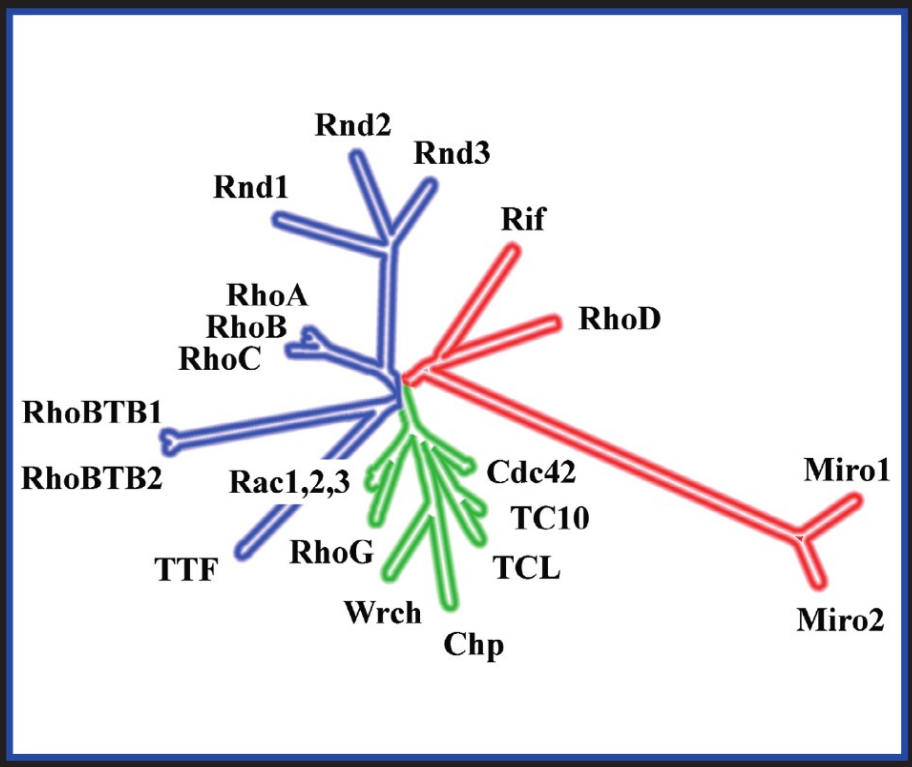
PROTEINS AND CELL REGULATION
VOLUME 3

RHO Family GTPases

Edited by
Ed Manser



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RHO Family GTPases

PROTEINS AND CELL REGULATION

Volume 3

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Aims and Scope

Our knowledge of the ways in which a cell communicates with its environment and how it responds to information received has reached a level of almost bewildering complexity. The large diagrams of cells to be found on the walls of many a biologist's office are usually adorned with parallel and interconnecting pathways linking the multitude of components and suggest a clear logic and understanding of the role played by each protein. Of course this two-dimensional, albeit often colourful representation takes no account of the three-dimensional structure of a cell, the nature of the external and internal milieu, the dynamics of changes in protein levels and interactions, or the variations between cells in different tissues.

Each book in this series, entitled "*Proteins and Cell Regulation*", will seek to explore specific protein families or categories of proteins from the viewpoint of the general and specific functions they provide and their involvement in the dynamic behaviour of a cell. Content will range from basic protein structure and function to consideration of cell type-specific features and the consequences of disease-associated changes and potential therapeutic intervention. So that the books represent the most up-to-date understanding, contributors will be prominent researchers in each particular area. Although aimed at graduate, postgraduate and principle investigators, the books will also be of use to science and medical undergraduates and to those wishing to understand basic cellular processes and develop novel therapeutic interventions for specific diseases.

RHO Family GTPases

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Preface

THE RHO GTPASE FAMILY

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1. THE CELL CYTOSKELETON

All organisms have cellular machinery dedicated to controlling cell shape, and the ability to dynamically change shape plays important roles in many biological processes. Muscle-based mechanisms are the best understood because the machinery is highly ordered and exerts force in a single dimension (ie. pulling between the two fixed ends of the cell). This utilizes interlaced actin and myosin II filaments that are distributed in fixed arrays. By contrast motile cells or cells with plastic processes (for example neurons) respond additionally by remodeling their internal actin cytoskeleton into many types of array. Actin, one of the most abundant eukaryotic proteins, is clearly a key protein in promoting structural change, being conserved from yeast to humans. Just recently proteins distantly related to actin have been discovered in prokaryotes such as *Bacillus subtilis* where they also regulate cell shape.

In the absence of internal structures the fluid lipid bilayer cell membrane would be spherical, but assymetry arises from both rigid elements within the cell (as exemplified by the well studied red blood cell) and external attachments. The internal cytoskeletal components work in combination with adhesive tension developed against other cells or the extracellular matrix. Actin-based structures are chiefly organized by Rho proteins, as appears to be the case for other important cytoskeletal components in mammalian cells, namely intermediate filaments, septin filaments and microtubules. All these are assembled from component protein subunits that under appropriate conditions can form various types of filaments. For many decades

microinjection of flourescently-labelled subunits, which incorporate into these filaments in vivo, has revealed a dynamic rather than static cytoskeleton. In the case of actin microfilaments and the microtubules, energy derived from hydrolysis of ATP or GTP respectively provides the filaments with polarity, and subunit addition occurs predominantly at one end. This produces a phenomena known as tread-milling whereby new monomers are added primarily at the 'plus' end to match loss from the 'minus' end. Recently exciting new discoveries are being made about the way in which Rho proteins control microtubules (Chapter 12), and we expect to similar levels of control to be found for intermediate filaments and the poorly characterized septin system.

2. SOME BACKGROUND

It is a decade since Alan Hall and his colleagues described how active versions of small GTP-binding proteins Rac1 and RhoA could assemble distinct actin arrays in mammalian cells. Significantly it was found external signals acting on transmembrane cell surface receptors use these GTPases to initiate similar changes in the actin cytoskeletal to promote changes in cell shape. Specifically Rac1 acting downstream of receptor tyrosine kinases generates peripheral extensions known as membrane ruffles or lamellipodia, while RhoA assembles more internally located actin stress fibres, and associated adhesion complexes. Shortly afterwards a related GTPase Cdc42, first described in budding yeast, was found to organize cell structures known as filopodia in mammalian cells - finger like projections containing an actin core.

Work from many laboratories including our own has established that Rho-GTPases alter the cytoskeleton by recruiting protein effectors. We discovered that kinases that belong to the ACK, PAK, MRCK and ROK families bind selectively to the GTP-form of certain Rho proteins. These kinases in turn act in phosphorylation cascades that are responsible for some of the changes to the actin, intermediate filament and microtubule networks initiated by the GTPases (see Chapters 6, 7 and 10). Non-kinase effectors such as WASP and formins play distinct but complementary roles. A working model is that many signals that impinge on cells to alter their shape route through a common sets of Rho switches. These proteins also have important functions beyond the cytoskeleton - in cell cycle control and transcriptional regulation, but such issues are less understood and not dealt with in this volume. Lessons learned about pathways regulated by Rho proteins are beginning to tell us how cells coordinate the complex internal reorganization required for processes such as cell movement.

3. THE BEHAVIOUR OF RHO PROTEINS

The reason small GTPases are referred to as molecular switches is that they exist in the cell in two forms, one bound to GDP and the other to GTP. In what is often referred to as the "active" state a GTPase may bind and allosterically activate an enzyme such as a PAK kinase (Chapter 10), alter the cellular location of effectors, or promote assembly of a multisubunit complex. The two inter-convertible states of these GTPases results from nucleotide-sensitive conformational changes in two regions of the protein referred to as switches I and II. The interaction of these regions with various types of protein are beautifully described and illustrated in Chapter 3. The presence of a permanent, covalently attached 16 or 20 carbon fatty acid at the C-terminal is essential to both membrane association and biological effects of Rho proteins (except perhaps RhoBTB).

The high degree of structural and functional conservation between mammalian Rho proteins and the orthologs in flies and even yeast is remarkable. More complex organisms in general have correspondingly increased numbers of Rho proteins although significant diversification can occur in apparently 'simple' organisms. These and other aspects of the evolution of the human Rho family are considered in Chapter 2.

The interconversion between the two states of a GTPase is promoted by two types of activities: guanine nucleotide exchange factors (GEFs) increase the level of activated (GTP-bound) GTPase and GTPase activating proteins (GAPs) speed the intrinsic hydrolysis of the bound GTP. Some of these Dbl-related RhoGEFs are discussed in Chapter 1 while a completely unrelated class of GEFs known as Dock180 proteins are covered in Chapter 4. The importance of diversity in the regulation of Rho proteins is evident for the GTPase activating proteins (RhoGAPs), whose bewildering diversity of domain structures is described in Chapter 5.

The Ras and Rho proteins behave in some ways like the alpha subunit of heterotrimeric G-proteins. Researchers initially applied techniques used in the heterotrimeric G-protein field to test whether small G-proteins behave as three-component systems (activator, G-protein, and effector) as for hormone- and light-activated heterotrimeric G systems.

The generally accepted view regarding guanine nucleotide dissociation inhibitors (GDIs) is that these proteins play a chaperone or negative regulatory role, though in one case the GDI can confer the Rac.GDP.GDI complex with Rac.GTP-like behaviour *in vitro*.

The overwhelming majority of studies to date have focused on roles and activities Rac1, Cdc42 and RhoA particularly since the latter two have orthologues well conserved across eukaryotes (see Chapter 2). These proteins play conserved roles in regulating actin assembly via effectors that

regulate myosin II, formin, and Arp2/3 pathways (as covered in detail in Chapters 6 and 7).

4. FINAL THOUGHTS

It is apparent that although the last 10 years have brought tremendous progress, really assessing how Rho and their partners regulate the cytoskeleton requires an understanding of how the numerous effectors actually integrate in the cellular context. Efforts recently underway to investigate global changes in phosphorylation promise to provide much more complete understanding of the role of kinase effectors of Rho. It is also important to appreciate that the cytoskeleton and membrane compartments are closely linked. Thus we are likely to see more input from colleagues working in the area of membrane trafficking (covered in Chapter 9).

We have plenty of work ahead to complete the molecular description of Rho actions in eukaryotes, and more particularly how these functions relate to vertebrate development (see Chapter 11). The use of knock-down as well as knockout techniques will no doubt produce a leap in the number of studies addressing Rho function in the whole animal.

ACKNOWLEDGEMENTS

Thanks must go to all the authors of this volume who have laboured to produce insightful chapters in their respective area of expertise. This effort will no doubt aid the expanding community of researchers who have an interest in Rho proteins. I would like to thank Jackson Zhao for excellent assistance in putting together and editing text and figures for each of the chapters in this book. I am grateful for support from the GSK-Singapore Research Fund.

Ed Manser
November 2004

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Chapter 1

THE RHO GTPASES
Critical Regulators In Cell Biology

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Abstract: Rho-family GTPases (p21s) are molecular switches related to the proto-oncogene Ras: these function in complementary pathways to orchestrate the actin cytoskeleton, regulate cell polarity, microtubule dynamics, membrane transport pathways and modulate a variety of transcriptional events. Rho GTPases are biochemically and structually simple proteins: they cycle between two conformational states, with the GTP-bound form regarded as active, while the GDP-bound state is essentially inactive. Conversion between these states is brought about by hydrolysis of bound GTP. The GTPases in their active form can recognize a variety of target proteins and thereby generate a response until GTP hydrolysis returns the switch to an 'off' state. The Rho family of GTPases is now intensively studied by cell biologists, as its members have turned out to be key regulators of many aspects of cell behaviour. In this introductory chapter I discuss how Rho proteins become activated by GEFs in response to extracellular signals, and in particular how integrin signaling might impinge on Rhos. At this stage our knowledge of many of these pathways is rudimentary, thus much still needs to be done to uncover specific pathways that regulate of Rho proteins *in vivo*. This volume covers what is known of Rho proteins in vertebrate systems with some reference to lower model organisms. The molecular and structural data is covered in some depth; in this 'post genomic' era we are also able to provide a panoramic view of the various protein families that have emerged as key players in Rho GTPases signaling.

1. INTRODUCTION

The Ras homology or Rho gene identification (Madaule and Axel, 1985), set the stage for the uncovering of other members of the family including RhoA,

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Cdc42 and Rac1. The ability to derive constitutively activated (GTPase deficient) or conversely dominant inhibitory versions of Rho proteins derived from analysis of the prototype Ras itself, and has provided invaluble means to investigate these GTPases. It was found in the early 1990s that active Rho proteins could elicit changes to the actin cytoskeleton consistent with structures found in cultured cells, namely with RhoA actin stress fibres (Ridley and Hall, 1992b), with Rac1 membrane extension or lamellipodia (Ridley and Hall, 1992a), and with Cdc42 finger like filopodia (Nobes and Hall, 1995). That Rho, Rac and Cdc42 regulate three such signal transduction pathways linking plasma membrane receptors to the assembly of distinct filamentous actin structures has since been confirmed in a wide variety of mammalian cell types, as well as in model systems such as flies and worms.

Like Ras these proteins are molecular switches that cycle between GTP-bound (active) states and GDP-bound (inactive) states. The molecular and biological details of this transition in terms of the proteins that catalyze the GTP loading (guanine nucleotide exchange factors) and GTP hydrolysis (GTPase activation proteins) are covered in detail in Chapters 3, 4 and 5. The active forms of the GTPases adopt a conformation that allows them to bind to and influence many protein 'effectors' as described in Chapters 6-12, of which the prototype is the serine/threonine kinase PAK (Chapter 10). These effectors have been found to have wide ranging effects on cell biology including re-organization of the actin cytoskeleton (Chapters 6 and 7), changes in cell-cell contacts (Chapter 8), alteration of the microtubule network (Chapter 12), and intracellular trafficking (Chapter 9). In the next few sections I consider a number of the key cellular processes in which Rho GTPases have been implicated and review some of the outstanding questions in the field.

2. HOW DO SIGNALS PASS TO RHO?

2.1 The Rho Guanine Nucleotide Exchange Factors

The first mammalian GEF to be identified was termed Dbl (diffuse B-cell lymphoma) was found to contained a region of ~180 amino acids with significant sequence similarity to Cdc24p, a protein identified genetically as an upstream activator of Cdc42p in yeast (Ron et al., 1991). Dbl was subsequently shown to catalyze nucleotide exchange on human Cdc42 in vitro and thus the conserved DH (Dbl homology) domain is the minimum region required for GEF activity (Hart et al., 1994). Many DH-domain-

containing proteins have since been isolated through a variety of screens. The genome-sequencing projects, 6 GEFs have been identified in Saccharomyces cerevisiae, and ~60 in humans (Schmidt and Hall, 2002).

There are three conserved regions in the DH domains. X-ray and NMR derived structures of DH domains of Sos1, Trio, and Tiam-1 reveal a highly related three-dimensional structure that is composed of a flattened, elongated bundle of 11 α-helices (Aghazadeh et al., 1998; Soisson et al., 1998; Worthylake et al., 2000). Two of these helices, termed CR1 and CR3, are exposed on the surface of the DH domain and participate in the formation of the GTPase interaction pocket. Interestingly GEFs and certain effectors can bind to the GDP-bound form and while stabilizing a nucleotide-free reaction intermediate (Cherfils, 2001). Under these conditions the GDP is released and replaced with cellular GTP leading to GTPase activation.

Many mammalian GEFs have been analyzed for their ability to catalyze exchange on a limited number of Rho GTPases. Ideally the activity of GEFs should be analyzed with respect to all Rho GTPases (except those that are constitutively active). This can be done by measuring their ability to stimulate nucleotide exchange in vitro, or by analyzing their effects after over expression in vivo. Several GEFs appear to be specific toward a single GTPase, for example, Fgd1 towards Cdc42 or p115RhoGEF towards RhoA (Hart et al., 1996; Zheng et al., 1996); whereas others may activate several members for example Dbl and Vav1 which act on Cdc42, Rac1 and RhoA (Hart et al., 1994; Olson et al., 1996). As yet the in vivo specificity cannot be predicted from primary sequence.

Almost all Rho GEFs possess a pleckstrin homology (PH) domain adjacent and C-terminal to the DH domain and such a DH–PH module often represents the minimal structural unit that can promote nucleotide exchange in vivo. PH domains are known to bind to phosphorylated phosphoinositides (Lemmon et al., 2002). It seems likely they are able to also directly affect the catalytic activity of the DH domain while also targeting GEFs to their appropriate intracellular location. There are four GEFs that lack an obvious PH domain (Schmidt and Hall, 2002). It is suggested some PH domains directly participate with the DH domain in GTPase binding (Rossman et al., 2002). Apart from the DH–PH module, most GEFs contain additional functional domains such as SH2, SH3, protein kinase, Rho-GAP, Ras-GEF, or additional PH domains. These then allow coupling of GEFs to upstream receptors and signaling molecules.

Aside from the issue of location (ie bringing GEFs to Rho GTPases) GEFs are tightly regulated via a variety of mechanisms. Some general principles have emerged for GEF regulation such as relief of intramolecular inhibitory sequences, stimulation by protein–protein interactions, and modulation of GEF activity. Often GEFs contain a regulatory domain that

blocks activity via intramolecular interactions. For example Ost, Tiam1, Vav, Dbl, , and Tiam1 activated by the removal of N-terminal sequences (Hart et al., 1994; Horii et al., 1994; van Leeuwen et al., 1995). In the case of p115RhoGEF and Lbc, removal of C-terminal sequences activates the protein (Sterpetti et al., 1999; Wells et al., 2001). In such cases, it is assumed that activation of full-length GEF is through the relief of autoinhibition by phosphorylation or by binding to other proteins, but these mechanisms are largely uncharacterized.

2.2 Pathways from cell surface receptors

We have a fairly mature understanding of the biochemical events initiated at the cell surface by soluble external factors acting on either receptor tyrosine kinases (RTKs) or seven-transmembrane serpentine receptors. RTKs phosphorylate themselves (and other substrate proteins) on tyrosine residues, and proteins containing Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains can therefore bind at these residues. The resultant signaling complex that forms around the RTK contains a variety of tyrosine phosphorylated proteins. Serpentine receptors activate heterotrimeric guanine-nucleotide binding (G)-proteins and signal via 'classical' second messengers such as cyclic AMP and calcium ions, as well as recruiting non-receptor tyrosine kinases such as c-Src (Ram and Iyengar, 2001). Like the prototype small GTPase Ras, which plays a ubiquitous role in cell proliferation and differentiation downstream of cellular growth factors, Rho GTPases become activated by guanine nucleotide exchange factors (GEFs) or the more recently uncovered DOCK180 family (see Chapter 4) which are surprisingly unrelated by sequence. RhoGEF and DOCK proteins exhibit a guanine nucleotide exchange activity *in vitro* (ie catalyzing GDP loss and GTP association) under appropriate conditions. These tend to be complex multi-domain species as for the classical RasGEF son-of-sevenless (mSos) which is also a Rac GEF Sos. Because most Rho GTPases contain a lipid-targeting domain, signaling takes place at membrane-cytosol interfaces (not only at the plasma membrane), where the small GTPases recruit distinct sets of target proteins, also known as effectors. The diversity of Rho effectors has the subject of a number of reviews (Etienne-Manneville and Hall, 2002) of which the prototype PAK is discussed in some detail in Chapter 10.

Specifically what signals can activate Rho family GTPases? Many trophic factors, such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF), can induce activation of Rac1. A marker for activation is membrane ruffling, which is associated with cell movement (Ridley et al., 1992).

