* in these cells also blocked PDGF and TGFβ stimulation of ERK activity, but did not affect the degree of ERK stimulation by angiotensin II. Similar to the effects displayed by the Ha-*ras* inhibitor, Ki-*ras* inhibition did not affect phorbol ester-mediated stimulation of ERK in vascular smooth muscle cells. These results are consistent with the conclusion that, at least for the cells described above, protein kinase-C stimulation of ERK activity occurs in a *ras*-independent manner. We have made similar observations in other cell types on the ability of *ras* antisense inhibitors to block transcriptional activation of specific genes (e.g., C-fos, cell adhesion molecules) in response to growth factors, cytokines and phorbol esters (B. Monia, unpublished results). These results demonstrate that, at least for some cell signaling pathways, different *ras* isoforms possess unique functions in signal transduction.

The antiproliferative effects of *ras* antisense inhibitors have been investigated by a number of groups and it have been shown that these inhibitors can block the proliferation of a

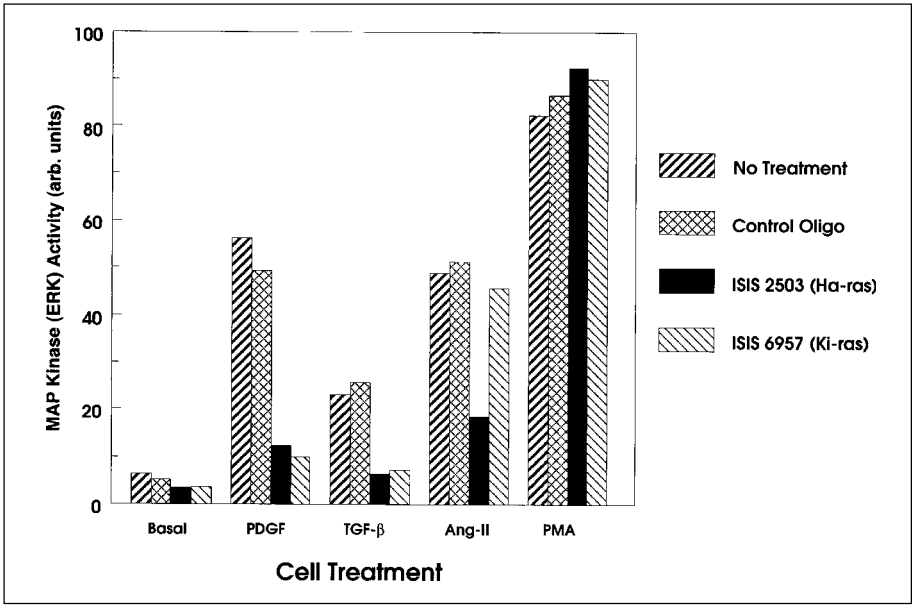


Fig. 24.4. Inhibition of MAP kinase stimulation by antisense oligonucleotides targeted to Ha-*ras* and Ki-*ras.* Serum-starved vascular smooth muscle cells were treated with the indicated Ha-*ras* or Ki-*ras* antisense oligonucleotides, a control (scrambled) oligonucleotide, or were left untreated. 30 hours following oligonucleotide treatment, cells were stimulated with the indicated agents for 10 minutes and analyzed for MAP kinase activity against myelin basic protein substrate using a ERK1/ERK2 immunoprecipitation assay. Quantitation was by phosphorimage analysis.

variety of tumor types in cell culture.[8,33,54-56] We have previously reported that ISIS 2503, a phosphorothioate antisense inhibitor against human Ha-*ras,* inhibits the proliferation of the Ha-*ras* transformed T24 bladder carcinoma line in a dose-dependent and oligonucleotide sequence-specific manner, displaying an IC_{50} for this effect that is very similar to the IC_{50} for inhibiting Ha-*ras* mRNA and protein expression.[8,33,56] We have also demonstrated apoptotic responses in T24 cells in vitro following administration of ISIS 2503. Again, these effects are highly sequence-specific and correlate well with reduction of target gene expression. In general, we observe that the time required to induce apoptosis by these oligonucleotides is significantly longer that the time required to inhibit proliferation (B. Monia, unpublished studies).