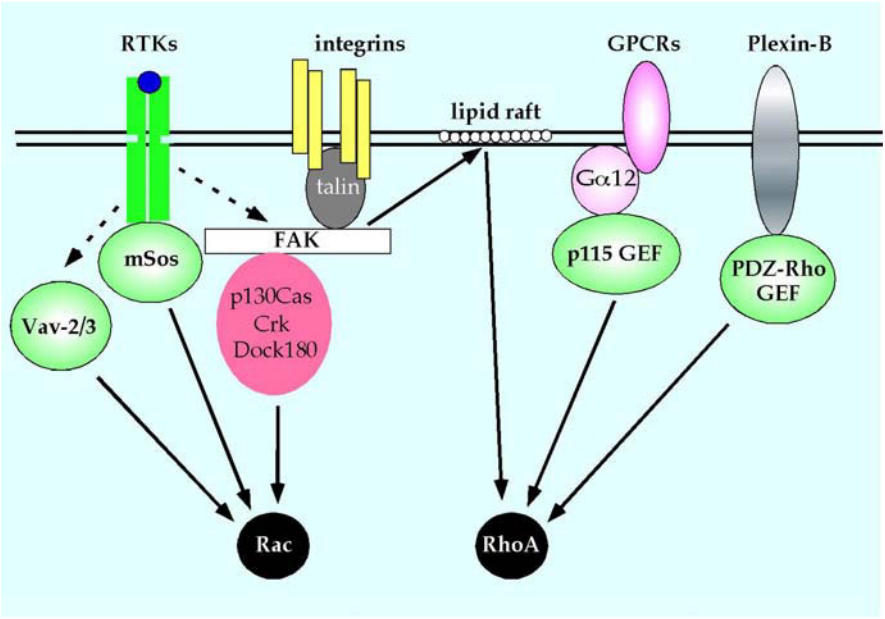

Figure 1. Extra cellular signals activate RhoGTPases via a variety of guanine nucleotide exchange factors (GEFs). Some GEFs can be recruited to the plasma membrane by receptor tyrosine kinases (RTKs) such as the EGF receptor. These RTKs generate phosphotyrosine residues (PY) that bind SH2 containing proteins (such as adaptors or Vav family GEFs), which in turn recruit other protein partners. Sos can promote exchange of GDP for GTP on both Ras and Rac. Focal adhesion kinase (FAK) is not necessary for assembly of focal adhesion complexes but the presence of FAK in focal adhesion complexes contributes significantly to cell signaling. Following integrin ligation, FAK is recruited to focal adhesion complexes in part by interaction with talin, and becomes tyrosine phosphorylated. The N-terminal domain of FAK is thought to allow coupling to activated RTKs. FAK is associated with p130Cas, and the SH2 containing adaptors Nck and Crk. The latter associates with Dock180 that in turn activates Rac1. The integrin-FAK pathway leads to activation of RhoA in a lipid raft-dependent manner. (Palazzo et al., 2004). Interestingly coupling of Rac1 to effector targets requires participation of lipid rafts (del Pozo et al., 2000). The p115 RhoGEF interacts with activated Gα12/13 via its RGS domain and signals from G-protein coupled receptors (such as the LPA receptor) to Rho activation (Hart et al., 1998). Some receptors such as Plexin-B direct bind to RhoGEFs, in the case shown via a PDZ interaction. (Perrot et al., 2002).

A number of laboratories have shown that Dbl-like GEFs of the Vav family are implicated in the signaling cascade from EGF and PDGF receptors. There are three Vav isoforms, all of which co-precipitate with EGF and PDGF receptors (Moores et al., 2000). The ubiquitous Vav-2 has been identified as a tyrosine-phosphorylated component of the EGF complex (Pandey et al., 2000), and significantly a carboxy-terminal (non-DH) fragment of Vav-2 inhibits Rac1 activation in response to EGF (Liu and Burridge, 2000). The Vav-3 protein has been shown to respond to a variety of growth factors *in vivo*, and it activates both Rac1 and Cdc42 although

recent knockout studies suggest Vav proteins are required primarily in the immune system (Fujikawa et al., 2003; Zeng et al., 2000). A different RacGEF, Tiam1, also becomes tyrosine phosphorylated in response to PDGF under conditions of growth factor treatment (Buchanan et al., 2000). These pathways are illustrated in figure 1.

While these instances involving Vav proteins and Tiam1 indicate how a signal arriving at the plasma membrane might be translated into GTPase activation, there are clearly many details that remain to be uncovered. The clearest understood mechanism of RhoGEF activation by extra cellular signals involves the p115 RhoGEF which can interact with heterotrimeric Gα12/13 (Kozasa et al., 1998). Although this protein is a GAP for the Gα subunit, it behaves essentially as an effector, activating RhoA via a Dbl homology domain, as reviewed (Kozasa, 2001). It is likely that a variety of other plasma membrane receptors are capable of directly recruiting RhoGEFs: one such example is the recruitment by Plexin-B of the Rho exchange factor PDZ-RhoGEF/LARG which causes RhoA activation and thus neurite retraction (Perrot et al., 2002).

2.3 Cross talk among small GTPases pathways

To coordinate cellular processes, pathways regulated by GTPases must interact with each another. This was apparent in early studies (Ridley et al., 1992) where Ras was found to mediate Rac1 activation leading to membrane ruffling (a common observation when cultured cells are treated with many growth factors). Ras directly activates phosphatidylinositol 3-OH kinase (PI(3)K) and thereby Rac1 perhaps via mSos while among Rho GTPases RhoG is reported to activate both Cdc42 and Rac1 (Blangy et al., 2000). The ability of Cdc42 to activate Rac1 (Nobes and Hall, 1995) is suspected to underlie coupling of filopodia to lamellipodia production. The RacGEF PIX which binds to PAK is implicated in this process as PIX accumulates in Cdc42-driven focal adhesion complexes. In many cell types, Cdc42 and Rac clearly antagonize RhoA function, as evidenced by the loss of actin stress fibres. Ras–GTP can block RhoA by p120 RasGAP-mediated activation of its partner p190 RhoGAP which appears to be a critical player in regulating RhoA in many cells. At present, because few RhoGEFs and RhoGAPs are understood in any detail, the mechanisms of cross-talk between various pathways are largely unchartered.

3. THE ROLE OF RHO GTPASES IN CELL MIGRATION

3.1 Movement of isolated cells

Many studies have pointed to Rac1 playing a critical role in cell migration (Keely et al., 1997). This is seen in most systems by a block to formation of leading edge membrane ruffles by a dominant-inhibitory Rac1 mutant, Rac1N17. In Boyden chamber assays activation of Rac1 only (and not of Cdc42 or RhoA) was found to be required for PDGF stimulated locomotion (Banyard et al., 2000). Real-time imaging techniques have allowed the production of active Rac1.GTP at the leading edge to be visualized directly (Kraynov et al., 2000). The front of the migrating cell generates protrusive force, generally associated with the extension of a lamellipodium in the direction of migration, coupled to formation of new cell adhesions to the extra cellular substrate (Raftopoulou and Hall, 2004). New evidence suggests that expansion at the leading edge of the cell is coupled to cell contractility in lamellipodia, such that extension occurs as a series of waves (Giannone et al., 2004). Through its ability to promote both protrusion and cell contraction, the actin cytoskeleton provides the driving force for cell migration (Raftopoulou and Hall, 2004). Rac induces actin polymerization and integrin adhesion complex assembly at the cell periphery essential for cell migration (Kaverina et al., 2002).

The biochemical mechanisms by which Rac catalyses actin polymerization are only beginning to be understood, direct Rac targets including the WAVE complex which regulates the Arp2/3 complex (Smith and Li, 2004), as well as key regulators phosphatidylinositol-4-phosphate 5-kinase, PAK and LIM kinases (Raftopoulou and Hall, 2004) as discussed in Chapter 7. Figure 2 illustrates how Rho GTPases are thought to engage a number of effectors to drive lamellopodia production and couple this to contractile forces of myosin II. The Cdc42 and Rac1 effector PAK (Chapter 11) localizes to adhesion complexes (Manser et al., 1998) and is associated with increased fibroblast migration (Sells et al., 1997). Cell migration is normally directed and controlled by extracellular cues: the stabilization of directional movement (chemotaxis) requires input from Cdc42 to maintain direction of movement (Allen et al., 1998). There are, however, variations on the theme. For example, macrophages require both Rac1 and RhoA activity to move (Allen et al., 1997; Allen et al., 1998). In epithelial cells however, because Rac1 increases adhesion between epithelial cells it counteracts cell scattering and subsequent migration (as discussed in Chapter 8).

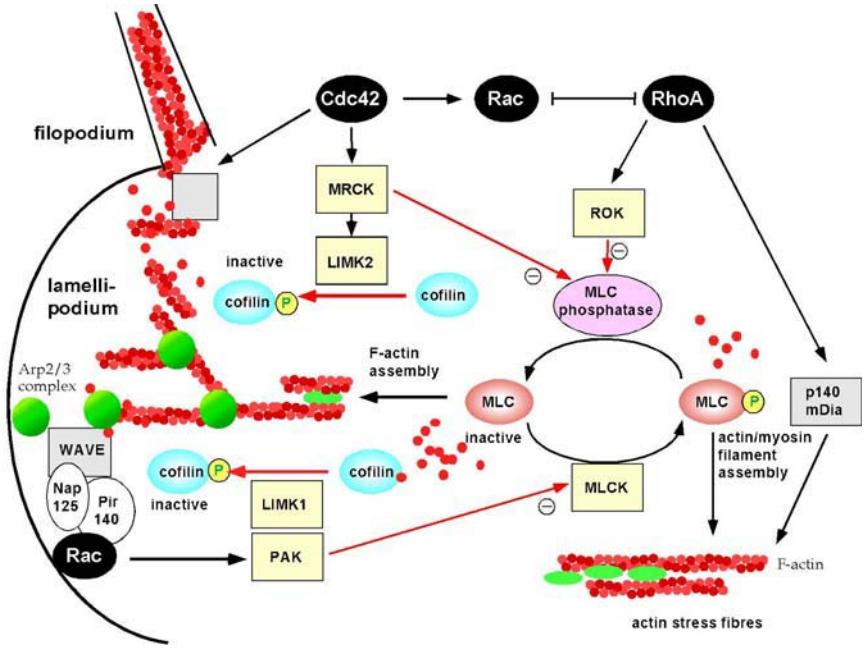

Figure 2. Biochemical roles of Rho GTPase effectors in actin organization. Rac1 is present at the leading edge of the cell to drive lamellipodia formation via the WAVE complex (green circles). WAVE can generate Arp2/3-dependent actin polymerization which allows assembly of sufficient actin to drive the lamellipodium forward. Rac also serves to activate PAK which downn-regulates antagonizes ROK/MRCK effects on myosin regulatory light chain (MLC). RhoA regulation of actin organization occurs via Rho-associated kinase ROK that blocks the myosin light chain phosphatase complex, preventing inactivation of phosphorylated MLC~P. Myosin II (green ovals) assembles into stress fibres and favours focal adhesion complex formation. Myosin light chain kinase (MLCK) is a key enzyme for maintaining the myosin heavy–light chain complex in an active state, but is negatively regulated by Rac via the p21-activated kinase (PAK). The formin p140mDia promotes filamentous actin formation in cooperation with ROK which promotes myosin containing stress fibres. At peripheral locations Cdc42 is thought to recruit the Arp2/3 complex and thereby promotes initiation of filopodia, but the effector for this function is not yet resolved. Cdc42 regulates myotonin-related Cdc42-binding kinase (MRCK), which has a similar effect to ROK on MLC phosphatase and probably also LIM-kinase 2 (LIMK2): its activity blocks cofilin's ability to sequester actin monomers, releasing a local pool for polymerization. Rac1 similarly uses PAK to regulate LIMK1. Phosphorylation events that have inhibitory effects are marked (-).

3.2 Membrane dynamics and cell movement

Growth factor induced ruffles are enriched for endosomal marker proteins indicating the intracellular origin of these membranes. Rac1 also induces membrane uptake by macropinocytosis (Ridley et al., 1992). Are membrane trafficking events also coordinated by Rho GTPases? Cdc42 is a good candidate as a regulator of trafficking as it is primarily found in the vicinity

of the Golgi apparatus (Erickson et al., 1996), the distribution centre for membrane vesicles. In epithelial cells active Cdc42 mutants inhibit the exit of basolateral proteins from the trans-Golgi network, but stimulate apical markers (Musch et al., 2001). Moreover, may also regulate intra-Golgi transport, as the coatomer protein γCOP is a target of Cdc42 (Wu et al., 2000), The specific roles of a number of other Rho proteins in relation to membrane trafficking are discussed in Chapter 9.

3.3 Coordinated movement of cell sheets

During embryogenesis, many cells move as sheets or associated groups rather than as individuals. One of the best-studied *in vivo* examples of this is during *Drosophila* embryonic dorsal closure, where Rho GTPases have been implicated in a number of key steps (Harden et al., 1999). Rho (acting through effectors PKN and ROK) controls the assembly and the contraction of the actin and myosin cables that tightens a 'purse-string', while Cdc42 and Rac promote filopodia and lamellipodia, which interact with apposing cells and facilitate movement.

Directed cell migration can be induced *in vitro* by scratching a confluent mammalian cell monolayer. Using either primary fibroblasts or primary astrocytes, Rac is essential for cell migration in these assays (Raftopoulou and Hall, 2004). Migrating sheets of cells recognize the space formed by the scratch and polarize to restrict their protrusive activity to the front for the cell. In this process the microtubule organizing centre (MTOC) re-orients in front of the nucleus to face the direction of migration (see Chapter 9) as does the Golgi apparatus. In both astrocyte and fibroblast scratch assays, inhibition of Cdc42 leads to misdirected protrusive activity and a random orientation of the MTOC (Palazzo et al., 2001). In astrocytes at least, both these aspects of polarity depend on a Par6–atypical PKC complex localized at the leading edge (see above). The initiating external cue in this assay is integrin engagement at the front of leading edge cells, causing Cdc42 activation. Thus, the molecules that control the establishment of polarity in migrating monolayers are the same as those that control asymmetrical cell division in *C. elegans* and *Drosophila*.

4. CDC42 ESTABLISHES CELL ASSYMETRY

Animal cells adopt a huge diversity of shapes, ranging from simple epithelial cells through to the complex branched structures of neurons. Shape is often dependent on the external environment, which provides directional cues that drive the establishment of intracellular polarity. Genetic analysis of budding

yeast provided the first evidence linking Cdc42 to cell polarity (Johnson and Pringle, 1990). In the absence of this GTPase, *Saccharomyces cerevisiae* cannot establish a defined site for daughter cell growth and therefore cells expand isotropically. Similarly, during mating, yeast cells extend surface protrusions (shmoos) towards a gradient of secreted pheromone; without Cdc42p (yeast Cdc42 protein) the protrusions are no longer oriented correctly. Cdc42p is not needed for cell growth or protrusion formation *per se*, but it is needed for these processes to localize correctly. Once the bud site has been established, the organization of the actin and microtubule cytoskeletons and the direction of vesicular transport pathways can follow.

Cdc42 is the principal determinant in establishing correct cell polarity: establishing intracellular asymmetry is often a prerequisite for morphogenesis. The biochemistry of polarity establishment has been aided by studies of Cdc42p function in yeast, although mammalian PAK was the first common Cdc42 target that might coordinate downstream events (Manser et al., 1994; Zhao et al., 1995). Pheromone induces an asymmetrical, stochastic accumulation of shmoo components on the membrane surface dependent on the PAK-related kinase Ste20p and Cla4p (Weiss et al., 2000). Cdc42 is stabilized at this location via a positive feedback loop (Butty et al., 2002) however Cla4p activity at a later stage is required to break this complex (Gulli et al., 2000). Such observation underlines the dynamic nature of Rho signaling in all organisms.

The genetic analysis of the first cell (zygote) division during *Caenorhabditis elegans* development has provided some of the best insights into how cellular asymmetry can be generated in multicellular organisms. The protein products of six *par* genes (PAR-1–6, partitioning defective) and an atypical protein kinase C (PKC-3, which does not directly bind Cdc42) are essential for establishing the anterior/posterior axis within this single cell. PAR-1 (protein kinase) and PAR-2 localize at the posterior end of the cell (where fertilization takes place), whereas PAR-3, PAR-6 and PKC-3 localize at the anterior end (Hung and Kemphues, 1999). PAR proteins are conserved throughout the animal kingdom with the PAR-6–atypical PKC complex being central to cell asymmetry during oocyte maturation and neuroblast division in *Drosophila* (Ohno, 2001). Cdc42.GTP interacts directly with the Par6 protein through a binding sequence which resembles that of other effectors such as PAK, leading indirectly to activation of atypical PKC (Joberty et al., 2000; Qiu et al., 2000). Inhibition of Cdc42 completely disrupts polarity and delocalizes all PAR proteins (Gotta et al., 2001).

What about mammalian systems? Macrophages retain their capacity to migrate when injected with a dominant inhibitory mutant form of Cdc42, Cdc42N17, but lose their ability to orientate correctly towards a chemotactic

gradient (Allen et al., 1998). Similarly, cell orientation requires Cdc42 function in wound healing assays (Nobes and Hall, 1999) and the biochemical pathways underlying these processes are some way towards being uncovered (Raftopoulou and Hall, 2004).

5. RELATIONSHIP BETWEEN RHO GTPASES AND CELL ADHESION COMPLEXES

The interaction of many trophic factors with their cell surface receptors leads to activation of Rac1, Cdc42 and RhoA, which in turn are able to drive formation of new integrin containing focal adhesion complexes (Nobes and Hall, 1995). The new adhesions in turn recruit proteins capable of further GTPase activation or inhibition. Integrin engagement itself leads to activation of Rac1 and Cdc42 (Clark et al., 1998), but down-regulation of RhoA. The persistence of forward movement in the case of motile cells might be maintained by such positive feedback cycles.

5.1 Linking focal adhesions to actin

Introduction of active Cdc42, Rac1 and RhoA can drive the formation of focal adhesion complexes, and part of this activity derives indirectly from the effects of Rho GTPases on the actin cytoskeleton (chapters 6 and 7). Focal adhesion complex formation is reinforced by tension between the extra cellular matrix, integrins and actin cytoskeleton and is very sensitive to myosin II inhibitors. The role of myosin II in sustaining focal adhesion complex structure requires components that link actin filaments to integrin complexes, including vinculin, talin and α-actinin. Vinculin is directly regulated by PtdIns(4,5)P_2, which disrupts intramolecular interactions to allow binding to talin and α-actinin (Gilmore and Burridge, 1995) key interactions in the assembly of focal adhesion complexes: indeed antibodies to PtdIns(4,5)P_2 block the formation of such adhesions. RhoA and ROK are known to increase cellular levels of PtdIns(4,5)P_2 through the action of PIPKs (Oude Weernink et al., 2000; Tolias et al., 1995). This promotes protein components to assemble with integrins into *bona fide* focal adhesion complexes.

5.2 Rho GTPases and focal adhesion kinase (FAK)

Focal adhesion kinase (FAK) is widely regarded as being permissive for cell motility suggesting it is at the interface between Rho GTPases and focal

adhesions. Experiments with FAK-null cells have established that efficient integrin-stimulated cell migration and focal adhesion complex turnover require FAK (Gilmore and Romer, 1996). FAK localizes to focal adhesion complexes, promotes changes in morphology by down-regulating RhoA (Ren et al., 2000). However FAK functions to integrate integrin signaling with receptor tyrosine kinases (Sieg et al., 2000) as part of the cytoskeletal network that includes Src family tyrosine kinases (Fig. 1) and adaptor proteins such as $p130^{cas}$, Shc, Nck and Grb2 (Schlaepfer et al., 1997). FAK can directly interact with p190RhoGAP which is suggested to down-regulate RhoA (Zhai et al., 2003).. _Comparison of FAK-/- fibroblasts with wildtype indicates FAK promotes a transient down-regulation of RhoA.GTP when cells are plated on fibronectin (Ren et al., 2000). However integrins also produce long-term RhoA activation and stabilization of microtubules, which requires FAK (Palazzo et al., 2004). Interestingly this study also indicates the ability of integrins and FAK to activate RhoA requires cholesterol containing lipid rafts.

Cell spreading (membrane protrusion) on solid matrices coated with fibronectin elevates Rac1.GTP and Cdc42.GTP. The adaptor $p130^{Cas}$ interacts with and is phosphorylated by FAK; such an interaction is required for cell migration (Cary et al., 1999). There is good evidence for physiological relevance of this FAK-$p130^{Cas}$ interaction. $p130^{Cas}$ in turn recruits CrkII and Dock180 (Kiyokawa et al., 1998). This signaling molecule is dealt with in Chapter 4. The ability of FAK autophosphorylation site PY297 to recruit phosphatidyl-inositol 3-kinase [PI(3)K] is also likely to lead to Rac1 activation (Rodriguez-Viciana et al., 1997). FAK phosphorylates and associates with G-protein-coupled receptor kinase-interactor (GIT1) a multi-domain protein that binds PIX and p21-activated kinase (PAK) thereby promoting migration (Turner et al., 1999; Zhao et al., 2000). Despite the importance of Rho GTPases for focal adhesion complex formation, presently PAK is the only known effector localized to focal adhesion complexes (via PIX/GIT1): this process is highly dynamic because kinase activation then breaks its association the complex (Zhao et al., 2000). Interestingly PAK is selectively activated by integrin, but not growth factor-induced, Rac1.GTP (del Pozo et al., 2000). As for FAK increasing the level of PAK enhances motility (Sells et al., 1999).

6. SUMMARY - THE WAY FORWARD

The analysis of signal transduction pathways controlled by Rho GTPases has made significant progress but poses many questions, particularly with so many GEFs, GAPs and targets involved in each biological response. How do

cells spatially localize the activation of GTPases? Once activated, how GTPases are able to 'choose' the correct target(s)? We are beginning to build a more detailed picture of how Rho GTPases coordinate cell function, but much of our knowledge is derived from work using cultured cells. It will be a challenge to understand how these processes are coordinated in whole organisms. To this end, some conserved Rho signaling pathways already studied in model organisms such as worms and flies, are beginning to be tackled the context of mammals (see chapter 11). In terms of guidance cues major challenges remain in the central nervous system, where a bewildering array of cues must be provided to ensure reproducible and precise connectivity of axons to target cells. It is already clear in *Drosophila* that Rho regulators such as the Rac1GEF Trio are critical to axonal pathfinding (Lin and Greenberg, 2000).

Pathways that control the actin cytoskeleton downstream of Rho GTPases are often controlled by phosphorylation. To find how this regulation occurs at the subcellular level researchers are already using tools such as phospho-specific antibodies and fluorescently labeled proteins. As more tools become available, investigating the dynamics of several proteins in moving cells is becoming feasible. Sensitive mass spectroscopy should also allow global cataloguing of protein complexes and regulatory phosphorylation sites in important cytoskeletal components.

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Chapter 2

EVOLUTION OF THE HUMAN RHO GTPASE FAMILY

Conservation And Diversity

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Abstract: The Rho GTPases form a relatively old family of signalling proteins, with representation across all branches of the eukaryote lineage. As a consequence of this, there is a large amount of sequence information relating to Rho GTPases in the rapidly increasing number of genome and cDNA databases. This kind of data is never a substitute for 'wet' experimental work in terms of understanding protein function; however, it can tell us something about the history of the proteins we work with. By understanding the history of these signalling proteins, we can more accurately draw together the growing body of experimental work on Rho GTPase function in various genetic model organisms. In this short review we look at the origins of the human Rho GTPase family and attempt to trace the emergence of specific isoforms. We also examine two other factors that contribute to the overall diversity of human Rho GTPase proteins: genetic polymorphism and alternative splicing.

1. INTRODUCTION – THE BIG PICTURE

RhoA, Cdc42 and Rac are by far the best characterised Rho GTPases (Bishop and Hall, 2000), and these are also the oldest and most conserved members of the family. The first Rho GTPase was almost certainly Rac, or a Rac-like protein. Rac is present in Plants (Arabidopsis) and in simple eukaryotes such as Dictyostelium, Leishmania, Entamoeba, Cryptosporidium and Giardia; however, all of these organisms lack a RhoA or Cdc42 orthologue. These two Rho GTPases first appear in fungi, i.e. after the divergence of the plant and animal lineages. The yeasts S. cerevisiae and S. pombe are the two best characterised members of this kingdom, and their RhoA (Rho1p) and Cdc42 proteins fulfil roles that are strikingly similar to

E. Manser (ed.), RHO Family GTPases, 19-29.

the functions of these proteins in higher organisms (Tanaka and Takai, 1998). Somewhat misleadingly, both of these yeasts lack a Rac GTPase; however, Rac is present in a wide range of other fungi (for example the corn smut fungus Ustilago maydis). It would seem that S. cerevisisiae and S. pombe have both lost their Rac gene at some point. Joining these three well-characterised Rho GTPases is another elderly family member – the recently identified Miro proteins. These mitochondrial Rho GTPases are extended to include two C-terminal EF-hands and appear first in plants (Arabidopsis) and fungi (Fransson et al., 2003). Finally, the RhoBTB GTPases are also worth comment for their relative age and unusual structure. The first RhoBTB GTPase identified was the RacA gene in Dictyostelium (Rivero et al., 2001). These proteins are also present in Drosophila and humans, and have an extended C-terminus containing two copies of the BTB/POZ motif – a domain involved in mediating protein-protein interaction. The function of the RhoBTB GTPases is unknown; however, their conservation across such a large section of the eukaryotic lineage suggests that this will be in a fundamental biological process.

In many old families of eukaryotic signalling proteins family size increases ploddingly as genome size increases. One example is the protein kinase C (PKC) superfamily, where a single PKC in S. cerevisiae is duplicated into 5 subfamilies in C. elegans, which are in turn each populated with multiple isoforms in humans (Mellor and Parker, 1998). The situation in the Rho GTPase family is very different. We have previously compared the Rho GTPase complement of a collection of widely differing eukaryotes – S. cerevisiae, C. elegans, Dictyostelium, Arabidopsis, Drosophila, and humans (Wherlock and Mellor, 2002). The overall picture of Rho GTPase evolution is one of plasticity – archetypal Rho GTPases such as Rac, Rho and Cdc42 are well conserved; however, most of these organisms also have divergent Rho GTPases that have no clear orthologues in the other species. Further, the number of Rho GTPases in these species is not correlated to the complexity of the organism or its genome – for example, Dictyostelium has fifteen Rho GTPases, whereas Drosophila has only eight. Expansion of the family has been the result of both gene duplication and retrotransposition there are also clear cases where isoforms have been lost during evolution (Wherlock and Mellor, 2002). This then is a comparatively rapidly evolving family of signalling proteins that has undergone significant recent change.

2. EVOLUTION OF CHORDATE RHO GTPASES

The human Rho GTPase family comprises some 23 genes (Figure 1). To date, only RhoA, Rac1 and Cdc42 have been studied in any detail. While

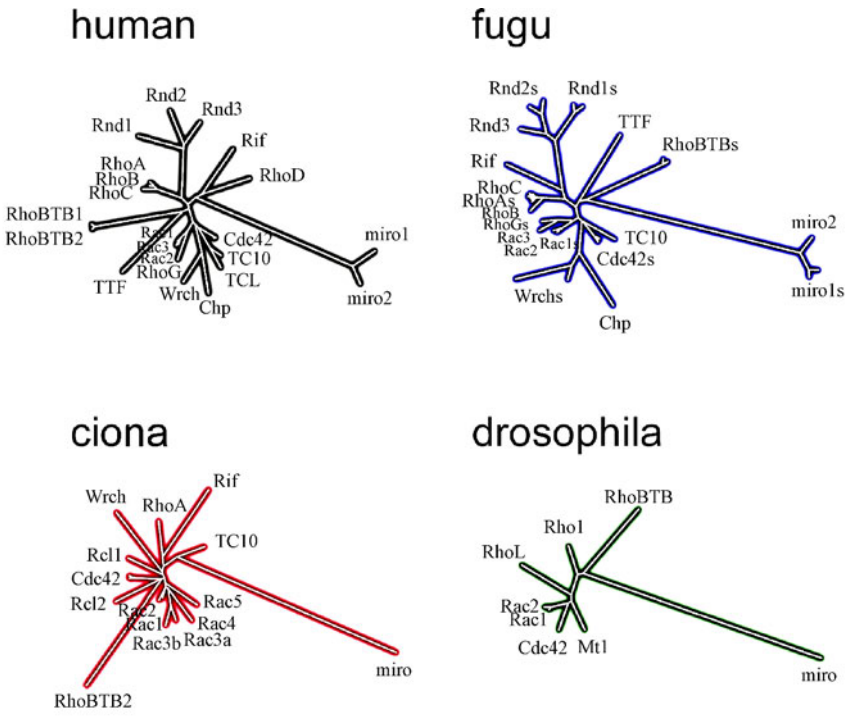

Figure 1. The development of the chordate Rho GTPases. The sequences of human Fugu, Ciona and Drosophila Rho GTPases were obtained as described in the text and aligned using the ClustalW algorithm. Sequence distances are displayed as unrooted dendrograms in TreeView. The existing Rho GTPase nomenclature is inconsistent between species; for example, Rac2 in Drosophila does not correspond to human Rac2. Naming of the Fugu Rho GTPases was based on the human orthologues where possible, and on the closest human homologue where unclear. Fugu have undergone an addition whole genome duplication and duplicated Rho GTPases are referred to in the plural; e.g. Rnd2s. All of the sequences used are available at http://www.bch.bris.ac.uk/staff/Mellor.

we know relatively little about the remaining isoforms, it is tantalisingly apparent that they are involved in a wide range of interesting cellular activities (Wennerberg and Der, 2004). One of the major tasks ahead is to characterise these other poorly understood Rho GTPases, and here it would clearly be helpful to know at what point in evolution these various proteins first appeared. The relatively rapid rate of development of the Rho GTPase family means that little information on the genetic histories of individual human Rho isoforms can be gained from comparing the diverse organisms surveyed in our previous analysis. To get a clearer picture of the evolution of the human Rho GTPases, we compared the development of this family across the chordate lineage.

We selected the invertebrate marine chordate Ciona intestinalis as the start point for our analysis. Ciona is a sea squirt (Ascidiacea), a member of the group of urochords that form the most basal clade of the chordate lineage. Ciona has therefore become an important organism for studies of the origins of modern chordates. The genome of Ciona has been sequenced and found to contain approximately 16,000 genes (Dehal et al., 2002), approximately half the number found in vertebrates. Fort and colleagues have recently completed an extremely thorough analysis of the Rho GTPases of this species (Philips et al., 2003) and we used their sequence information for our comparison. For a midpoint we searched the completed genome database of the tiger pufferfish, Fugu (Takifugu rubripes) (Aparicio et al., 2002) and compiled a complete list of predicted Rho proteins. Human Rho GTPases sequences were obtained previously (Wherlock and Mellor, 2002), with the addition of the recently identified Miro proteins (Fransson et al., 2003). Figure 1 shows the Rho GTPase complements of the three chordates, together with the arthropod Drosophila as a comparison. Figure 2 shows the relatedness of the human Rho GTPases to their orthologues in the three other species.

Comparing the four species, we can begin to untangle the histories of the human Rho GTPases. The most highly conserved proteins are the three oldest Rho GTPases Rac1, RhoA and Cdc42, which change very little across the chordate lineage, and which are also highly conserved in Drosophila. In humans RhoA has two highly related (87%) isoforms; RhoB and RhoC, which probably arose from retrotransposition and gene duplication of the RhoA gene, respectively (Wherlock and Mellor, 2002). Both proteins are clearly present in Fugu, but absent in Ciona. Similarly, humans have three highly related Rac genes (Rac1-3), all three of which are clearly identifiable in Fugu. Things before this are a little misty – Drosophila and Ciona have multiple Rac-like genes; however, it is unclear if any of these are orthologues/prototypes of human Rac2 or Rac3. Five of the six Ciona intestinalis Rac GTPases exist as a tandem array of duplicated genes and this appears to be a recent species-specific addition (Philips et al., 2003). Similar expansions of Rac-like GTPases have also occurred in Dictyostelium and Arabidopsis (Wherlock and Mellor, 2002). Overall, it seems that the division of Rac into the three human Rac-like GTPases occurred relatively late in chordate evolution. The human Cdc42 GTPase gene undergoes alternative splicing to produce two isoforms (discussed later). Interestingly, the organisation of the Cdc42 gene and its splice variants are extremely highly conserved in both Ciona and Fugu. The alternatively spliced Cdc42 is absent from Drosophila. Humans also have a number of Cdc42-like GTPases (TC10, Wrch, Chp, TCL) that interact with an overlapping set of binding partners. These are absent from Drosophila. TC10 and Wrch

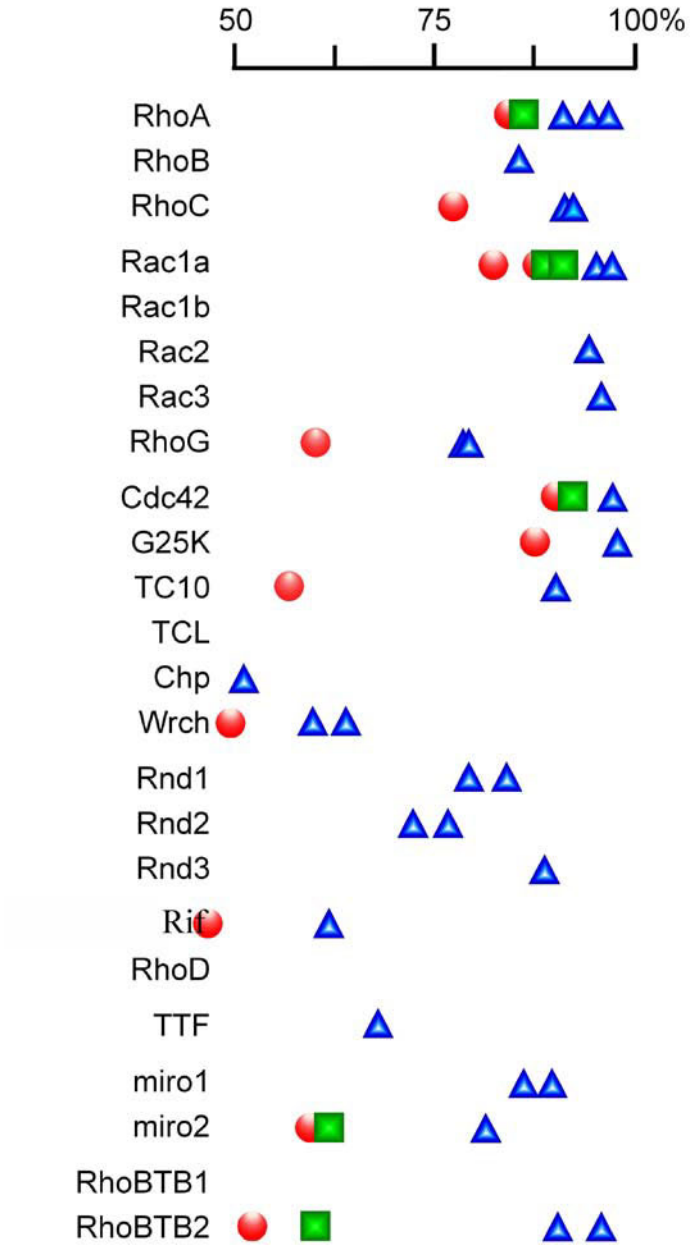

Figure 2. Human Rho GTPase orthologues. The diagram shows the percentage identity between Rho GTPases from Fugu (blue triangle), Ciona (red circle) and Drosophila (green square) and their human orthologue. A missing dot indicates an absence of that Rho GTPase. Double dots in Fugu correspond to duplicated Rho GTPases.

appear first in Ciona and are joined by Chp in Fugu. Ciona also has two Rac/Cdc42-like GTPases; Rcl1 and 2, which appear to be species specific. TCL appears to be the latest addition to the Cdc42-like GTPases, in that it is absent from all species shown here except humans.

Examination of the remaining poorly characterised human Rho GTPases shows that most of these are chordate-specific. Ciona is the first organism in which the Rif GTPase is present. Fugu contains a number of other human Rho GTPase orthologues that are absent from Ciona. The three Rnd GTPases are all present, as are TTF, a second Miro and the intronless RhoG GTPase. It has been suggested that RhoG is the human counterpart of the C. elegans Mig-2 and Drosophila Mtl GTPases; however, this is not supported by the sequence data. It should be noted that the ray-finned fish have undergone an additional whole genome duplication to mammals and as a consequence there are a number of duplicated Rho GTPases present in Fugu. Any duplicated copies of Rac2, Rac3, TC10, Chp, Rnd3, Rif, TTF and Miro2 once present appear to have been lost; divergent copies of the other isoforms have been retained.

Overall, the most striking observation is that the majority of human Rho GTPases are present in fish, and in many cases, also in the primitive sea squirt Ciona. The exceptions are the Cdc42-like TCL GTPase and RhoD – both of which are present here only in humans. We would suggest that the genetically tractable zebrafish (Danio rerio) is therefore a potentially powerful system in which to pursue the functions of the poorly characterised human Rho GTPases. The second key point is how few Rho GTPases are required by Drosophila. The majority of the human Rho GTPases have no orthologue in flies – it seems that we must look for functions of these signalling proteins in processes that are unique (or uniquely different) in chordates.

3. ADDITIONAL DIVERSITY OF RHO GTPASES

The diversity of the human Rho GTPases is further increased by missense mutation. Unlike the Ras GTPases, Rho GTPases rarely manifest clinically significant somatic mutations. Two exceptions are known to date: DBC2 (RhoBTB2) is mutated in breast and lung cancer (Hamaguchi et al., 2002), whereas TTF undergoes translocation in some non-Hodgkin lymphoma and multiple myeloma (Dallery et al., 1995; Preudhomme et al., 2000) and point mutation to its non-coding region in diffuse large-cell lymphomas (Pasqualucci et al., 2001). Germline mutation and polymorphism appears to be a much more frequent source of variation. The best characterised non-synonymous change is the point mutation identified in Rac2; D57N, which

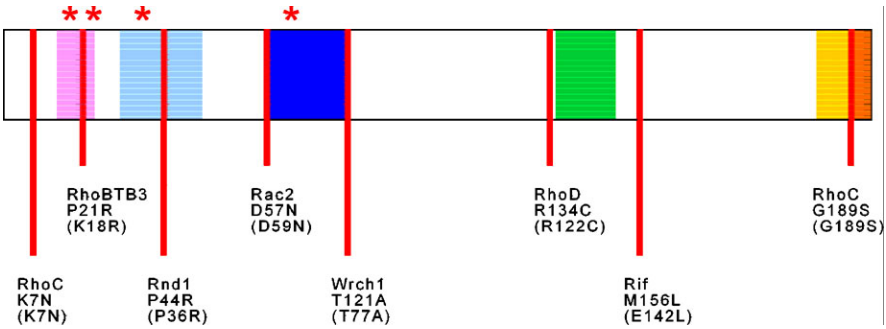

Figure 3. Human Rho GTPase non-synonymous SNPs. A diagram of the primary sequence of RhoA is shown and important regions, drawn to scale, are highlighted: the phosphate binding loop (residues 13-20, RhoA numbering, pink); switch I (residues 27-44, pale blue); switch II (residues 59-77, dark blue); Rho insert (residues 124-136, green); hypervariable polybasic region (residues 182-189, yellow) and the CAAX box (residues 190-193, orange). Residues that are often mutated to generate specific mutants used for functional studies are marked by an asterisk: RhoA G14V and Q63L, both constitutively GTP-bound; RhoA T19N, constitutively GDP-bound, and RhoA F30L which is fast-cycling. All of the available non-synonymous SNPs for human Rho GTPases are mapped schematically on the RhoA diagram. The Rho GTPase for which the mutation has been described is shown as well as the wild-type residue. The numbering of the equivalent RhoA residue is given in brackets below each mutation.

is associated with a human phagocyte immunodeficiency (Ambruso et al., 2000; Williams et al., 2000). The D57N mutation occurs in switch II (D59N RhoA numbering, Figure 3) and results in a decrease in GTP binding due to an enhanced rate of GTP dissociation (Gu et al., 2001). This suggests that D57N Rac2 may act in a dominant inhibitory fashion by sequestering endogenous Rac2 exchange factors (Gu et al., 2001). Many other non-synonymous single nucleotide polymorphisms (SNPs) in human Rho GTPases can be identified from the NCBI database and these are summarised in Figure 4. The biological effect(s) of these SNPs, if any, has not yet been studied and their prevalence is unknown. None actually correspond to the well described mutants – i.e. the GTPase-defective and thus constitutively GTP-bound mutants G14V and Q63L (RhoA numbering); the inactive GDP-bound T39N mutant or the fast-cycling F30L mutant ((Reinstein et al., 1991), (Lin et al., 1997)). Nevertheless, all the SNPs reside within or very close to the important regions of Rho GTPases, namely the phosphate-binding loop, the switch I and II, the Rho insert region and the CAAX box (Figure 3). One of the key goals of post-genomic research is to relate human genetic variation to disease susceptibility – it would seem that investigation of the frequency and functional consequences of these human Rho GTPase SNPs would be a worthwhile research activity.