Our results on T24 cell proliferation are consistent with a recent report demonstrating the antiproliferative effects of ISIS 2503 against tumor cell lines in vitro.[8] Interestingly, it was also shown in that report that a Ki-*ras* antisense inhibitor (ISIS 6957, described above) displays no significant antiproliferative effects against the T24 tumor line, but is a potent antiproliferative agent against normal diploid fibroblasts whereas the Ha-*ras* oligonucleotide has no effects on proliferation against this cell type. Neither oligonucleotide affected the proliferation of a bladder carcinoma tumor line (J-82) that does not contain a *ras* mutation. It will be interesting to determine whether a N-*ras* specific antisense inhibitor, or a combination of isotype-specific *ras* inhibitors, can block proliferation of this tumor cell line. Similar isotype-specific antiproliferative effects have been observed against other cell lines using *ras* antisense inhibitors (B. Monia, unpublished results).

Antisense inhibitors against specific *ras* family members have been used to demonstrate additional isoform-specific functions for these proteins. For example, Yan et al has recently reported that treatment of transformed colon epithelial cells with Ki-*ras* antisense oligonucleotides can prevent the upregulation of carcinoembryonic antigen (CEA), an important marker of malignancy, in human colon cancer, whereas a Ha-*ras* antisense inhibitor does not affect CEA upregulation.[9] In a related study, the same group of investigators have employed antisense technology to demonstrate that Ki-*ras* (but not Ha-*ras*) is critical for the proper maturation (glycosylation) of β_1 integrins in colon epithelial cells.[10] Thus, antisense targeting of *ras* family members appears to be a viable approach for determining the roles of different *ras* isoforms in tumor cell signaling, transformation, tumor progression, and proliferation.

Antitumor Activity of Ras *Antisense Oligonucleotides in Animal Models*

The obvious extension of the types of studies described above is to test the feasibility of using antisense inhibitors against *ras* family members to prevent tumor growth in vivo. A number of studies have recently been reported describing in vivo antitumor effects of antisense oligonucleotides targeted to various cell signaling molecules that are very consistent with an antisense mode of action.[29-31,33-37] One of the important observations that have been made in all of these studies, as well as in other studies using different in vivo models, is that, despite the fact that cationic lipids or other transfection techniques are generally required for efficient oligonucleotide uptake in cell culture, simple saline formulations of oligonucleotides is all that is normally required to produce antisense effects in vivo following systemic administration. Although the mechanisms underlying these observations are not well understood, they obviously indicate that the mechanisms of macromolecular uptake by cells in animals are very different from cellular uptake mechanisms in culture.

Antisense approaches against *ras* isotypes have been successfully employed to prevent the growth of a wide variety of human tumor types in animal models (Table 24.2). Initial studies focused on the utilization of vector-mediated antisense RNA methods designed to inhibit expression of Ki-*ras*.[57-59] Intratracheal delivery of the Ki-*ras* antisense constructs was shown to prevent the growth of human lung tumors in a orthotopic mouse model. These studies demonstrated that Ki-*ras* is essential, not only for initiation of tumor growth, but also for maintenance of the malignant phenotype in this particular tumor model.

Antisense oligonucleotides targeted against *ras* isoforms have also been successfully employed to prevent the growth of human tumors in mouse models (Table 24.2). Activity has been demonstrated against a wide variety of tumor types including tumors that express mutations in Ha-*ras* alleles, Ki-*ras* alleles, or tumors that only express normal (unmutated) *ras*. In all of these studies, appropriate control oligonucleotides were shown to exert little or no antitumor activity, supporting the conclusion that the antitumor activity displayed by these oligonucleotides is through an antisense mechanism of action. One of the most interesting observations from these studies has been the differential sensitivity displayed by different tumor types in vivo against isotype-specific *ras* antisense inhibitors. In some cases, antitumor activity can be demonstrated by targeting either Ha-*ras* or Ki-*ras* suggesting that both isoforms play an essential function in the growth of those particular tumor types. However, other tumor types have been shown to be preferentially sensitive to oligonucleotides targeted to a particular *ras* isoform. For example, antisense oligonucleotides targeted against Ha-*ras* are far more potent than Ki-*ras* antisense oligonucleotides in preventing the growth of MDA-MB231 tumors in mouse xenograft models.[63] Moreover, although isoform-specific tumor sensitivity often correlates with *ras* mutation status (Table 24.2), a number of notable exceptions exist. For example, antisense inhibitors targeted against human Ha-*ras* have been reported to exert potent antitumor effects against tumor types known to contain

Table 24.2. Examples in which antisense approaches against* ras *isoforms have been successfully employed to prevent tumor growth in animals