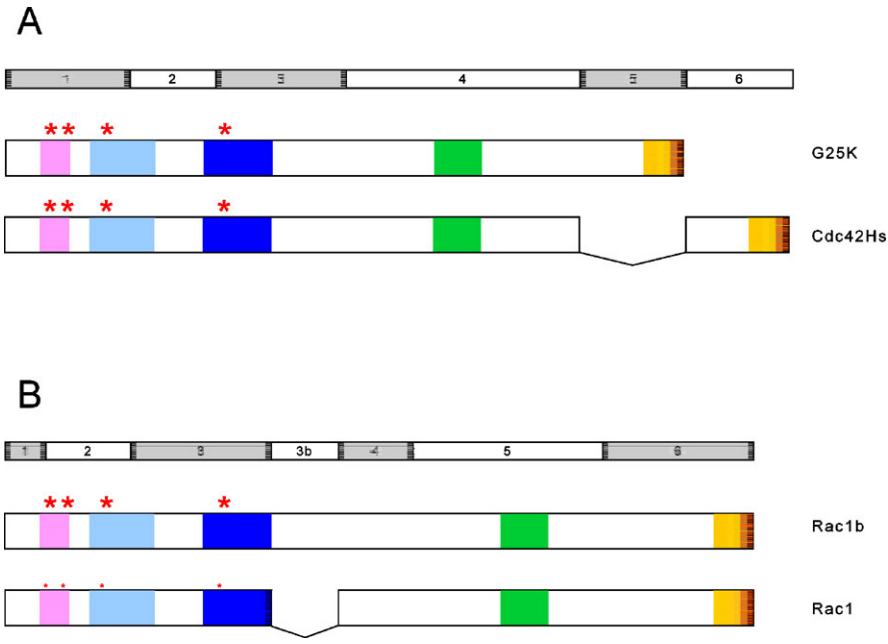

Figure 4. Exon boundaries of Cdc42 and Rac1 splice variants. A diagram of the primary sequences of Cdc42 and Rac1is shown and the important regions, drawn to scale, are highlighted as in Figure 3. Residues that are often mutated to generate specific mutants are marked by an asterisk and include: Cdc42/Rac1 G12V and Q61L, GTPase-defective; Cdc42/Rac1 T17N, GDP-bound, and Cdc42/Rac1 F28L which are fast cycling. The exons are shown above the two splice variants, each exon being represented by a white or grey rectangle. A. The last 29 amino acids at the C-terminus of the two Cdc42 isoforms are encoded by alternative exons: exon 5 for G25K and exon 6 for Cdc42H. The C-termini only differ by 9 amino acids overall and 8 out of the 9 differences occur in the last 10 amino acids. B. Rac1b has an extra 19 amino acids just after the switch II region (VGETYGKDITSRGKDKPIA), which corresponds to the 4th exon usually referred to as exon 3b.

A second way that human Rho GTPase diversity is increased is through alternative splicing. Three isoforms have been reported to exhibit splice variation; Cdc42, Rac1 and TCL. We screened the human EST database for variation in other human Rho GTPases, but were unable to find additional examples. The alternative splicing of TCL appears to be misreported. Two isoforms of mouse TCL/ARHJ (also known as TC10-like, TC10beta or RhoT) have been cloned (Chiang et al., 2002). However, Abe and colleagues (Abe et al., 2003) later showed that the shorter mouse isoform, which lacks 10 amino acids at the N-terminus and corresponds to the mouse TCL cloned by Vignal et al (Vignal et al., 2000), is not generated by alternative splicing and is therefore likely to result from polymorphism or be an artefact. Thus there appears to be only one mouse TCL with a N-terminal extension very similar to that of the unique human TCL first described (Vignal et al., 2000).

Two transcript variants have also been cloned for human Rac1; Rac1 and Rac1b ((Jordan et al., 1999), (Schnelzer et al., 2000)). Rac1b has an extra exon, exon3b, and therefore contains 19 additional amino acids, inserted at the carboxyl terminus of switch II (Figure 3b). The Rac1b splice form is not present in Fugu or Ciona and in humans has only been detected in carcinoma cell lines ((Jordan et al., 1999), (Schnelzer et al., 2000)). This had led to the hypothesis that Rac1b is an oncogenic variant. The alternatively spliced form is constitutively active ((Matos et al., 2003), (Fiegen et al., 2004)) but interacts with a only small subset of Rac1 effectors (Matos et al., 2003). Its role in oncogenesis is unclear at present.

Finally, perhaps one of the most interesting and overlooked aspects of the Rho GTPase literature is the alternative splicing of human Cdc42 (Figure 3a). Cdc42Hs or transcript variant 1 (NM_001791) was originally cloned from a placental cDNA library (Shinjo et al., 1990) whereas Munemitsu and colleagues (Munemitsu et al., 1990) isolated G25K from a foetal brain library (transcript variant 2, NM_044472). The last 29 amino acids at the C-terminus of the two Cdc42 isoforms are encoded by alternative exons (Figure 3a). Eight out of the nine differences between the two corresponding proteins occur in the last ten amino acids. The exon/intron pattern of the Cdc42 gene is strikingly conserved in Fugu and Ciona, as are the corresponding protein sequences of the two splice forms. The conservation of these proteins across the chordate lineage suggests that there are significant biological differences between the two splice variants. Currently we have no information as to what these may be. No distinction is made between these two proteins in the bulk of the Rho GTPase literature and sequencing of the Cdc42 constructs in common circulation shows that these comprise a random mixture of Cdc42Hs and G25K isoforms. The C-terminus of Rho GTPases often directs cellular localisation – a key aspect of Cdc42 function. It would seem that there is important unfinished business in the characterisation of these two Cdc42 isoforms.

4. SUMMARY

The majority of work on human Rho GTPase signalling has focussed on the three big players of the family – RhoA, Rac1 and Cdc42. It is easy to overlook the fact that these proteins make up only a fraction of the human complement of Rho GTPases. Analysis of the evolution of the Rho GTPase family shows that most of the uncharacterised human Rho GTPases appear during the development of the chordate lineage – we suggest that we should look to chordate specific functions to find roles for these poorly characterised human Rho GTPase isoforms.

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Chapter 3

STRUCTURAL ANALYSIS OF RHO PROTEIN COMPLEXES

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Abstract: The structural GDP/GTP cycle of Arf proteins is implemented by switch regions that change their conformations in response to the nature of their interactions. They include the classical switch regions in the nucleotide-binding site, and a unique switch region located on the opposite side of the protein. These switch regions communicate with each other and couple activation of Arfs by GTP to their recruitment to membranes. Variations in this cycle determine the specificity and differences in Arf1 and Arf6 signaling. The role of guanine nucleotide exchange factors (GEFs) as key players in the implementation of the nucleotide/membrane coupling is also described. A particular emphasis is given to the inhibition of GEF-activated nucleotide exchange by the drug Brefeldin A and by a GEF point mutation, as tools to trap intermediate states of the reaction. Taken together, structural and biochemical studies yield a comprehensive model for one of the most remarkable nucleotide cycle found in G proteins.

1. INTRODUCTION: RHO FAMILY PROTEIN STRUCTURES

The Rho family were originally identified by their similarity to the Ras protein (Rho = Ras homology). They differ only from the latter by an extra sequence, known as the insert region. Extensive studies on the Ras family members, particularly Ras itself, have established the basic fold of the small G proteins both in the active and inactive forms (reviewed in Kjeldgaard et al., 1996) and (Vetter and Wittinghofer, 2001)). The core of the 20 kDa domain consists of 5 β-strands, which form a mixture of parallel and anti-parallel β-sheet, surrounded by five α-helices. The overall topology is

E. Manser (ed.), RHO Family GTPases, 31-72.

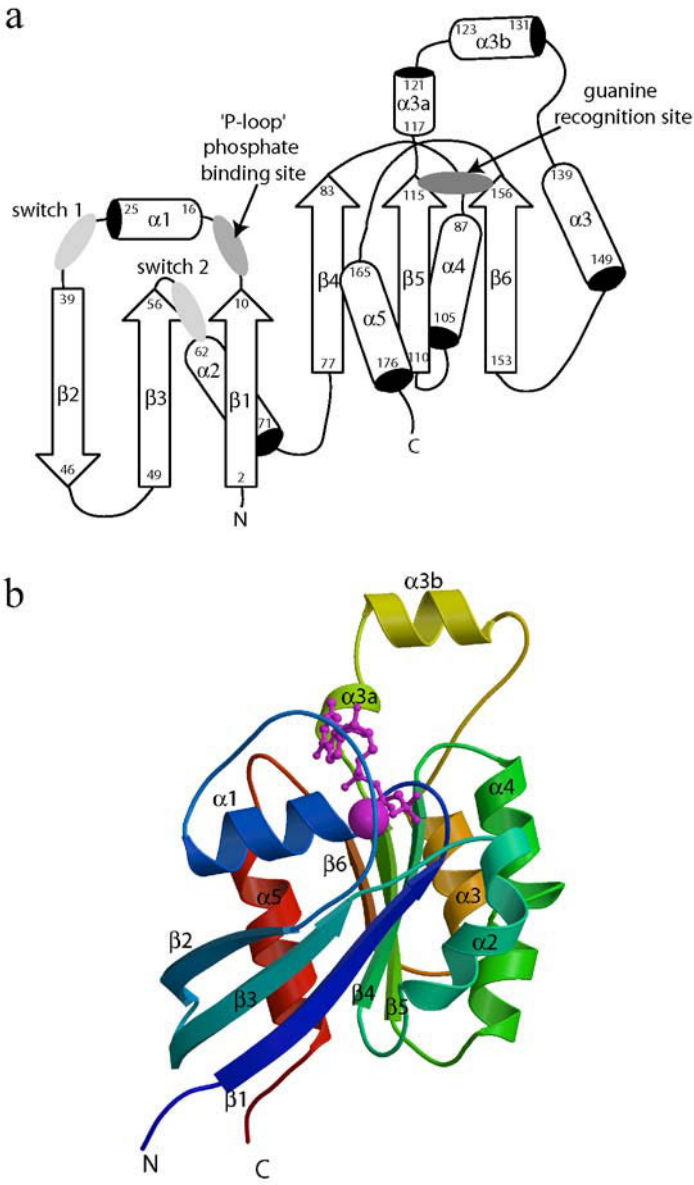

Figure 1. **a**. Topology diagram of Rac1.β-strands are represented by arrows and α-helices as cylinders. The number of the first and last residue in each secondary structure element is labelled. The main regions involved in nucleotide binding and the nucleotide sensitive switches are indicated by ovals and are labelled accordingly. **b**. Structure of Rac1 bound to GMPPNP. Rac1 is coloured from blue at its N-terminus through to red at its C-terminus, GMPPNP is shown in pink and the Mg^{2+} ion is represented by a pink ball. The secondary structural elements are labelled as in figure 1a. All figures were produced using Molscript (Kraulis, 1991) and Raster 3D (Merritt and Bacon, 1997).

similar to that of most nucleotide binding proteins. All the small GTPases share certain common features that are involved with binding and specificity for GTP and GDP (Figure 1a). The phosphate binding site (β1-α2 loop or P-loop) has the consensus sequence GxxxxGKS/T. The Ser/Thr sidechain of this sequence forms a hydrogen bond with the γ-phosphate of the nucleotide. The other major consensus sequence for nucleotide binding is the guanine recognition site, that, as its name implies, binds the guanine base and has the consensus sequence N/TKXD. The major differences between the GDP- and GTP-bound forms lie in two loops known as switch 1 (or the effector loop) and switch 2. These switches change conformation dramatically when GTP and its analogues bind, due to the formation of hydrogen bonds involving Thr-35 and Gly-60 (Rac1/Cdc42 numbering) and the γ-phosphate. The switch regions are relatively unstructured and are often absent or highly flexible in X-ray and NMR derived structures. The formation of the new interactions with GTP is thus sufficient to change both the dynamics and structure of the loops. This results in a reorientation of the effector loop, allowing it to adopt a conformation where it can interact with downstream effector proteins.

The first Rho family protein structure solved was that of Rac1, bound to the GTP analogue GMPPNP (Hirshberg et al., 1997). This structure showed that the insert region forms 2 extra α-helices (denoted α3a and α3b), which are mobile and highly exposed on the surface of the molecule (Figure 1b). Subsequent structures of RhoA.GDP, RhoA.GTPγS (Wei et al., 1997), (Ihara et al., 1998) and Cdc42 (Feltham et al., 1997) showed that the insert region is not sensitive to the bound nucleotide. Finally, a mutagenesis study of Rac, with the insert region deleted, showed that removal of these residues had no effect on the overall structure of the protein (Thapar et al., 2002).

2. RHOGDI COMPLEXES

The Rho and Rab families of the Ras superfamily are unique in their utilization of guanine nucleotide inhibitors (GDIs) for regulation. RhoGDIs are negative regulators of the Rho family that have three distinct biological functions; they block the dissociation of guanine nucleotides from Rho, Rac and Cdc42 (Leonard et al., 1992), (Fukumoto et al., 1990), they inhibit GTP hydrolysis on the G proteins (both intrinsic and GAP stimulated)(Hart et al., 1992; Chuang et al., 1993), and they are able to mediate the release of G

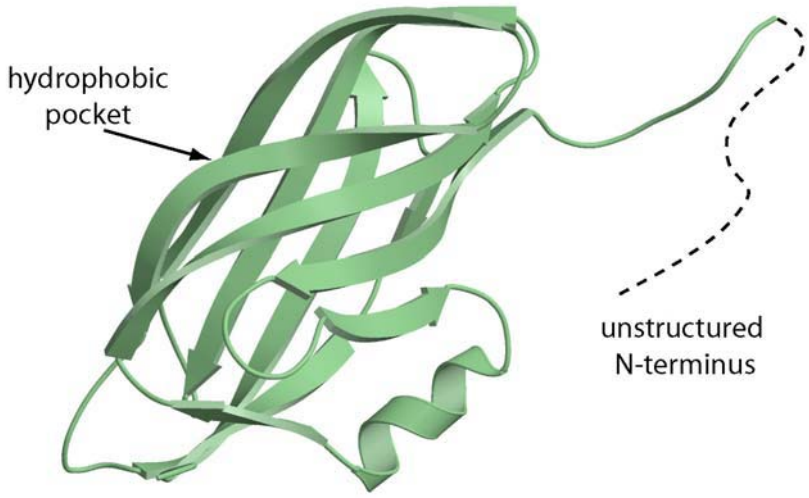

Figure 2. Structure of RhoGDI Ribbon representation of RhoGDI showing the Ig fold of the C-terminal domain, containing the hydrophobic pocket. The unstructured 60 residues at the N-terminus are represented as a dashed line, although their precise location is not fixed.

proteins from membranes (Bokoch et al., 1994). Three Rho family GDIs have been identified; the ubiquitously expressed Rho-GDI (RhoGDI-1) that has broad specificity for Rho family members (Ueda et al., 1990), LyGDI (D4GDI or RhoGDI-2) that is expressed primarily in haematopoietic cells but retains broad specificity (Scherle et al., 1993), (Lelias et al., 1993) and RhoGDI (RhoGDI-3) that is only active towards RhoB and RhoG and in contrast to the other, cytosolic GDIs is membrane associated (Zalcman et al., 1996). Two structures of free RhoGDIs have been solved (Keep et al., 1997), (Gosser et al., 1997) and four X-ray crystal derived structures are available of Rho family proteins in complex with their GDIs; Rho A/RhoGDI (Longenecker et al., 1999), Cdc42/RhoGDI (Hoffman et al., 2000), Rac2/LyGDI (Scheffzek et al., 2000) and Rac1/RhoGDI (Grizot et al., 2001). Uncomplexed RhoGDI has been shown to consist of two distinct domains (Figure 2). The C-terminal domain comprises the final 140 residues of the protein and adopts an immunoglobulin-like fold consisting of two anti-parallel β-sheets packed against each other to form a β-sandwich. The β-sandwich is closed at the top and bottom by loops and on one side by a short 310 helix. The fourth side is solvent exposed and contains a deep pocket between the β-sheets that is largely hydrophobic, with a patch of negative potential at its opening (Gosser et al., 1997), (Keep et al., 1997). This pocket was proposed to be the binding site for the C-terminal

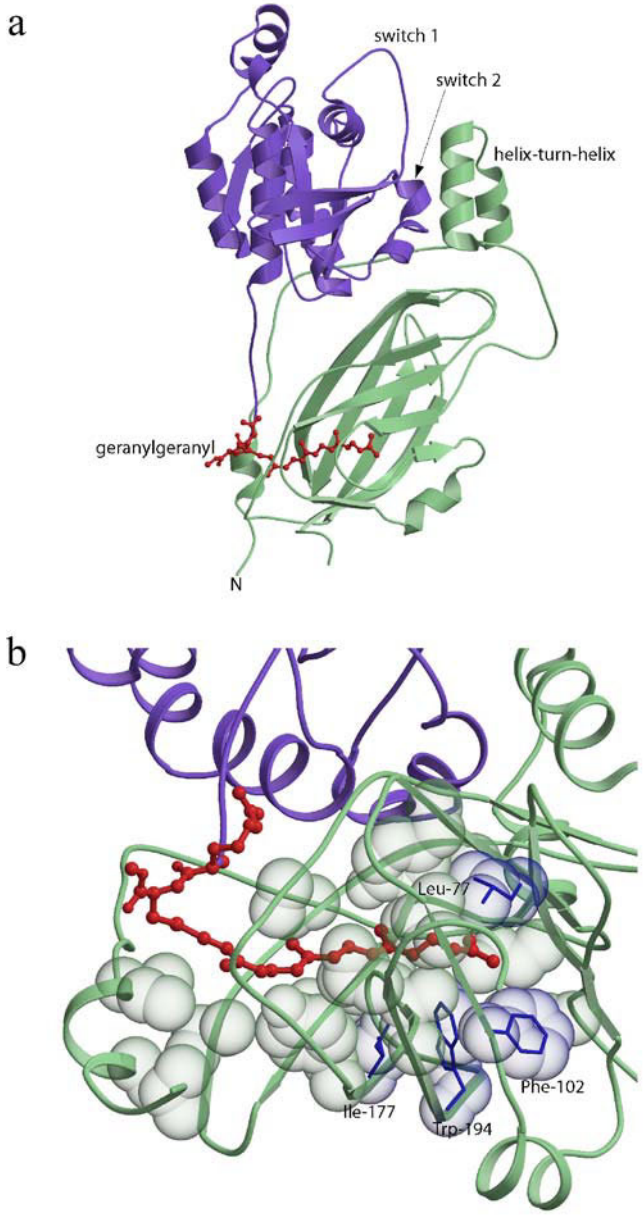

Figure 3. **a**. Structure of RhoGDI complexed with Cdc42. Cdc42 is shown in purple (top) with the switch regions labelled, RhoGDI surrounds the geranylgeranyl group (red ball and spoke). The lipid moiety inserts into the C-terminal Ig domain of RhoGDI. The other major interaction is between the N-terminus of RhoGDI and switch 2. **b**. Expanded view of the lipid-binding domain of RhoGDI. Cdc42 elements are shown in blue with the geranylgeranyl moiety ball and spoke is coloured red. The C-terminal Ig domain of RhoGDI is shown in green with side-chains that contact the lipid moiety in a cpk representation. Residues referred to in the text are coloured blue and are shown as CPK overlaid with a stick representation.

geranylgeranyl modification on the G protein (Gosser et al., 1997; Keep et al., 1997), (Lian et al., 2000). The N-terminal 60 amino acids are flexible and largely unstructured, although NMR studies indicated the tendency of residues 48-58 to form a helix (Lian et al., 2000). This region was shown, however, to contribute both to G protein binding and to be necessary for inhibiting nucleotide dissociation (Lian et al., 2000; Gosser et al., 1997).

The structures of the GDIs in complex with their cognate, prenylated G protein have gone a long way to explaining many of the functions of GDI action. The first structure of a GDI/G protein complex was a low-resolution structure of RhoA/RhoGDI (Longenecker et al., 1999). This structure revealed for the first time that the N-terminal domain of the GDI is bound to switch 1 and 2 of RhoA, providing a possible explanation for the nucleotide dissociation inhibitor activity of the molecule. This structure however did not show the C-terminal residues of RhoA or the lipid modification, leaving the details of this important interaction to later high-resolution structures. Three high-resolution structures are available; Cdc42/RhoGDI (Hoffman et al., 2000) and two representations of Rac/RhoGDI (Scheffzek et al., 2000; Grizot et al., 2001) (Figure 3a shows the Cdc42/RhoGDI complex). These structures confirm the two domain architecture of the GDI and reveal that the N-terminal domain becomes ordered in the presence of the G protein. The N-terminal domain contains a helix-loop-helix motif (residues 35-55, Cdc42/ RhoGDI numbering) which interacts with both switch 1 and 2 of the G protein. The two helices in the N-terminal domain have complimentary hydrophobic surfaces that interact to stabilize the structure and provide a binding surface for the G protein. The extreme N-terminus (residues 5-25) forms a weakly ordered extended loop that contains a short α-helix (residues 10-15) that contacts the C-terminal domain. Leu-11 and Ile-14 form a hydrophobic patch on the N-terminal helix that functions to cover the geranylgeranylbinding pocket (Hoffman et al., 2000) (Grizot et al., 2001). The first 21 residues in the Rac/LyGDI structure are flexible and therefore not visible, indicating that differences may occur between the different GDI/G protein complexes (Scheffzek et al., 2000). The contacts made between switch 2 and the GDI N-terminal domain make the largest contribution to the interaction, with the exception of the lipid-binding cavity. Tyr-64 forms hydrogen bonds to two conserved residues in the GDI (Asp-42 and Lys-51 in RhoGDI) while Arg-66 bridges the two GDI domains, by making hydrogen bond contacts to Asp-185, Pro-30 and Ala-31. Leu- 67, Leu-70 and Pro-73 in the G protein provide hydrophobic interactions with residues in the helix-turn-helix motif at the N-terminus of the GDI. The switch 2 regions of the Rho family are more similar in the GDP and GTP bound forms than for example switch 2 in the Ras family proteins (Hoffman et al., 2000). It is possible therefore that the extensive contacts between

switch 2 and the GDI (Figure 3a) provide a mechanism for GDI to bind to both inactive and active forms of the G protein.

The C-terminal, Ig-like domain of GDI (residues 74-204 in RhoGDI and 59-203 in LyGDI) is largely unchanged in the complex when compared with the free GDI. Rho family/RhoGDI interactions require isoprenylation of the Rho protein but only the Cdc42/RhoGDI and Rac1/RhoGDI structures have direct data pertaining to the lipid moiety binding. These structures show that the geranylgeranyl- moiety inserts, as predicted, into the hydrophobic pocket in the C-terminal GDI domain. The pocket is lined with hydrophobic residues that form complimentary contacts along the length of the isoprenoid (Figure 3b). The Cdc42/ RhoGDI structure also elegantly explains the 20-fold decrease in affinity seen between Cdc42/RhoGDI-1 and Cdc42/ RhoGDI-2 (Platko et al., 1995). RhoGDI-2 (Ly-GDI) has an Ile Asn substitution at residue 177RhoGDI-1 that lies at the centre of the geranylgeranylbinding pocket: the presence of a polar side chain would be disruptive to binding of the hydrophobic lipid. Although the overall fold of the Ig domain remains unaltered on G protein binding, differences are observed in the lipid-binding cavity. The floor of the recess expands due to a displacement of Trp-194, Leu-77 and Phe-102, and a shift in strands β1, αH and the 310 helix (Figure 3b). The combination of these changes opens a constriction at the base of the pocket and deepens it, providing space to accommodate the entire lipid. This provided proof of previous observations made by NMR spectroscopy indicating changes in the lipidbinding cavity on G protein binding (Gosser et al., 1997). The paucity of contacts between the IgG-like domain and the G protein confirmed the lack of nucleotide dissociation inhibition activity associated with this domain.

A feature of many G proteins is the presence of a short sequence of basic residues directly preceding the isoprenylated C-terminal cysteine. These amino acids are thought to act as a secondary membrane-anchoring signal for the G protein by interacting with the acidic head groups of the membrane lipid bilayer. An acidic patch was identified in both GDI/G protein complexes at the neck of the lipid binding cavity, which was demonstrated to interact with the basic residues of Cdc42 in the Cdc42/RhoGDI structure. It is likely that this acidic patch on the GDI aids membrane release of the G protein by competing with the lipid head groups for the basic region of the G protein. Kinetic studies on the association of RhoGDI-1 with membrane bound Cdc42 have described a two-step mechanism (Nomanbhoy et al., 1999; Newcombe et al., 1999). The first stage is a rapid association of the two proteins, which is followed by a second, slow, isomerization that is the rate-limiting step in the dissociation of Cdc42/RhoGDI from the membrane. This scenario can be explored in relation to the structures of the GDI/G protein structures. The G protein, located at its target membrane, would have its geranylgeranyl- moiety inserted into the lipid bilayer and its basic amino

acid patch, preceding the lipid attachment site, would interact with the membrane head groups. The initial fast stage of the interaction would be mediated by the interaction between the incoming N-terminal domain of the GDI with the switch regions of the G protein. This would juxtapose the GDI lipid-binding cavity with the membrane surface in readiness to initiate the second, slow phase of binding, when the geranylgeranyl- moiety exchanges its membrane domicile for the hydrophobic environment of the GDI C-terminal domain. Thus, the lipidated C-terminus of the G protein would never contact the aqueous cytosol.

Both high-resolution complex structures report the similarity between the GDI-bound G protein and the GDP-bound free forms. Notably, Cdc42/ Rac Thr-35 is positioned such that its sidechain is solvent exposed and available to interact with the GDI. This allows the main chain carbonyl of Thr-35 to co-ordinate with the Mg^{2+}, which enhances the affinity of the G protein for the nucleotide. While it was originally thought that GDIs interact exclusively with the GDP form of the G protein it has been shown that the active form of G proteins can bind GDIs with equal affinity (Nomanbhoy and Cerione, 1996) although possibly the GDI has a decreased activity towards the GTP bound form (Sasaki et al., 1993). As the Rac/GDI complex would require significant rearrangement to accommodate Rac.GTP, an explanation for the equally high affinity of GDI for GDP or GTP forms awaits the structure determination of GDI complexed with an activated form of a G protein.

A previous study had indicated the importance of the Rho-family insert loop in GDI mediated nucleotide dissociation inhibition (Wu et al., 1998). The structures of both GDI complexes show that the insert loop of the G proteins is distant from the RhoGDI interface (Figure 3a) suggesting that this defining region of the Rho family is not involved in the GDI interaction. However, it has been suggested that the insert loop stabilizes the proximal TQID motif of Cdc42 that co-ordinates the guanine base of the nucleotide, providing a plausible explanation for the previous observations (Hoffman et al., 2000).

These structures have provided vital details pertaining to the action of RhoGDIs and the regulation of Rho family proteins. Still to be uncovered is the mechanism by which the RhoGDI/G protein complex dissociates to allow activation of the G protein by a GEF. A clue to this comes from the work that has demonstrated the direct interaction of ERM proteins with RhoGDI, resulting in a decrease in their GDI activity towards G proteins and a concurrent increase in the activity of any GEFs present (Takahashi et al., 1997). RhoGDI/G protein complexes have also been shown to have some signalling capacity e.g. with lipid kinases, although the precise nature of this interaction is yet to be elucidated, with various phospholipids enhancing the release of G protein from a GDI complex (Tolias et al., 1998; Faure et al., 1999). Other evidence indicates a role for RhoGDIs in both the activation of

NADPH by Rac, in exocytosis (Olofsson, 1999) and in phospholipase C stimulation (Illenberger et al., 1998). It thus appears that the categorization of GDIs as simple negative regulators of the Rho family proteins is premature.

3. RHO FAMILY GEFS

In order to transmit an incoming signal the G proteins have to exchange their bound GDP for an activating GTP. The main purpose of exchange factors, in structural and mechanistic terms, is to stabilize the nucleotide free state of the G protein while the nucleotide exchange takes place. Such a state is a necessary intermediate in the exchange process and is likely to be extremely unstable. The exchange factors for the various subfamilies of small G proteins do not show any significant sequence homology, implying that there is no common, unifying mechanism by which they perform their role. Recently, the structures have been determined for exchange factors for the Ras (Boriack-Sjodin et al., 1998), Arf (Goldberg, 1998) and Ran (Renault et al., 2001) families with their cognate GTPases, which confirm the diversity in their mode of action.

3.1 Structures of free GEFs

The exchange factors for the Rho family proteins all contain a unique domain of approximately 200 amino acids, the Dbl homology (DH) domain. The minimum unit for a Rho family GEF is usually a DH domain with a pleckstrin homology (PH) domain immediately C-terminal to it (reviewed in (Schmidt and Hall, 2002)). Since PH domains are known to bind to phosphoinositides, it was presumed that the PH domain was required for membrane localization. The first structural data on DH domains were two NMR structures of isolated DH domains, one from βPIX (Aghazadeh et al., 1998) and one from Trio (Liu et al., 1998), and an X-ray structure of a the DH-PH pair from the protein Sos (Soisson et al., 1998). These structures showed that the DH domain is a flattened elongated molecule that is unrelated to the exchange factors for the other small GTPases. The DH domain of βPIX contains a total of 11 α-helices, which are arranged into a pseudo five-helix bundle, with the five segments of the bundle being made

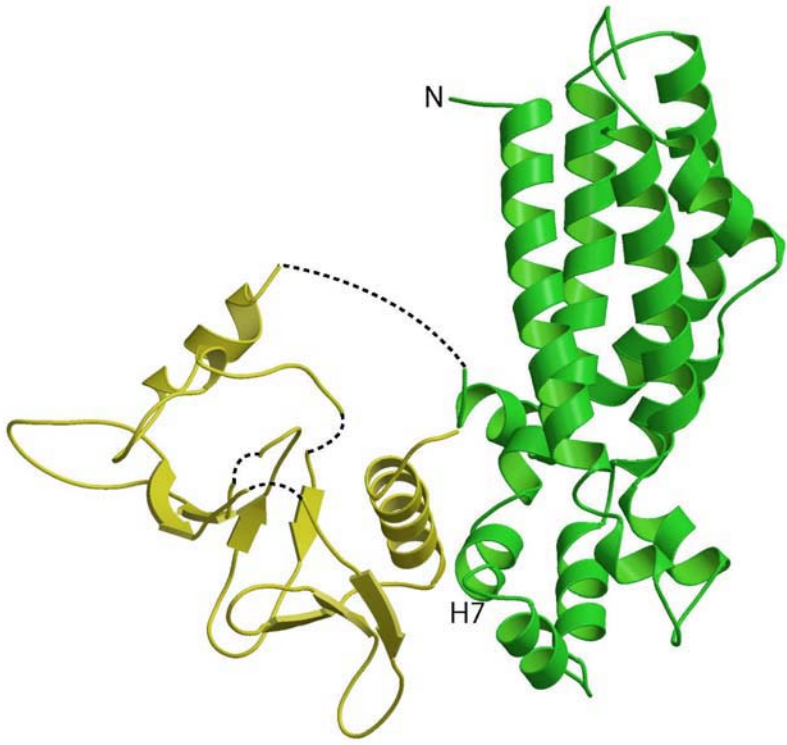

Figure 4. The structure of the DH/PH domain from Sos. DH domain is shown in green and the PH domain in yellow. Regions that were absent in the electron density map are shown as dotted lines. The DH helix, H7, which contribute most to the interface with the PH domain, is labelled.

up of; helix A, helix D, helices E, F and G, helices I and J and helix K. The remainder of the helices (B, C and H) are shorter and are arranged at one end of the bundle. The structure of Trio is essentially the same, although the nomenclature and length of the smaller helices varies. Sequence analysis of the DH domains had shown that there are three highly conserved regions, CR1, CR2 and CR3. These three regions are within the pseudo five-helix bundle and comprise helices A, D and I/J. The βPIX DH domain appears to fold independ-ently of the PH domain and examination of the DH/PH pair by NMR has shown that the interactions between the two domains are minimal (Aghazadeh et al., 1998). This is not surprising, since a splice variant exists that lacks the PH domain. A similar study of the Trio DH/PH interactions, again by NMR, gave a similar result: the chemical shifts of the DH domain in the isolated DH and in a DH/PH pair were essentially the same (Liu et al., 1998). It was thus unexpected when the first structure of a

DH/PH pair, that from Sos, revealed that the two domains interacted significantly, burying 1,100 Å2 (Figure 4) (Soisson et al., 1998).

The interaction site on the DH domain involves the end of the bundle where the shorter helices are arranged, the helix inDH that contributes most to the interaction being H7 (equivalent to helix H in βPIX). In this structure, the linker between the two domains is disordered and thus invisible in the electron density map, but the linker length of 13 amino acids means that only one possible DH/PH pair in the crystal lattice is possible. Interestingly, the residues in the DH and PH domains that interact with each other are the least conserved between different Rho family exchange factors. This raises the possibility that the interaction between the DH and PH may change significantly in different proteins and may have an effect on the specificity of G protein binding. One other notable feature from the Sos DH/PH structure is that the PH domain contains extra sequences at the N-terminus relative to PH domains in other proteins. In Sos, the extra residues contain an α-helix, a β-strand and a turn of 310 helix. These structural elements were packed against the remainder of the PH domain and although they were predicted to be present in all DH/PH pairs, they adopt different secondary structures in different GEFs.

3.2 Rho family/GEF complexes

The first structure of a Rho family protein/GEF complex solved was that of Rac1 with its specific exchange factor Tiam1 in a complex comprising nucleotide-free Rac1 and the DH/PH domains of Tiam1 (Worthylake et al., 2000). The interface between Rac and its GEF is extensive, burying a surface area of more than 3,000Å2. The regions of Rac1 involved in the interaction are primarily switch 1 (25-39) and switch 2 (57-75) and those of Tiam1 are in or near the conserved regions CR1 (1040-1066) and CR3 (1180-1207) (Figure 5a). The DH domain in Tiam1 has essentially the same structure as in the free GEFs that have been solved, with some minor changes in the C-terminal α-helix, which has a kink in Tiam1 that is absent in the other structures. The PH domain of Tiam1, like that in the other DH/PH proteins, contains extra residues at the N-terminus, which in this case form a 310 helix.

The P-loop of Rac1 (residues 10-17), the region required for binding the phosphate groups of the bound nucleotide, is not directly in contact with Tiam1. This is similar in the other G protein/GEF complexes solved and suggests that the GEF does not directly alter the P-loop/nucleotide interactions. The helical insert region of Rac does not make any contacts

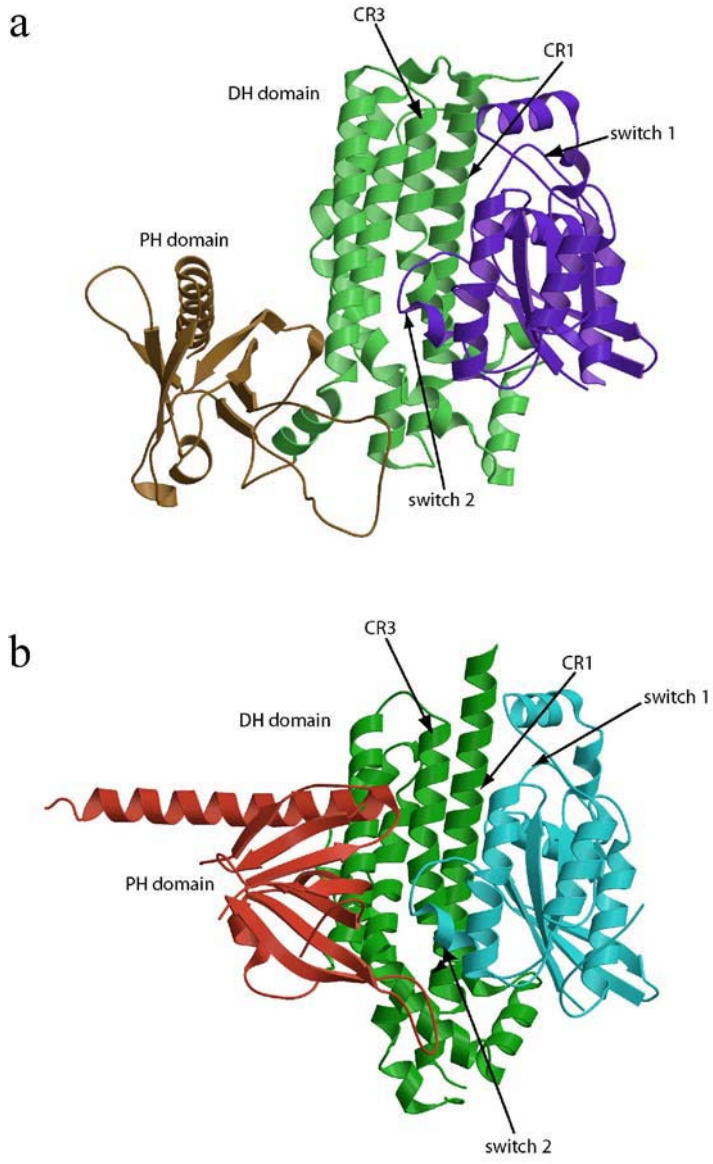

Figure 5. **a**. Structure of the Tiam1/Rac1 complex. Rac1 is shown in purple with switch 1 and 2 labelled. The DH domain of Tiam1 is shown in green with the PH domain in brown. The helices that comprise the CR1 and CR3 are involved in the interactions with the G protein. **b**. Structure of the Dbs/Cdc42 complex. Cdc42 is shown in blue with switch 1 and 2 labelled. The DH domain of Dbs is shown in green, with the PH domain in brown. As in the Tiam1/Rac1 structure, the CR1 and CR3 helices are the major site of interaction between the proteins. The reorientation of the PH domain with respect to the DH domain, in comparison with the Tiam1 PH domain (panel a), can be seen.

with Tiam1, implying that the insert is not involved in the mechanism of nucleotide exchange. Switches 1 and 2 make the major contacts with Tiam1, although the β1, β2 and β3 strands, which lie close to the switches in the G protein (Figure 1), are also involved in the interface. The interaction between Tiam1 and switch 1 results in a shift of switch 1 relative to the nucleotide binding cleft. This shift has several consequences that make the binding of nucleotide unfavourable. Firstly, the essential Thr-35, whose sidechain hydroxyl group usually co-ordinates the Mg^{2+}, is shifted relative to the GDP and GMPPNP-bound forms of Rho family proteins, thus disrupting the metal ion co-ordination. The shift in switch 1 also results in the movement of the sidechain of Ile-33 such that it would sterically clash with the ribose moiety, preventing nucleotide access to the binding pocket.

The movement of Ile-33 also alters the position of the sidechain ofCys-18, which forms a hydrogen bond with the γ-phosphate in the nucleotide-bound form. Phe-28, also in switch 1, interacts with the guanine nucleotide: the position of this residue is shifted in the Tiam1 complex. The interaction with Tiam1 causes a shift in residues in switch 2, leading to a rearrangement of Ala-59, which moves to block the magnesium-binding site. Other residues in switch 2 that are affected include Gly-60, Gln-61 and Glu-62, which form hydrogen bonds with each other and with Lys-16 from the P-loop, partially blocking the nucleotide binding pocket.

3.3 Role of the PH Domain

In the Tiam1/Rac complex, all of the interactions between the G protein and the exchange factor are made through the DH domain: the PH domain does not make any contacts with the G protein. The orientation of the PH domain relative to the DH domain is, however, significantly different to the orientation of the DH/PH pair that was observed in the structure of Sos. This difference may be indicative of a general variation in orientation of these domains in the different proteins. The PH domain in the Tiam1 complex is thought to be required for structural stabilization of the protein, since its removal radically reduces the in vitro exchange rate i.e. it has a structural rather than functional role. In addition, the evidence that binding of phosphoinositides by the PH domain affects the exchange activity by the GEFs is controversial.

The idea that the PH domain is a bystander in the exchange process was radically changed when the structure of another GEF, Dbs, was solved in a complex with Cdc42 (Rossman et al., 2002). The Dbs/Cdc42 buried surface area is similar in size to that between Tiam1 and Rac and the relative orientations of the G protein and DH domains are almost identical, as are the changes in switches 1 and 2 caused by the GEF binding. The PH domain of Dbs, like Sos, has an extra β-strand and a turn of 310 helix compared to

canonical PH domains. In this complex, unlike in Tiam1/Rac, the PH domain directly contacts the G protein (Figure 5b). This interaction is achieved by a shift in the orientation of the PH domain with respect to the DH, which allows it to contact switch 2. The crystal structure highlighted an essential residue in the Dbs PH domain, Tyr-889, which in involved in both Cdc42 and DH domain interactions. Mutation of this residue in Dbs resulted in a loss in the transforming ability of this GEF in vivo (Rossman et al., 2002). The importance of Tyr-889 seems to hinge on its ability to stabilize His-814 within the DH domain, allowing it to make the necessary interaction with Asp-65 in switch 2. A structure of the Dbs/RhoA complex has shown that in this case the PH domain also contacts the GTPase, while the structure of a third GEF/G protein complex, Intersectin/Cdc42, shows no such interaction (Snyder et al., 2002). Thus, it remains to be seen whether direct contact by the PH domain is a feature peculiar to the Dbs interaction or will be found in other exchange factors.

The lack of concrete evidence for the effects of inositol phospholipids on the exchange rate of the DH/PH proteins is interesting, given the role of PH domains in membrane localization. One reason for the lack of effect of phospholipids may be that many experiments have been performed in vitro. It has been suggested that the role of inositol phospholipid interaction is to orient the PH and DH domains in the correct position with respect to the G protein, which is attached to the membrane via a geranylgeranyl moiety (Rossman et al., 2002). This membrane positioning may be aided by the electrostatic surface: in the Dbs/Cdc42 complex, the putative membrane-proximal surface is positively charged and the membrane-distal surface is negative. Such a charge dichotomy would necessarily aid orientation.