***ras* Target**	**Tumor**	**Tissue of Origin**	***ras* Genotype**	**Reference**
Ki-*ras*	H460a	Lung	Ki-*ras* mutated	Mukhopadhyay et al (1991)[57]
Ki-*ras*	H460a	Lung	Ki-*ras* mutated	Zhang et al (1993)[58]
Ki-*ras*	H460a	Lung	Ki-*ras* mutated	Georges et al (1993)[59]
Ha-*ras*	Engineered (T24)	Bladder	Ha-*ras* mutated	Gray et al (1993)[60]
Ha-*ras*	T24	Bladder	Ha-*ras* mutated	Schwab et al (1994)[61]
Ha-*ras*	T24 MDA-MB231 A549	Bladder Breast Lung	Ha-*ras* mutated Normal Ki-*ras* mutated	Bennett et al (1995)[3]
Ki-*ras*	A549 SW480	Lung Colon	Ki-*ras* mutated Ki-*ras* mutated	Bennett et al (1995)[3]
Ha-*ras*	BEL-7402	Liver	Normal	Liao et al (1997)[62]
Ha-*ras*	Mia Paca II MDA-MB231 H69 A549 SW620 T24	Pancreas Breast Lung Lung Colon Bladder	Ki-*ras* mutated Normal Unknown Ki-*ras* mutated Ki-*ras* mutated Ha-*ras* mutated	Cowsert (1997)[63]

mutations in Ki-*ras* genes (e.g., Mia Paca II pancreas, A549 lung, SW620 colon). Studies attempting to determine the mechanisms of isotype-specific antitumor activity of *ras* antisense oligonucleotides are underway in which in vitro and in vivo models are being employed. Nevertheless, studies of this nature demonstrate that antisense targeting of specific *ras* family members can produce potent antitumor effects in animals and that antisense compounds may represent a novel class of drugs for the treatment of human malignancies in the clinic.

Perspectives

The studies described in this document demonstrate that antisense inhibitors can be successfully employed for abrogating the function of *ras* signaling pathways both in vitro and in vivo. Furthermore, these studies support the conclusion that antisense is a valuable approach for both target validation and for the discovery of novel therapeutic agents for the treatment of human cancer. This may be best illustrated by the fact that one of the first anticancer drugs to enter the clinic that is specifically designed to inhibit *ras* function in tumors a Ha-*ras* targeted antisense oligonucleotide (B. Monia, unpublished information). This is despite the fact that *ras* has been known to be a critical regulator of human tumorigenesis for over 20 years and that heroic efforts have been made over this period of time attempting to discover novel *ras*-specific anticancer drugs using traditional approaches.

Nevertheless, it is clear that we are only at the earliest stages of understanding and exploiting antisense technology to serve both therapeutics as well as basic research. Some of the important issues that this technology has the potential to particularly address relate to the functions of highly related members of multigene families within cell signaling pathways in both normal and diseased settings. Understanding the function of such pathways will undoubtedly lead to a much more profound understanding of such critical processes as "cross-talk" between seemingly unrelated signaling pathways, tissue-specific and disease-specific functions for signaling proteins, and cellular compensation mechanisms to inhibitors of cell signaling, which will undoubtedly yield important insights to potential mechanisms of drug resistance for cell signaling inhibitors. Thus, antisense technology has the potential to provide the tools for significantly advancing our current understanding of the molecular events which underlie signal transduction processes which will very likely result in a better understanding and therapeutic approach for human disease.

Acknowledgments

The author would like to thank a number of research colleagues who have contributed substantially to the findings and conclusions described in this chapter. Doriano Fabbro of Novartis Pharmaceuticals (Switzerland), Dennis Stacey and Guan Chen of the Cleveland Clinic, and Lex Cowsert and Joseph Johnston of Isis Pharmaceuticals. The author would also like to acknowledge Mary D'Acquisto for her outstanding assistance in the preparation of this chapter.

References

1. Watson JD, Crick FHC. A structure for doexyribose nucleic acid. Nature 1953; 171:737-739.
2. Chiang M-Y, Chan H, Zounes MA et al. Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. J Biol Chem 1991; 266:18162-18171.
3. Crooke ST. Therapeutic applications of oligonucleotides. Annu Rev Pharmacol 1993; 32:329-376.
4. Monia BP, Lesnik E, Gonazalez C et al. Evaluation of 2' modified oligonucleotides containing deoxy gaps as antisense inhibitors of Ha-*ras* gene expression. J Biol Chem 1993; 268:14514-14522.