3.4 Discrimination between G proteins

The switch regions of Rac1 are stabilized by the GEF into a conformation that forces the disruption of both Mg^{2+} and guanine nucleotide interactions. The discrimination between the different Rho family proteins by the GEFs may lie, however, outside the switches, since these are highly conserved between Rho, Rac and Cdc42. One suggestion was that the discrimination lies within the less-conserved β2 and β3 strands, which also engage the GEF, although they do not appear to be involved in the mechanism of nucleotide exchange (Worthylake et al., 2000). It is interesting to note that the regions of the GEFs that contact the β2 and β3 strands also vary between different exchange factors, which is in agreement withthis hypothesis. Work with Cdc42/Rac chimaeras and mutagenesis of Rac highlighted Trp-56within theβ3 strand as a potential hotspot for specificity for the exchange factors TrioN,GEF-H1 and Tiam1. Furthermore, mutation of the equivalent residue in Cdc42, Phe-56, toTrp is sufficient to allow these Rac-specific

GEFs to exchange the nucleotide on Cdc42 as efficiently as wild-type Rac (Gao et al., 2001). Similarly, mutation of Trp-56 in Rac to Phe allowed it to bind the Cdc42 specific GEF, Intersectin (Karnoub et al., 2001b). Trp-56 is however, conserved between Rac1 and RhoA, so this residue cannot be the sole determinant of specificity. A detailed structural study of Cdc42, Rac1 and RhoA with Intersectin and Dbs has defined a 'specificity patch' on the G proteins, which includes residues from β1 (residue 3, Cdc42/Rac numbering), β2 (residues 41 and 43) and β3 (residues 52, 54 and 56) (Snyder et al., 2002). These 6 residues are thought to hold the key to the discrimination of the various GEFs for Cdc42, Rac or Rho. Residues in Intersectin that interact with this specificity patch were then mutated and its specificity was changed so that it could promote exchange on either RhoA or Rac1.

3.5 Regulation of Exchange Factors

An NMR-based investigation of the Vav exchange factor has shown that there is a region, N-terminal to the DH domain, that forms two turns of an α-helix, which is then connected to the DH via a flexible linker (Aghazadeh et al., 2000). The α-helix binds to the DH domain, blocking the region that is proposed to interact with the G protein. Phosphorylation of Tyr- 174, within the α-helix, activates the GEF by disrupting the helical structure and causing its release from the DH domain. The regulation of Vav is more complicated however, since the DH domain and part of the N-terminal helix are also thought to interact with the PH domain, inhibiting the GEF activity. The DH/PH interaction is also disrupted by phosphorylation of Tyr-174 or by the binding of phosphatidyl inositol-3,4,5-trisphophate (PIP3) to the PH domain (Das et al., 2000). This has led to a model, whereby the PH domain is not bound to the DH domain, but rather interacts with the N-terminal α-helix, which interacts in turn with the DH domain (Aghazadeh et al., 2000). Interaction of the PH domain with PIP3 disrupts its binding to the N-terminal α-helix, allowing it to be phosphorylated. Phosphorylation of Tyr13 174 is then sufficient to complete the disruption of the intramolecular complex. The PH domain may then go on to play a role in the interaction with the G protein, as in the Dbs-like structures, or may simply be necessary for maintaining the correct conformation of the DH domain, as in the Tiam1 and Intersectin complexes.

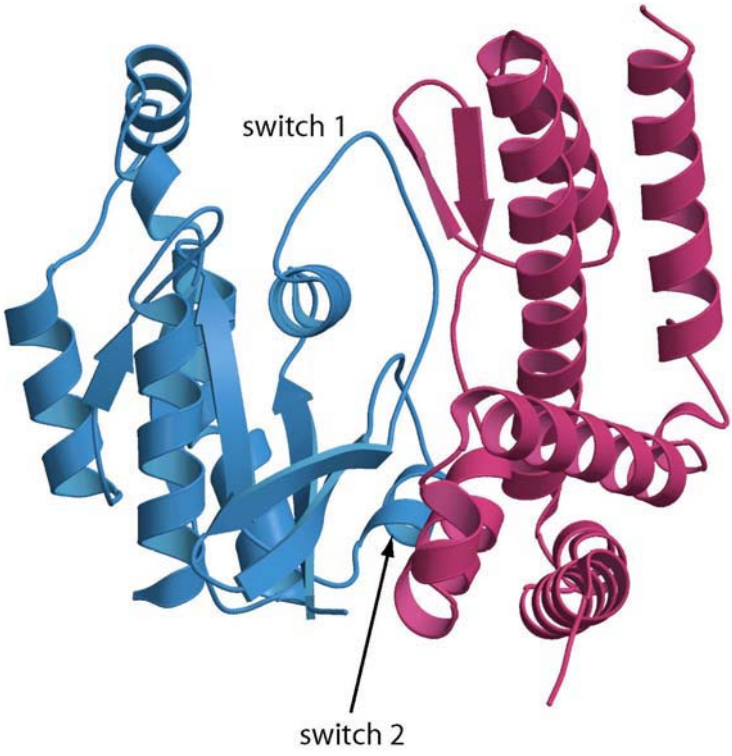

Figure 6. Structure of the SopE/Cdc42 complex. The Salmonella typhimurium toxin, SopE, is coloured dark pink (right side) with Cdc42 coloured blue (left). The conformationally plastic 'switch' regions of the G protein are labeled.

3.6 Non-DH Domain Exchange Factors

Most of the GEFs for the Rho family G proteins contain the DH domain and interact with the G protein in the manner described above. Recently, a class of proteins, the DOCK180 family, has been described that possess exchange activity but appear to utilize a different structural motif to stabilize the nucleotide-free form of the G protein (Brugnera et al., 2002; Cote and Vuori, 2002). As yet, there is no structural information available for the complexes that the DOCK family make with the Rho family proteins. The exchange reaction has also been hijacked by a bacterial toxin. The SopE toxin from Salmonella typhimurium activates Rho family GTPases to induce cytoskeletal rearrangements, although it does not share sequence homology with the DH domain GEF family. A structure of SopE in complex with Cdc42 confirms that SopE is not a DH domain protein (Buchwald et al.

2002). It is, like the DH domain, an α-helical structure, composed of six α-helices arranged into two three-helix bundles (Figure 6). The complex with Cdc42buries approximately 2,800 Å2 of surface area, similar to the Rac/Tiam1 structure. SopE does not interact directly with the P-loop, nor does it bury the pocket where the guanine base binds. As in the DH/Rho family structures, the major changes in the G protein lie in the switch 1 and switch 2 regions. In the SopE complex, these changes are caused by the insertion of a 4-residue loop from SopE between the two switches, which pushes aside switch 1 and pulls switch 2 towards SopE. The N-terminal region of switch 1 (residues 26-31) becomes disordered, opening the nucleotide-binding pocket and Phe-28, which is involved in interacting with the nucleotide, is pushed out of the pocket. In switch 2, Ala-59 blocks the magnesium binding site, in an analogous manner to its position in the DH/Rho family complexes. Thus, the SopE system uses a distinct structural motif to bring about the same consequence as the DH family of GEFs.

4. EFFECTOR COMPLEXES

The variety of cellular processes controlled by the Rho family proteins are mediated through a growing number of downstream effector proteins, many of which are still being characterized. The members of the Rho subfamily have a far greater number of effector proteins than other G protein subfamilies. Effectors can be loosely divided based upon their specificity: they may bind Cdc42, Rac or Rho-like proteins or a combination of more than one group. The effectors are both functionally and structurally diverse, as is the nature of their interactions with the G proteins. Removal of the insert region in Rac expressed in vivo disrupts the formation of membrane ruffles (Karnoub et al., 2001a) and prevents transformation (Joneson and Bar-Sagi, 1998). Thus, it is likely that at least one downstream effector of the Rho family will interact with the insert region, although no such interactions have been identified to date. The structures of several, diverse Rho family effectors and their complexes have been studied so far and will be described here.

4.1 CRIB Proteins

Many downstream effectors for Cdc42 and Rac contain a small (16 amino acid) consensus sequence known as the CRIB motif (Cdc42/Rac Interactive Binding), which is essential for the interaction with the G proteins (Burbelo et al., 1995). In several effectors it has been shown that sequences C-

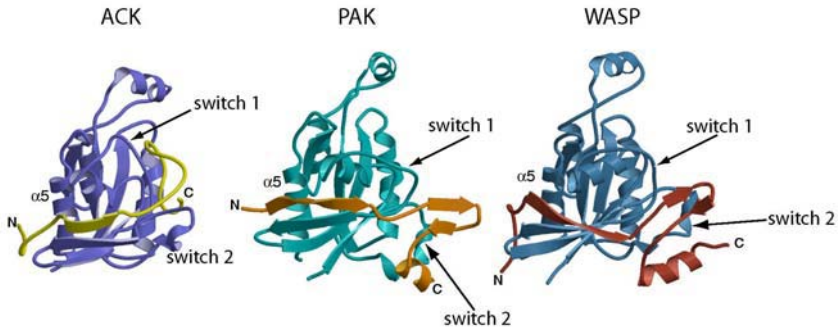

Figure 7. Comparison of CRIB/Cdc42 structures. In each case, the G protein is shown in a shade of blue and the CRIB fragment in a shade of orange (in front). The positions of the two switch regions that become fixed on effector binding are shown, as is the position of the α5 helix, which interacts with the CRIB effector in all three cases and is the location of the mutation that specifically disrupts Cdc42/ACK and Cdc42/WASP binding.

terminal to the CRIB are also necessary for tight binding, making the full GBD (G protein binding domain) 40-45 amino acids. All the CRIB proteins bind to Cdc42•GTP and some of them bind to Rac•GTP. Structural studies of the CRIB family proteins have addressed two fundamental questions: how do some CRIB proteins discriminate between the closely related Cdc42 and Rac and how does binding the Rho family protein alter the effector structure, leading to activation of the downstream pathways?

4.1.1 ACK, PAK and WASP

The solution structures of three different Cdc42/CRIB complexes have been solved: ACK (activated Cdc42 kinase), a tyrosine kinase, which has been implicated in integrin signalling and endocytosis (Mott et al., 1999); WASP (Wiscott-Aldrich syndrome protein), which interacts with the cytoskeleton (Abdul-Manan et al., 1999) and PAK (p21 activated kinase), a serine/threonine kinase involved in JNK signalling and cytoskeletal rearrangements (Morreale et al., 2000). Comparison of the three structures reveals interesting differences in the way that the effectors contact the G proteins (Figure 7). In addition, since ACK and WASP are specific for Cdc42 while PAK binds to both Rac and Cdc42, the structures shed light on how the CRIB proteins may discriminate between two such similar molecules. In each structure, the CRIB consensus region binds in a similar manner to the G protein, forming an intermolecular β-sheet with the β2 strand of Cdc42 and then interacting with switch 1. An intermolecular β-sheet has also been observed in the complex of Ras with its effectors Raf-1 (Nassar et al., 1995) and Ral-GEF (Vetter et al., 1999), and Ral with its effector Sec5 (Fukai et al., 2003). The regions outside the CRIB consensus

interact with the same regions of Cdc42, helix α5 and switch 2, although the details of the interactions are all different. ACK does not form any extra secondary structure but wraps around the G protein, forming an irregular hairpin at the top of switch 1. WASP and PAK both form a regular β-hairpin that interacts with switch 1 and switch 2, followed by a short piece of α-helix, which interacts with switch 2. In WASP and PAK the relative orientation of the hairpin and α-helix are different, as is their orientation with respect to the switch 2 helix.

The short CRIB consensus sequence includes several invariant residues that interact similarly in the three complexes. At the N-terminus of the CRIB, an Ile residue packs against hydrophobic residues in the α5 helix and Ile-46 in the β2 strand of the G protein. The other conserved CRIB/G protein interactions include Phe-81/His-83 in PAK, Phe-518/His-520 in ACK and Phe-244/His-246 in WASP, which interact with Tyr-40, a residue that is highly conserved in the Ras superfamily. There are also a pair of conserved His residues at 83/86 in PAK, 520/523 in ACK and 246/249 in WASP that form ionic interactions with the conserved Asp-38 in Cdc42. Conserved hydrophobic residues C-terminal to the CRIB region interact with Leu-67 and Leu-70 in switch 2 of the G protein. Thus, the conserved interactions are mediated by a combination of hydrophobic and charged residues. NMR studies on the free GBDs of these three proteins revealed no significant tertiary structure. In both PAK and WASP there was evidence for the formation of the short section of α-helix that is seen in the complex with Cdc42 (Morreale et al., 2000; Rudolph et al., 1998). In ACK no secondary structure could be discerned in the free GBD (Mott et al., 1999).

It seems likely that the GBD will be structured in the context of the full-length protein, given the structural data available on longer fragments of these effectors (see below), but that it is exhibits a certain degree of structural plasticity. The switch regions of Cdc42 and Rac are highly conserved, the only difference being a single (conservative) substitution. It is therefore clear that, like the GEFs, the basis of the selectivity of CRIB effector proteins between Cdc42 and Rac must lie outside the switches. Mutagenesis studies combined with measurements of Kd suggested that interactions with Leu-174 in helix α5 of Cdc42 contribute to binding of WASP and ACK to Cdc42 but do not contribute to PAK binding (Owen et al., 2000). Position 174 is an Arg residue in Rac and may represent one of the points of discrimination between G proteins. Analysis of the Kd of point mutations may not lead us to a complete understanding of the discrimination between Cdc42 and Rac, since in all the Cdc42/CRIB protein complexes solved the buried surface area is large (2,500-4000 Å2). Furthermore, since

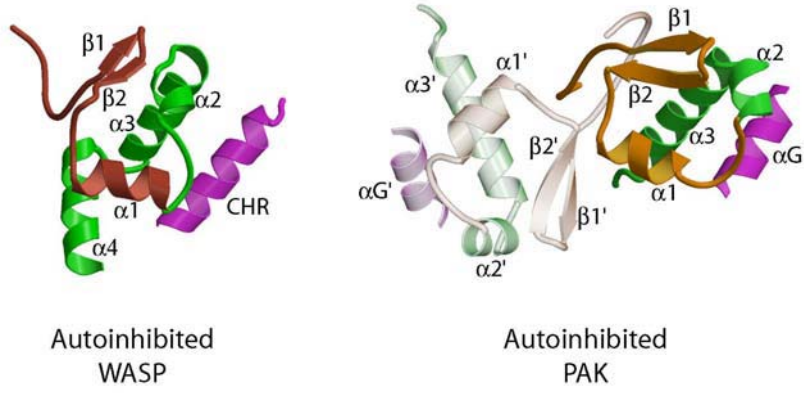

Figure 8. Structure of auto-inhibited WASP and PAK fragments The layers of the structures are shown in different colours. In both cases the 1/ 2 and 1 elements are part of the GBD and are involved in the interaction with Cdc42; 2 and 3 are in the region immediately C-terminal to the GBD that is involved in negative regulation of the C-terminus of the molecule. In WASP, helix α4 follows α3: this helix has no counterpart in PAK. The CHR and G are the cofilin homology region (WASP) and helix G from the kinase domain (PAK) respectively. The second PAK monomer is shown in pale shades corresponding to the first PAK monomer and labelled α1´, β1´ etc.

there is no Rac/CRIB fragment complex solved so far, the details of these interactions are unknown.

An insight into the activation mechanisms of the CRIB proteins came when the structures of both PAK and WASP in autoinhibited forms were solved (Lei et al., 2000; Kim et al., 2000). WASP has a region at the C-terminus that binds to both the Arp2/3 complex and actin. It was found that a short sequence at the C-terminus, the cofilin homology region (CHR), interacts with residues within and C-terminal to the GBD. The structure of a molecule comprising the CHR tethered by a flexible linker to the GBD extended at the C-terminus by approximately 20 residues has been solved (Figure 8). In this tethered complex, both the β-hairpin and the α-helix observed in the Cdc42 complex are present but they now pack against three α-helices formed by the region immediately C-terminal to the GBD, mainly via hydrophobic interactions. The four helices form a hydrophobic surface, against which a helix from the CHR is packed. It is clear that in this form the protein can bind neither the Arp2/3 complex (via the CHR) nor Cdc42 (via the GBD). To bind Cdc42, the protein has to undergo a conformational change that results in release of the CHR, allowing it to bind to other partners. The thermodynamic cost of this conformational change is a lower binding affinity between the autoinhibited form of the protein and Cdc42 than between isolated GBD fragments and Cdc42. Thus, the Kd of the tethered construct is approximately 100-fold higher than that of the fragment (Kim et al., 2000).

Yeast-two hybrid experiments with PAK-1 fragments have shown that a region Cterminal to the GBD, the kinase inhibitory (KI) domain, binds directly to the kinase domain inhibiting its catalytic activity. An X-ray structure of a GBD/KI fragment bound to the kinase domain revealed that the GBD/KI domain is strikingly similar to the equivalent region in WASP (Figure 8). One of the helices from the kinase domain packs against the three helices of the KI domain in a manner closely resembling the CHR helix of WASP packing against its autoinhibitory domain. There is one noticeable difference in PAK: in the crystal, there were two PAK complexes, with the dimer interface forming between the CRIB/KI regions. Given this structure it would seem to be impossible to form a Cdc42/PAK complex without breaking this dimer. This was in fact demonstrated in a later paper by the same authors (Parrini et al., 2002). However, others have shown that PAK forms a stable dimer, even in the presence of Cdc42 (Buchwald et al., 2001), suggesting that dimerization may also involve other regions of the protein.

The activation mechanism of ACK is not known at present but it is likely to involve a similar, intra-molecular inhibition. This is implied by the discovery that mutation of Leu-543 of ACK causes constitutive activation of the kinase (Kato et al., 2000). This Leu interacts with Leu-67 and Leu-70 in switch 2 in the Cdc42/ACK complex (Mott et al., 1999) and mutation of the equivalent Leu residue in PAK (Leu-107) or WASP (Leu-270) also disrupts their auto-inhibitory interactions (Frost et al., 1998), (Devriendt et al., 2001). The activation mechanism of ACK may be different to that of PAK: the kinase domain is N-terminal to the GBD. It has also been suggested that Cdc42 binding disrupts an intra-molecular interaction between the SH3 domain and a Pro-rich region (Yang et al., 1999), both of which are Cterminal to the GBD. An intra-molecular SH3/Pro interaction is also thought to exist in another CRIB-containing protein, mixed lineage kinase 3 (MLK3) (Zhang and Gallo, 2001). In both ACK and MLK3 the role of Cdc42 binding in activation is still unclear.

4.1.2 Par6

The partition-defective protein Par6 is involved in a multi-subunit complex with Cdc42, atypical protein kinase C (aPKC) and Par3. Par proteins are required for formation and maintenance of tight junctions, cell polarity establishment and developmental changes that depend on polarized cell division or polarized cell movement (Joberty et al., 2000; Lin et al., 2000). The region of Par6 that interacts with Cdc42 and TC10 contains a ‘semi-CRIB’ motif that includes the Ile, Pro and Phe consensus residues at the N-

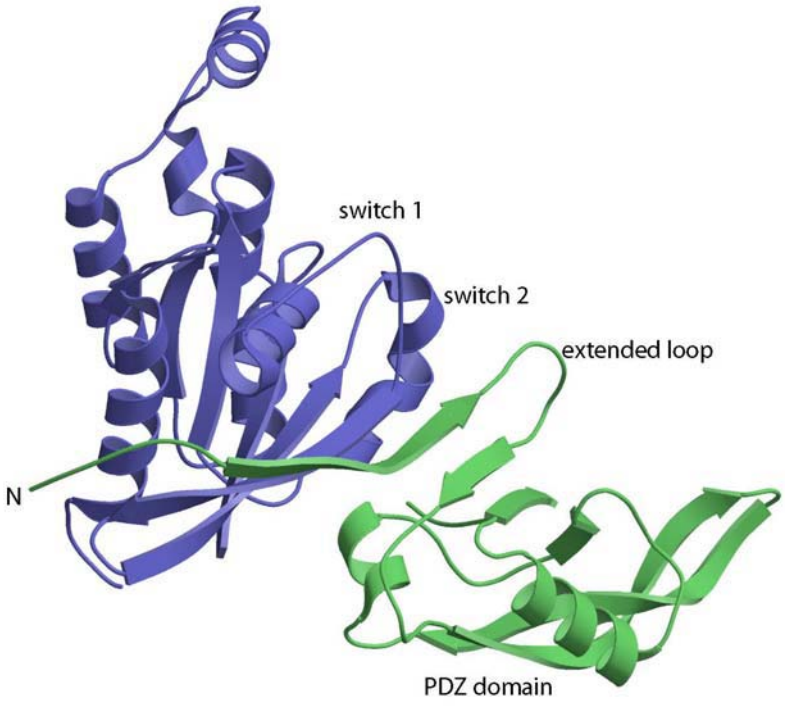

Figure 9. Structure of the Par6/Cdc42 Complex. Cdc42 is coloured blue (left) and the two switches are labelled. Par6 is shown in green (right); the semi-CRIB is at the N-terminus and is followed by an extended loop that leads into the PDZ domain.

terminus of the CRIB, but is lacking the two conserved His residues that were shown to interact with Asp-38 in the conventional CRIB effector/Cdc42 complexes. This semi-CRIB is not sufficient for G protein binding and a PDZ (PSD95/Discs Large/ZO-1) domain that lies C-terminal to the semi-CRIB is also required (Joberty et al., 2000). The crystal structure of the complex formed between Cdc42 and the semi-CRIB/PDZ fragment of Par6 has been solved (Garrard et al., 2003). The semi-CRIB region at the N-terminus of the Par6 fragment interacts with helix α5 and then forms an intermolecular β-sheet with the β2 strand of Cdc42, in a manner analogous to the other CRIB proteins (Figure 9). The CRIB consensus residues: Ile-133, Ser-134, Pro- 136 and Phe-138 make similar interactions to those made by the equivalent residues in the ACK, PAK and WASP complexes. C-terminal to the semi-CRIB, the Par6 does not form a hairpin, like PAK or WASP. Rather, the chain reverses via an extended loop that leads into the first β-strand of the PDZ domain, which continues the inter-molecular β-sheet. The four stranded β-sheet in the PDZ domain, along with the strand in

the semi-CRIB and the three stranded β-sheet in Cdc42 thus come together to form an inter-molecular, eight stranded, anti-parallel β-sheet. The semi-CRIB region makes contacts with helix α5, helix α1, the β2 strand and switch 1, while the extended loop between the semi-CRIB and the PDZ domain interacts with switch 2. Residues 204-208, which are part of a turn in the PDZ domain contact switch 1 and switch 2.

In the other Cdc42/CRIB complexes, the interface involves many more residues from the effector GBD, with the consequence that the buried surface areas are large, at least 2,500 Å2. The number of residues in the Par6 that contribute to the Cdc42 interaction is much smaller and this is reflected in the buried surface are of only ~1,100 Å2. The size of this interface is more reminiscent of that between Ras and its effectors Raf-1 and Ral-GEF, where the major interaction is also via an intermolecular β-sheet. NMR studies of the Par6 CRIB/PDZ fragment suggest that the semi-CRIB is likely to be structured in the absence of Cdc42, extending the PDZ β-sheet by one strand. Despite this, fluorescence spectroscopy indicates that there is a conformational change when Cdc42 binds to Par6. Par6 binds to aPKC via its N-terminal region and stimulates the aPKC catalytic activity (Yamanaka et al., 2001, Etienne-Manneville, 2001). The aPKC activation can be inhibited by addition of the GBD of Par6 and this inhibitory effect is alleviated by Cdc42. Thus, the conformational change induced by Cdc42 binding could be important for the mechanism of aPKC activation

4.2 Non-CRIB Rac effectors

4.2.1 p67phox

The p67phox protein is a component of the NADPH oxidase multi-protein enzyme complex. This complex,found in phagocytes, forms the principal defence mechanism against microbial infection inhumans. Binding of Rac to p67phox is a critical step in the activation of the latent NADPH oxidase. The Rac binding domain (RBD) of p67phox had been localised to the N-terminal 200amino acids, which contains four copies of the 34 residue tetratricopeptide repeat (TPR). TPRdomains are found in a number of proteins of diverse function, generally contain at least threeTPR motifs (Lamb et al., 1995) and often mediate protein-protein interactions. Each TPRmotif forms a helix-turn-helix motif, so that a TPR domain consists of a series of anti-parallel α-helices (A and B) (Das et al., 1998). This arrangement creates an amphipathic groove on one face of the TPR domain that can bind to peptides in an extended conformation. In the Hsp70-Hsp90

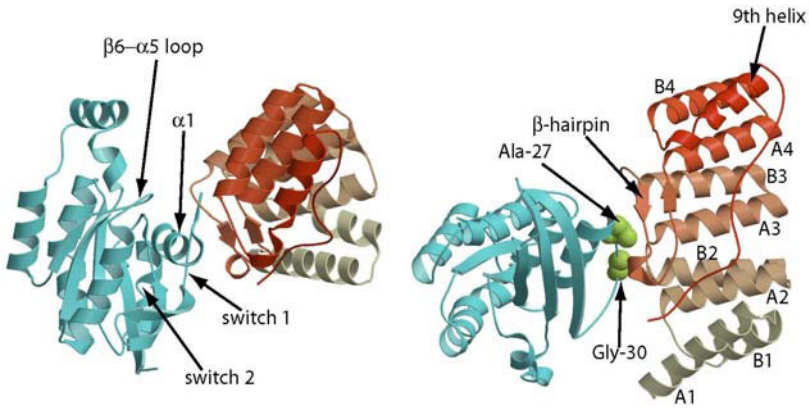

Figure 10. Structure of the Rac1/p67phox complex. Rac1 is shown in blue (left side) and the TPR domain of p67phox is shown in shades of orange, becoming darker towards the C-terminus. The switches, the β6-α5 loop and helix α1, which contact p67phox are labelled. The panel on the right represents a 900 rotation around the horizontal axis with respect to the structure on the left, to show the TPR motifs. The repeated helical dyads are labelled A1, B1 to A4, B4 and the 9th helix is indicated. Ala-27 and Gly-30 in Rac1 are shown as green spacefill representations: these residues are thought to contribute to the specificity of the interaction.

chaperone system, peptides bind in the TPR groove, making contacts only with the first helix of the first TPR motif (helix A) (Scheufler et al., 2000). The structure of Rac•GTP complexed with the RBD of p67phox revealed a set of interactions distinct from those of CRIB family of effectors and also a novel use of the TPR repeats as a binding motif (Lapouge et al., 2000).

The p67phox RBD comprises 9 α-helices, the first 8 of which form 4 TPR motifs, while the 9th helix packs against the B helix of TPR4 (Figure 10). The TPR groove is filled by a stretch of residues C-terminal to the 9th helix, which bind in an extended conformation. Thus, the TPR groove is not utilized for intermolecular interactions. Instead, contacts are made between Rac and one edge of the TPR domain. The Rac does not contact any of the TPR helices but makes contacts with a β-hairpin insertion between TPR 3 and 4 and the loops between TPR 1-2 and TRP 2-3. The regions of Rac1 that contact the effector include residues from helix α1, the N-terminal end of switch 1 and the loop between strand β6 and helix α5. The TPR domain does not contact all of switch 1 and no contacts are seen with switch 2 or the insert region. This is in contrast to the complexes between Cdc42 and the CRIB effectors, where extensive contacts are made with both switches 1 and 2. The TPR/Rac complex also differs from all the CRIB effector complexes in that no intermolecular β•sheet is formed. Only 1,170 Å2 of surface area is buried in the TPR/Rac complex, less than that in the conventional

CRIB/Cdc42 complexes. This may partially account for the lower affinity observed for the TPR/Rac complex.
p67phox binds specifically to Rac rather than Cdc42. Analysis of the Rac residues involved in the interface showed that all were conserved between Rac and Cdc42, except Gly-30. Ala-27 and Gly-30 have been defined as critical residues for the specificity of the interaction between Rac and p67phox: mutation of these residues in Cdc42 to the corresponding residues in vRac results in a Cdc42 protein that binds p67phox with a relatively high affinity (Lapouge et al., 2000). Ala-27 does not directly contact the TPR domain but in Cdc42, this residue is a Lys, whose longer sidechain is predicted to interfere with binding.

4.2.2 Arfaptin

Arfaptin (or POR, Partner of Rac) was identified independently as an effector for both the Rac and Arf small G proteins and consequently was proposed to be a facilitator of crosstalk between signalling pathways. Arfaptin (residues 118-341) has been crystallised alone and in complex with both Rac•GDP and Rac•GMPPNP (Tarricone et al., 2001). The Arfaptin domain consists of 3 α-helices (A-C), which form an anti-parallel α-helical bundle. Two of these domains associate via interactions between helices A-C of one monomer with helices A and B from the other, to form an elongated, crescent-shaped dimer. A single Rac molecule binds to the concave surface of the Arfaptin crescent, close to the dimer interface, burying 1,600 Å2 of surface area. Contacts are predominantly with a single monomer of Arfaptin (Figure 11), where switch 1 packs against helix αA and switch 2 interacts with helix αB. A single contact is seen to the second monomer, at His 57 in helix αA´. This is sufficient to prevent binding of a second Rac molecule to Arfaptin, accounting for the observed stoichiometry of 1 Rac: 1 Arfaptin dimer.

Arfaptin has a similar binding affinity for both the GDP- and GTP-bound forms of Rac, which implies it is not a conventional effector, although it binds to Arf1 and Arf6 in a GTP-dependent manner. Comparison of the Rac•GDP/Arfaptin and Rac•GMPPNP/Arfaptin complexes reveals the basis for their similar affinities. Thr-35, which co-ordinates to the Mg^{2+} in Rac.GTP, is in contact with Arfaptin, so that the structure of switch 1 is closer to the GDP-like form. It is predicted that a canonical Rac•GTP conformation could not be accommodated in the Arfaptin complex (Tarricone et al., 2001). Presumably, in the Arf/Arfaptin complex the G protein can take up its usual GTP-like conformation, allowing nucleotide discrimination. It is possible that Arfaptin's ability to bind both forms of Rac allows it to sequester Rac until Arf is activated, whereupon Arf displaces

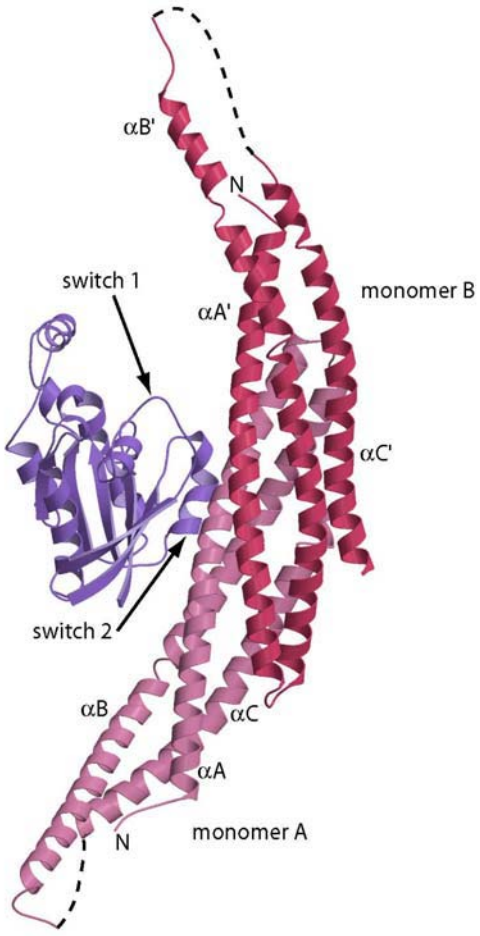

Figure 11. The structure of Rac1/Arfaptin 2. The monomers in the Arfaptin 2 dimer are shown in different shades of pink (right) with the helices labelled A, B, C and A', B' and C' in monomers A and B respectively. The regions where no electron density was observed are represented by dotted lines. Rac1 is shown in purple (left side). The major contacts in this complex are between monomer A and switch 2.

Rac freeing it to signal appropriately. The role of Arfaptin in this model is thus to co-ordinate Rac and Arf activation

4.3 Rho Effectors

Effectors for Rho include at least eleven proteins: DAG Kinase, PLD, PIP5-kinase, Kinectin, Rhotekin, Rhophilin, p140 Diaphanous, MBS, Citron, ROK and PRK. These proteins can be msubdivided by the homology of their

Rho binding motifs. The REM proteins (or Class 1 Rho binding motif) include the PRKs, Rhophilin and Rhotekin, while RKH proteins (REM2 or Class 2 Rho binding motif) include the ROKs and Kinectin (reviewed in (Bishop and Hall, 2000)). Structural information is limited at present to one of the REM proteins, PRK1, in complex with RhoA.

PRK1 (PKN) and 2 are serine/threonine kinases with a catalytic domain homologous to thatnof the protein kinase C family in their C termini and a unique regulatory domain in their N termini (Mukai and Ono, 1994), (Palmer et al., 1995). The N terminal region of PRK1 includes three HR1 repeats, one of which, HR1a, incorporates an inhibitory pseudo-substrate site (Kitagawa et al., 1996). Kinase activity is enhanced by binding of GTP-bound Rho or Rac to the N-terminal region (Amano et al., 1996; Lu and Settleman, 1999; Vincent and Settleman, 1997; Watanabe et al., 1996).

The X-ray structure of RhoA in complex with the HR1a repeat of PRK1 showed that HR1a forms an anti-parallel coiled-coil (ACC) finger domain (Maesaki et al., 1999) (Figure 12). The ACC finger domain is distinct from other G protein binding domains and Rho family effectors whose structures are known. The coiled-coil secondary structure is reminiscent of the Arfaptin structure, but the contacts that the effectors make with the G proteins are quite different (Tarricone et al., 2001). The structure of the complex between HR1a and RhoA indicated two possible contact sites on RhoA for HR1a. The major site, Contact 1, buries a surface area of 2,080Å2 and mainly involves hydrophilic interactions. This contact involves residues in the β2 and β3 strands of RhoA, the N-terminus of helix α5 and residues at the ends of switch 1. Contact 2, which involves more hydrophobic residues, buries a total surface area of 1,640Å2 and involves residues in switch 1 (Val38-Asn41), strand β3 and switch 2 (Trp58 and Asp65-Asp76). The size of Contact 2 means that it is unlikely to be an artefact of crystal packing. It is also noteworthy that it involves more residues in the switch regions of RhoA, which are likely to be involved in effector binding.

5. GAP COMPLEXES

To complete the GDP/GTP cycle it is necessary for small G proteins in the active conformation to hydrolyse the bound GTP to GDP and return to the resting state. The intrinsic GTPase activity of Rho-family proteins, like that of all small G proteins, is relatively low, so to terminate the signaling process efficiently GTPase activator proteins (GAPs) are required. In fact, RhoGAP domains can accelerate the GTPase activity of Rho family proteins

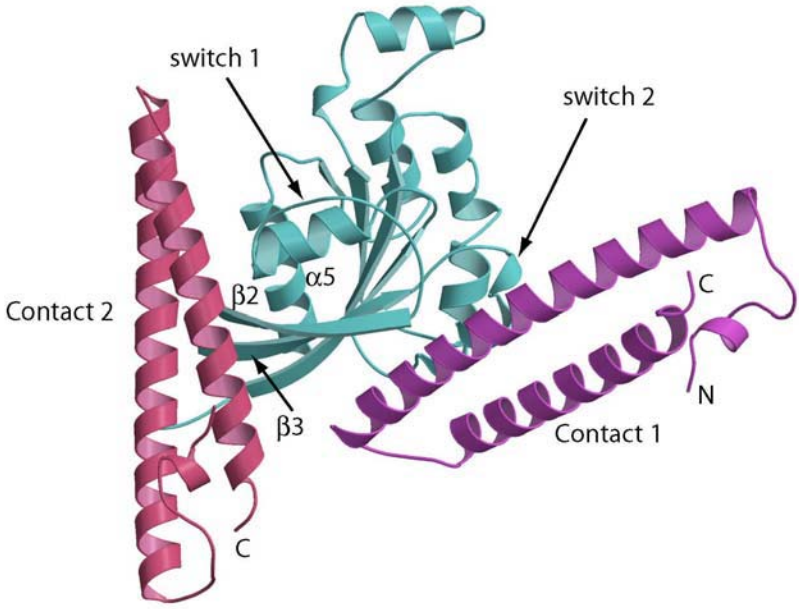

Figure 12. The structure of the RhoA/PRK1 HR1a complex. The two potential HR1a binding sites are labelled Contact 1 and Contact 2. Contact 1 was defined as the primary contact site on the basis of the buried surface area, although the HR1a in Contact 2 make more interactions with the switches. RhoA is shown in blue (centre) , with both switches and the main contacts with HR1aContact2 (2, 3 and 5) labelled. The two HR1a molecules are shown in shades of pink (in front).

by up to 105 fold. An analysis of the human genome has identified 53 cDNAs encoding putative RhoGAP domain containing proteins (Peck et al., 2002). These proteins contain a region of approximately 200 residues, which has been termed the RhoGAP or Bcr-homology (BH) domain (Zheng et al., 1993)). A feature of all the RhoGAP proteins is the large number of other modules they contain, indicating that they may function to link Rho family signalling to other pathways (Moon and Zheng, 2003).

Structures are available for uncomplexed RhoGAP domains from three proteins: the p85 subunit of PI3kinase (Musacchio et al., 1996), p50-RhoGAP (Barrett et al., 1997) and Graf (Longenecker et al., 2000). Four small G protein/RhoGAP domain structures have also been solved: Cdc42•GMPPNP/ p50RhoGAP (Rittinger et al., 1997a), Rho•GDP•AlF4-/ p50RhoGAP (Rittinger et al., 1997b), Cdc42•GDP•AlF3/ RhoGAP (Nassar et al., 1998) and Cdc42R305A•GDP•AlF3/ RhoGAP (Nassar et al., 1998).

The three structures of free RhoGAP domains show that it is helical and comprises a core of four roughly parallel helices, with a diverse number of accessory helices: PI3Kp85 has a total of 7 helices (Musacchio et al., 1996),

while p50RhoGAP (Barrett et al., 1997) and Graf (Longenecker et al., 2000) have a total of 9 helices each (Figure 13). . An analysis of the conserved residues in the RhoGAP domain family showed that they are clustered within a shallow depression on the structure that is formed by helices B and F, creating a hydrophobic patch (Musacchio et al., 1996), (Barrett et al., 1997). This patch was therefore predicted to be the site of G protein interaction. In particular it was noted that Arg-305 and Asn-414 (p50RhoGAP) were likely to be involved in the G protein interaction (Barrett et al., 1997). The structures of the RhoGAP domain in complex with

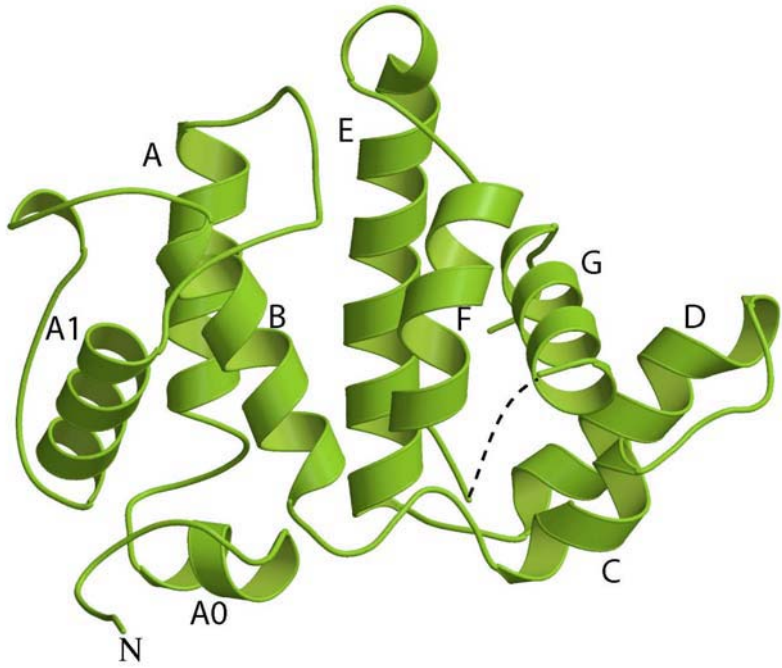

Figure 13. The structure of the RhoGAP domain of p50RhoGAP. The RhoGAP domain of p50RhoGAP is shown as a ribbon representation. The core of 4, approximately parallel helices, that is common to the RhoGAPs is made up of helices A, B, E and F. Helices A0, A1, C, D and G are accessory helices. Regions that have no electron density in the X-ray structure are represented by a dashed line.

a small G protein have provided significant insights into the molecular mechanism underlying GAP-mediated GTPase activity of the Rho family of small G proteins. The first complex to be solved was that of Cdc42•GMPPNP and the RhoGAP domain of p50RhoGAP (Rittinger et al., 1997a). This structure confirmed the hypothesis that the shallow depression

on RhoGAP involving the hollow formed between helices B and F, the A-A1 loop and the F-G loop is the G protein interaction site (Figure 14a). The complex buried 1,807Å of accessible surface area. The predominant contacts on the G protein involved switch 1, especially Val-36 and Phe-37 (both contact the F-G loopRhoGAP) and switch 2, especially Asp-63 (Lys-122RhoGAP), Tyr-64 (Asn- 220RhoGAP) and Leu-67 (F-G loopRhoGAP), which both insert into the hydrophobic pocket on p50RhoGAP. The Cdc42•GMPPNP/p50RhoGAP complex can be considered as the ground state for the hydrolysis reaction and it was proposed that, in analogy with the heterotrimeric G protein Giα1, Arg-305, which interacts with the P-loop (Gly-12) of Cdc42, adopts a different conformation during the catalytic process in order to stabilize the transition state. This hypothesis was corroborated by biochemical studies, which demonstrated that mutation of this conserved arginine in various RhoGAP domains decreased GAP enhanced GTPase activity dramatically, despite little change in the affinity for the G protein (Ahmed et al., 1994), (Hoffman et al., 1998), (Leonard et al., 1998), (Graham et al., 1999).