5. Hodges D, Crooke ST. Inhibition of splicing of wild type and mutated luciferase-adenovirus Pre-mRNAs by antisense oligonucleotides. Molec Pharmacol 1995;48:905-918.
6. Crooke ST, Bennett CF. Progress in antisense oligonucleotide therapeutics. Annu Rev Pharmacol Toxicol 1996; 36:107-127.
7. Baker BF, Lot SS, Condon TP et al. 2'-0-(2-Methoxy) ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. J Biol Chem 1997; 272:11994-12000.
8. Chen G, Oh S, Monia BP et al. Antisense oligonucleotides demonstrate a dominant role of C-Ki-*RAS* proteins in regulating the proliferation of diploid human fibroblasts. J Biol Chem 1996; 271:28259-28265.
9. Yan Z, Deng X, Chen M et al. Oncogenic c-k-*ras*, but not oncogenic c-H-*ras* upregulates CEA expression and disrupts basolateral polarity in colon epithelial cells. J. Biol. Chem. 1997; 272:27902-27907.
10. Yan Z, Chen M, Perucho M et al. Oncogenic K-*ras* but not oncogenic H-*ras* blocks β1 integrin maturation in colon epithelial cells. J Biol Chem 1997; 272:30928-30936.
11. Schulte TW, Blagosklonny MV, Romanova L et al. Destabilization of *Raf*-1 by geldanamycin leads to disruption of the *Raf*-1-MEK-Mitogen-activated protein kinase signaling pathway. Molec Cell Biol 1996; 16:5839-5845.
12. Cioffi CL, Garay M, Johnston JF et al. Selective inhibition of A-*raf* and C-*raf* mRNA expression by antisense oligodeoxynucleotides in rat smooth muscle cells: Role of A-*raf* and C-*raf* in serum-induced proliferation. Mol Pharmacol 1997; 51:383-389.
13. Schumacher C, Sharif H, Haston W et al. Exposure of human vascular smooth muscle cells to *Raf*-1 antisense oligodeoxynucleotides: cellular responses and pharmacodynamic implications. Mol Pharmacol 1997; 51:383-389.
14. Duff JL, Monia BP, Berk BC. Mitogen-activated protein (MAP) kinase is regulated by the MAP kinase phosphatase (MKP-1) in vascular smooth muscle cells. J Biol Chem 1995; 270:7161-7166.
15. Liao DF, Monia BP, Dean N et al. Protein Kinase C-zeta mediated angiotensin II activation of ERK1/2 in vascular smooth muscle cells. J Biol Chem 1997; 272:6146-6150.
16. Traub O, Monia BP, Dean NM et al. PKC-epsilon is required for mechano-sensitive activation of ERK1/2 in endothelial cells. J Biol Chem 1997; 272:31251-31257.
17. Dean NM, McKay R, Condon TP et al. Inhibition of Protein Kinase C-α expression in human A549 cells by antisense oligonucleotides inhibits induction of Intercellular Adhesion Molecule 1 (ICAM-1) mRNA by phorbol esters. J Biol Chem 1994; 269:16416-16424.
18. Ziegler A, Luedke GH, Fabbro D et al. Induction of apoptosis in small-cell lung cancer cells by an antisense oligodeoxynucleotide targeting the Bcl-2 coding sequence. J Natl Cancer Inst 1997; 89:1027-1036.
19. Xu L, Owens LV, Sturge GC et al. Attenuation of the expression of the Focal Adhesion Kinase induces apoptosis in tumor cells. Cell Growth Diffn 1996; 7:413-418.
20. McKay R, Dean NM. Inhibition of metalloproteinase expression in human keratinocytes using antisense oligonucleotide targeting human c-jun. Mol Pharmacol 1998; (in press).
21. Chen Y, Baez M, Yu L. Functional coupling of the 5-Ht_{2C} serotonin receptor to G proteins in Xenopus oocytes. Neurosci Lettr 1995; 179:100-102.
22. Quick MW, Simon MI, Davidson N et al. Differential coupling of G-protein α subunits to seven helix receptors expressed in Xenopus oocytes. J Biol Chem 1994; 269:30164-30172.
23. Kasahara J, Sugiyama H. Inositol phospholipid metabolism in Xenopus oocytes mediated by endogenous G_o and G_i protein. FEBS Lettr 1994; 355:41-44.
24. Tang T, Kiang JG, Cote TE et al. Antisense oligodeoxynucleotides to the G_{i2} protein α subunit sequence inhibits an opioid-induced increase in the intracellular free calcium concentration in ND8-47 neuroblastoma x dorsal root ganglion hybrid cells. Mol Pharmacol 1995; 48:189-193.
25. deMazancourt P, Goldsmith PK, Weinstein LS. Inhibition of adenylate cyclase activity by galanin in rat insulinoma cells is mediated by the G-Protein G_{i3}. Biochem J 1994; 303:369-375.

26. ffrench-Mullen JMH, Plata-Salaman CR, Buckley NJ et al. Muscarinic modulation by a G-protein alpha-subunit of delayed rectifier K^+ current in rat ventromedial hypothalamic neurones. J Physiol 1994; 474:21-26.
27. Roshak A, Sathe G, Marshal LA. Suppression f monocyte 85-kda phospolipase A_2 by antisense and effects on endotoxin-induced prostaglandin biosynthesis. J Biol Chem 1994; 269:25999-26005.
28. Robinson CJM, Scott PH, Allan AB et al. Treatment of vascular smooth muscle cells with antisense phosphorothioate oligodeoxynucleotides directed against p42 and p44 mitogen-activated protein kinases abolishes DNA synthesis in response to platelet-derived growth factor. Biochem J 1996; 320:123-127.
29. Monia BP, Johnston JF, Geiger T et al. Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against C-raf Kinase. Nature Med 1996; 2:668-675.
30. Gray GD, Hernandez OM, Hebel D et al. Antisense DNA inhibition of tumor growth induced by C-Ha-*ras* oncogene in nude mice. Cancer Res 1993; 53:577-580.
31. Schwab G, Chavany C, Duroux I et al. Antisense oligonucleotides adsorbed to polyalkylcyanoacrylate nanoparticles specifically inhibit mutated H-ras-mediated cell proliferation and tumorigenicity in nude mice. Proc Natl Acad Sci 1994; 91:10460-10464.
32. Stepkowski SM, Tu Y, Condon TP et al. Blocking of heart allograft rejection by Intercellular Adhesion Molecule-1 antisense oligonucleotide alone or in combination with other immunosuppressive modalities. J Immunol 1994; 153:5336-5346.
33. Bennett CF, Dean N, Ecker DJ et al. Pharmacology of antisense therapeutic agents; Cancer and Inflammation. In: Agrawal S, ed. Methods in Molecular Medicine: Antisense Therapeutics. New York: Humana Press, 1995:13-46.
34. Nesterova M, Cho-Chung Y. A single injection of Protein Kinase A-directed antisense treatment to inhibit tumor growth. Nature Med 1995; 1:528-533.
35. Monia BP, Sasmor H, Johnston JF et al. Sequence-specific antitumor activity of a phosphorothioate oligodeoxynucleotide targeted to human C-*raf* Kinase supports an antisense mechanism of action in vivo. Proc Natl Acad Sci 1996; 93:15481-15484.
36. Dean NM, McKay R, Miraglia L et al. (1996). Inhibition of growth of human tumor cell lines in nude mice by an antisense oligonucleotide inhibitor of Protein Kinase C-alpha expression. Cancer Res 56:3499-3507.
37. Yazaki T, Ahmed S, Chahlavi A et al. Treatment of glioblastoma U-87 by systemic administration of an antisense Protein Kinase C-alpha phosphorothioate oligodeoxynucleotide. Molec Pharmacol 1996; 50:242-263.
38. Bennett CF, Kornbrust D, Henry S, Stecker K et al. An ICAM-1 antisense oligonucleotide prevents and reverses dextram sulfate sodium-induced colitis in mice. J Pharmacol Exp Ther 1997; 280:988-1000.
39. McKay RM, Cummins LL, Graham MJ et al. Enhanced activity of an antisense oligonucleotide targeting murine PKC-α by the incorporation of 2'-O-propyl modifications. Nucleic Acids Res 1996; 24:411-417.
40. Crooke RM, Graham MJ, Cooke ME et al. In vitro pharmacokinetics of phosphorothioate antisense oligonucleotides. J Pharmacol Exp Ther 1995; 275:462-473.
41. Stein CA, Does antisense exist? Nature Med 1995; 1:1119-1121.
42. Altmann KH, Dean NM, Fabbro D et al. Second generation of antisense oligonucleotides: From nuclease resistance to biological efficacy in animals. Chimia 1996; 50:168-176.
43. Hepler JR, Gilman AG. G proteins. TIBS 1992; 17:383-387.
44. Cuadrado A, Carnero A, Lacal JC. *Ras* Proteins: Switch devices for signal transduction. In: Lacal JC, McCormick F, eds. The *Ras* Superfamily of GTPases. Boca Raton: CRC Press, 1993:119-153.
45. Kalkbrenner F, Wittig B, Schultz G. Inhibition of G proteins by antisense drugs. In: Crooke ST, ed. Antisense Research and Application: Handbook of Experimental Pharmacology. Springer-Verlag Co, 1997:341-369.
46. Lacal JC, Tronick SR. The *ras* oncogene. In: Reddy EP, Skalka AM, Curran T, eds. The Oncogene Handbook. Elsevier Science 1988:257.
47. Bishop JM. The molecular genetics of cancer. Science 1987; 235:305-306.

48. Bos JL. *ras* Oncogenes in human cancer: A review. Cancer Res 1989; 49:4682-4689.
49. Vogelstein B, Fearon ER, Hamilton SR et al. Genetic alterations during colorectal tumor development. N Engl J Med 1988; 319:525-532.
50. Reddy EP. Nucleotide sequence analysis of the T24 human bladder carcinoma oncogene. Nature 1983; 220:1061-1063.
51. Bos JL, Verlaan-de Vries M, Marshall CJ et al. A human gastric carcinoma contains a single mutated and an amplified normal allele of the Ki-*ras* oncogene. Nucleic Acids Res 1986; 14:1209-1217.
52. Bennett CF, Chiang M-Y, Chan H et al. Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. Mol Pharmacol 1992; 41:1023-1033.
53. Monia BP, Johnston JF, Esker, DJ et al. Selective inhibition of mutant Ha-*ras* mRNA expression by antisense oligonucleotides. J Biol Chem 1992; 267:19954-19962.
54. Saison-Behmoaras T, Tocque B, Rey I et al. Short modified antisense oligonucleotides directed against Ha-*ras* point mutation induces selective cleavage of the mRNA and inhibits T24 cell proliferation. EMBO J 1991; 10:1111-1118.
55. Chang EH, Miller PS, Cushman C et al. Antisense inhibition of *ras* p21 expression that is sensitive to a point mutation. Biochemistry 1991; 30:8283-8286.
56. Monia BP. Disruption of the MAP Kinase signaling pathway using antisense oligonucleotide inhibitors targeted to *ras* and *raf* Kinase. In: Krieg A, Stein C, eds. Applied Antisense Oligonucleotide Technology. New York: John Wiley and Sons Inc, 1998: (in press).
57. Mukhopadhyay T, Tainsky M, Cavender AC et al. Specific inhibition of K-*ras* expression and tumorigenicity of lung cancer cells by antisense RNA. Cancer Res. 1991; 51:1744-1754.
58. Zhang Y, Mukhopadhyay T, Donehower LA et al. Retroviral vector-mediated transduction of K-*ras* antisense RNA into human lung cancer cells inhibits expression of the malignant phenotype. Human Gene Therapy 1993; 4:451-460.
59. Georges RN, Mukhopadhyay T, Zhang Y et al. Prevention of orthotopic human lung cancer growth by intratracheal instillation of a retroviral antisense K-ras construct. Cancer Res 1993; 53:1743-1750.
60. Gray GD, Hernandez OM, Hebel D et al. Antisense DNA inhibition of tumor growth induced by c-Ha-*ras* oncogene in nude mice. Cancer Res 1993; 53:577-584.
61. Schwab G, Chavany C, Duroux I et al. Antisense oligonucleotides adsorbed to polyalkylcyanoacrylate nanoparticles specifically inhibit mutated Ha-*ras*-mediated cell proliferation and tumorigenicity in nude mice. Proc Natl Acad Sci 1994; 91:10460-10467.
62. Liao Y, Tang ZY, Liu KD et al. Apoptosis of human BEL-7402 hepatocellular carcinoma cells released by antisense Ha-*Ras* DNA-in vitro and in vivo studies. J Cancer Res and Clin Onc 1997; 123:25-39.
63. Cowsert LM. In vitro and in vivo activity of antisense inhibitors of *Ras*: Potential for clinical development. Anti-Cancer Drug Design 1997; 12:359-371.

Index

D

E

F

G

H

I

J

L

M

N

P

R

S

T

U

V

W

Y