The structure of RhoA•GDP•AlF4-/RhoGAP finally confirmed the importance of Arg305 in the stabilization of the transition state of the GAP mediated GTP hydrolysis reaction (Rittinger et al., 1997b). AlFx has been used as a tool to investigate the GTP hydrolysis reaction in G proteins since it was realised that AlF4- is able to stimulate the activation of the guanine nucleotide binding regulatory component of adenylate cyclase (Sternweis and Gilman, 1982). Structural and biochemical studies concluded that AlF4- bound in the - phosphate position to activate the G proteins ((Higashijima et al., 1991) but its unique coordination suggested that it more closely mimicked the transition state of the hydrolytic reaction (Coleman et al., 1994), (Sondek et al., 1994). In the RhoA•GDP• AlF4-/RhoGAP structure, a rotation of 200 was observed between the G protein and RhoGAP when compared with the Cdc42•GMPPNP/RhoGAP ground-state structure (Figure 14a and b). This reorientation allowed Arg-305 to contribute directly into the active site of the G-protein, The rotation, centred on switch 2, also allows the formation of hydrogen bonds between helix α3Rho and the C-terminal end of the A-A1 loopRhoGAP. These extra interactions account for the increase of 460Å2 of buried surface area found in the transition state complex compared to the ground-state complex. Three catalytically important residues were identified in this complex: Gln-63Rho is stabilized by an interaction with Arg-305RhoGAP (the so called 'arginine finger'), which allows it to hydrogen bond to a water molecule directly in line with the - oxygen of GDP and the aluminium ion. Thus, the water molecule is aligned for a nucleophilic attack on what would be the terminal phosphate in the

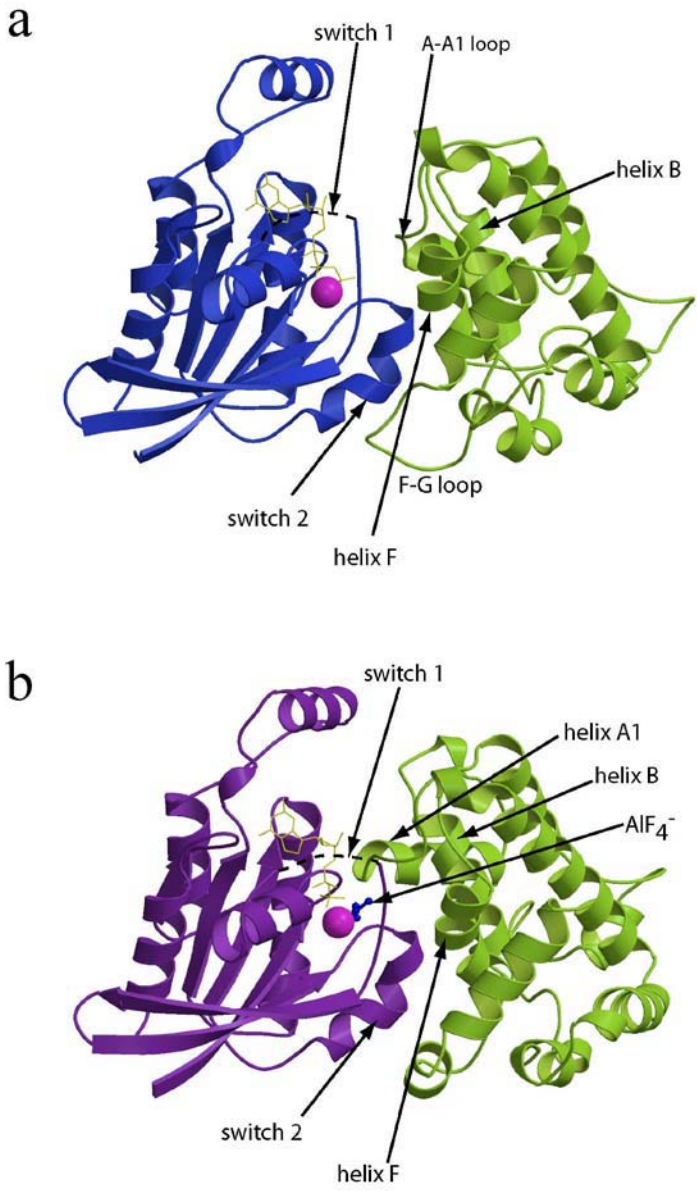

Figure 14. **a**. Structure of Cdc42·GMPPNP complexed with the RhoGAP domain of p50RhoGAP. Cdc42 is shown in blue (left) with its Mg^{2+} ion represented as a pink sphere and GMPPNP as an yellow stick. p50RhoGAP is shown in green (right) with the regions that interact with Cdc42; A-A1 loop, helix B, helix F and the F-G loop indicated. **b**. Structure of RhoA·GDP·AlF4 - complexed with the RhoGAP domain of p50RhoGAP. RhoA is shown in purple with its Mg^{2+} ion in pink, GDP as a yellow stick representation and AlF4- as a blue ball and stick representation. The RhoGAP domain of p50RhoGAP is shown in green and the main contact regions, helices A1, B and F, are indicated.

native reaction (Figure 15). The third important residue in the hydrolysis reaction is Asn-414RhoGAP, which interacts with the main chain carbonyl of Tyr34Rho thus stabilizing switch 1. Stabilization of switch 1 is probably necessary to fix the position of Thr-37, which co-ordinates to both the Mg^{2+} ion and the γ-phosphate, aligning them for hydrolysis. The second function of GAPs is to fix the position of the switches to allow the alignment of the catalytically important residues. Both switches 1and 2 are significantly more ordered in the transition state complex, which is indicative of this function.

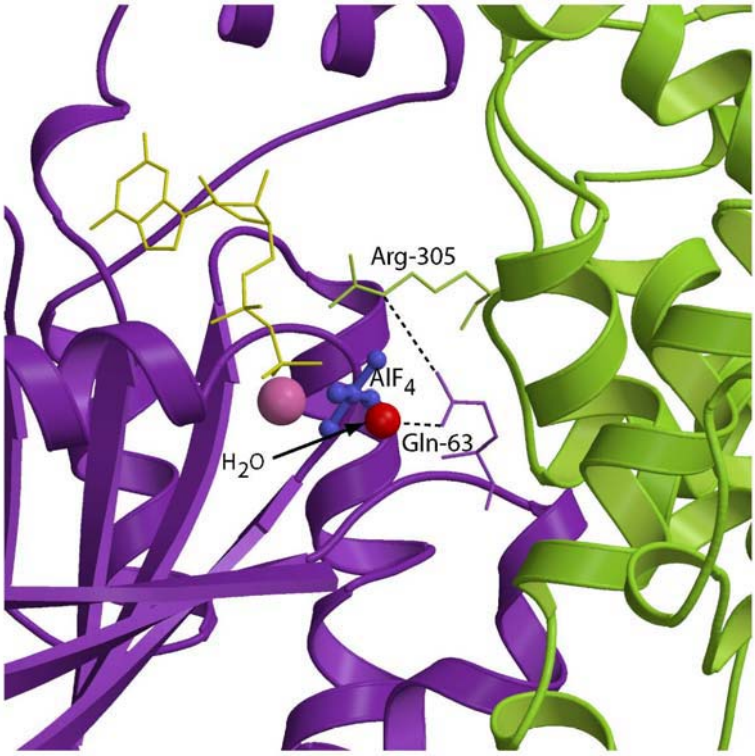

Figure 15. Expanded view of the catalytic centre of the RhoA·GDP·AlF4-/RhoGAP complex. RhoA is shown in purple (left). GDP in yellow, Mg^{2+} as pink sphere, AlF4- in blue and the nucleophilic water molecule in red (arrow). The catalytically active residue, Gln-63RhoA is highlighted and labelled while the interactions it makes with Arg-305RhoGAP and the water are represented by dashed lines. RhoGAP is shown in green with the catalytically active residue, Arg305, highlighted and labelled.

The mechanism proposed for GTP hydrolysis in the Rho family G protein/RhoGAP domain complex is very similar to that promulgated for both Ras/RasGAP (Scheffzek et al., 1997) and for the heterotrimeric G proteins (Coleman et al., 1994), (Sondek et al., 1994), despite the fact that in the heterotrimeric G proteins the 'arginine finger' is present in cis- in the

helical lobe of the α-subunit (Coleman et al., 1994; Sondek et al., 1994) and the stabilization of the switch regions is achieved by the RGS (regulators of G protein signalling) proteins (Tesmer et al., 1997). On first analysis, the GAP domains of RasGAP and RhoGAP do not appear to be homologous except at the most basic level of helicity. A more detailed examination revealed a similarity in the core of the molecules indicating a possible divergence from a common ancestor (Rittinger et al., 1998). The mode of binding adopted by the two GAP molecules with their cognate small G protein is also related, although the orientation of the helices in them two GAP molecules is different. Overall, however, the presentation of the catalytic arginine finger to the small G protein is very similar. The similarities seen in the Ras/RasGAP, Rho/RhoGAP and the heterotrimeric transition state structures led to the proposal of a 'universal' mechanism for GTP hydrolysis. However, the Ran/RanBP1/RanGAP complex structure revealed that GTP hydrolysis can occur in the absence of a catalytic arginine (Seewald et al., 2002). In this complex, it appears that Tyr-39Ran may fulfil the role normally occupied by the trans-arginines of the Ras/RhoGAPs and the cis-arginines of the heterotrimeric α subunits. The final two structures determined of Rho family G proteins in complex with their RhoGAPs are also interesting in this respect. Structures are available for Cdc42•GDP•AlF3/RhoGAP and Cdc42R305A•GDP •AlF3/RhoGAP (Nassar et al., 1998). It had been observed that the R305A mutant of p50RhoGAP, while lacking the catalytic arginine, retained the ability to increase GTP hydrolysis 10 fold (Nassar et al., 1998). The structure of the Cdc42R305A• GDP•AlF3/RhoGAP complex revealed that the active site in this complex had reoriented slightly to allow Tyr-32 Cdc42 to fulfil some of the stabilizing interaction performed by Arg-305 in the wild type transition complex (Nassar et al 1998). Tyr-32Cdc42 is equivalent to Tyr-39Ran. The R305A RhoGAP mutant is also capable of stabilizing the transition state conformations of switch 1 and 2, which explains some of its capacity to stimulate GTP hydrolysis. Thus, it has become apparent that the GAP molecules have a dual role, by supplying a catalytic residue to the active site but also, importantly, stabilizing the optimal conformation in the small G protein for hydrolysis.

Interestingly it has been reported that prenylated forms of the Rho family G proteins are better substrates for GAPs than non-prenylated forms (Molnar et al., 2001). As there are no GAP complex structures that include lipid-modified G proteins it remains to be seen if there is a structural basis for this observation.

6. CONCLUDING REMARKS

We have attempted to summarize here the pertinent details of all the Rho family complex structures determined at present. Several points emerge from this discussion. Rho family G proteins are under a tremendous regulatory load. Not only are they regulated by an additional class of proteins, the GDIs (the Rab family being the only other GDI-regulated class of small G proteins), the 21 identified Rho family G proteins have a total of 46 associated GEFs (Zheng, 2001) and 53 GAPs (Peck et al., 2002). In addition to this level of regulation, the Rho family G proteins also interact with a large array of effector proteins.

Of the structures of Rho family G protein complexes solved to date the three groups of regulatory proteins all display similar folds within each group, however the structural diversity in the Rho effectors is extensive. For example, the CRIB proteins, although they all have some similarities in the way that they interact, differ in their details, particularly outside the short CRIB consensus sequence. The other four effector structures are also completely different both to each other and to the CRIBs. It seems likely that families will emerge whose members interact with the G proteins in a similar, but not identical manner. There are also however, several other effector proteins with no sequence homology to Arfaptin, PRK, TPR domains or Par6/CRIBs. It is likely that these proteins will adopt different structures and will interact with the Rho family proteins in novel ways.

The manner in which the effectors contact the Rho family G proteins is multifarious. They utilize β-strands (the CRIBs), β-hairpins (the CRIBs), α-helices (Arfaptin, PRK-1 and the CRIBs), inter-helical loops (p67phox) and even a dimer interface (Arfaptin) to interact with the G protein. In some cases, such as the CRIB proteins, the effectors make an extensive set of contacts with the Rho family proteins, burying a large surface area (2,500-4,000 Å2) while in others, such as p67phox, the buried surface is only ~1200Å2. The region of the G proteins that interact with the regulatory proteins and downstream targets is also not conserved. In most cases, switch 1 or 2 is involved in the interactions, which is to be expected, given that effectors bind preferentially to the GTP-bound form of the G protein and the regulators by default need to interact with these nucleotide sensitive regions. It is usually the case that other regions of the G protein are also involved in binding to the interacting proteins, this may be important for specificity: the switches are relatively well conserved within the family while the diversity in the other regions is higher. In no case so far is the insert region involved in binding any interacting protein. As deletion of this region leads to a non-transforming mutant of Rac (Joneson and Bar-Sagi, 1998) we await the identification of the proteins that associate with this region of the Rho family proteins.

Structural information on Rho family complexes has moved at an exciting pace over the last six years. The next stage must be to determine the mechanisms of action of these Rho-family interacting proteins. Although many details have been elucidated of the mechanism of action of GEFs and GAPs it remains, for example, to see how binding of the GDIs to the prenylated, membrane bound G proteins, extracts them from the membrane and how the activating GEF molecules manage to make a productive complex with the GDP-bound G proteins. We also know very little about mechanisms of discrimination utilized by GAPs, as the only complex structures available contain the same GAP molecule. In addition, we do not know at present how the binding of the Rho family protein to its various regulators and effectors causes the downstream consequences that are seen in vivo. This point has begun to be addressed with the elegant work on PAK and WASP activation. We can look forward to many more such breakthroughs in the future, where a combination of structural and mechanistic studies will help us to understand fully these complex systems.

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Chapter 4

ACTIVATION OF GTPASES BY DOCK180 FAMILY OF PROTEINS

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Abstract: Members of the Dock180 superfamily of proteins have been recently identified as novel guanine nucleotide exchange factors (GEF) for Rho family of small GTPases. While conventional GEFs use a conserved DH-PH cassette for catalyzing GTP/GDP nucleotide exchange on Rho family GTPases, the Dock180 family members mediate nucleotide exchange via a novel evolutionarily conserved Docker/DHR2/CZH2 domain. The activation of distinct Rho-family GTPases by particular members of the Dock180 family have been linked to a multitude of biological processes. Based on sequence similarity, Dock180 family proteins can be broadly divided into four subfamilies, DOCK-A, DOCK-B, DOCK-C and DOCK-D, based on overall domain structure and substrate specificity. The function of proteins in DOCK-A and DOCK-B subfamilies is subject to regulation by their interacting partners such as ELMO and CrkII. In particular, the Dock180/ELMO complex functions as a bi-partite GEF for the GTPase Rac. This review discusses the features of the Dock180 family members, their regulation by binding partners and their relevance to biology.

1. INTRODUCTION

Rho family small GTPases are involved in a large number of cellular responses (Wennerberg et al., 2004). The best studied members of this family include Rho, Rac and Cdc42. These small GTPases function as molecular switches by alternating between a GDP-bound "off" state and a GTP-bound "on" state (Figure 1). The binding of either GDP or GTP results in structural change of the GTPases that allows their interaction with

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different set of proteins in the two states. The active, GTP-bound small GTPases bind to their specific effectors through their effector-binding loops exposed upon GTP binding and thereby elicit downstream signaling events (Bishop et al., 2000). Due to their intrinsic GTP hydrolysis activity, the GTPases cleave the phosphate group at γ position from the bound GTP and convert it to GDP, thereby turning the GTPases off. The intrinsic catalytic activity of the small GTPases can also be enhanced by GTPase-activating proteins (GAPs). Thus, the GTPase remains GDP-bound by default. To turn GTPase from the inactive state to active state, the bound GDP needs to be replaced by GTP, an event referred to as guanine nucleotide exchange. Guanine nucleotide exchange factors (GEF) mediate the exchange of GTP for GDP on these GTPases, usually in response to upstream signaling events.

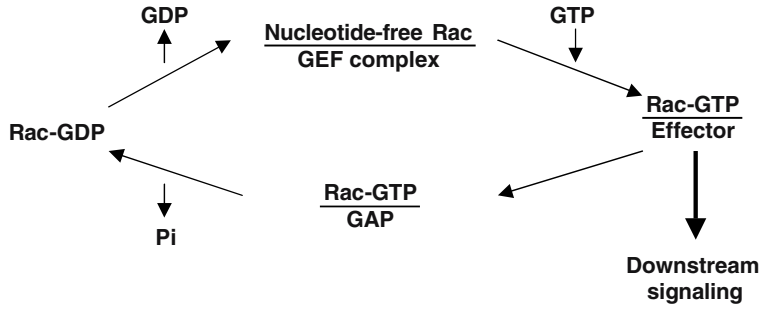

Figure 1. The cycling of Rac between the inactive GDP-bound and active GTP-bound states.

It is believed that the nucleotide exchange reaction mediated by GEFs occurs via multiple discrete steps (Schmidt et al., 2002). First, the GEF binds with low affinity to the inactive GDP-bound form of the GTPase (e.g. Rac), which then leads to the dissociation of the bound GDP. GEFs have the highest affinity for this nucleotide-free transition state, and thus stabilize GTPase in the nucleotide-free state. Due to the higher intracellular GTP levels (~10 fold higher than GDP), GTP is preferentially loaded on to nucleotide-free GTPase. The conformational change in the GTPase, upon GTP binding, results in a dramatic drop of affinity between the GTPase and GEF. As a result, the GTP-bound GTPase dissociates from the GEF and is free to interact with its downstream effectors.

Several families of GEFs for Rho family GTPases have been identified (Schmidt et al., 2002). Rho GTPases attract attention because of their fundamental importance in actin cytoskeleton reorganization, change in cellular morphology, cell migration and tumor cell metastasis (Burridge et al., 2004). Essentially all conventional Rho GEFs possess a Dbl homology (DH) domain along with a tandem pleckstrin homology (PH) domain

(Schmidt et al., 2002). The DH domain makes direct contact with the GTPase, and represents the 'catalytic domain' of each GEF. However, the tandem PH domain appears essential for function of the DH domain in cells (Erickson et al., 2004). In some cases, the PH domains bind to membrane phospholipids and may target the GEFs to the membrane where Rho GTPases are available for activation. In other instances, the PH domain makes direct contacts with the GTPase and assists the DH domain during guanine nucleotide exchange (Rossman et al., 2003).

The Dock180 superfamily of proteins represent newly identified GEFs for several Rho family GTPases (Brugnera et al., 2002; Cote et al., 2002; Meller et al., 2002). Unlike the conventional GEFs for Rho GTPases, members of the Dock180 family lack a typical DH domain. However, all members of this family possess a highly conserved Docker domain (also referred to as DHR2 or CZH2 domain) that is critical for interaction with small GTPases and for the nucleotide exchange. Based on the presence of the Docker domain, eleven Dock180 family members have been identified in the human and mouse genome. In addition, Docker domain containing proteins have been found in many other organisms throughout evolution, including nematode, fly, plant and yeast (Brugnera et al., 2002; Cote et al., 2002; Meller et al., 2002). Members of the Dock180 superfamily have been independently identified to regulate key biological processes in different organisms, confirming the importance of this new family of GEFs (Brugnera et al., 2002; Chen et al., 2002; Erickson et al., 1997; Fukui et al., 2001; Meller et al., 2002; Nolan et al., 1998; Wu et al., 1998; Yajnik et al., 2003).

In addition to the signature Docker domain, members of the Dock180 family also possess other domains and motifs. All of them contain a DHR1 (Dock180 homology region 1) domain (Cote et al., 2002; Meller et al., 2002). Based on their structural features and sequence similarity, the eleven human DOCK proteins can be divided into four subfamilies, DOCK-A, B, C and D (Table I and Figure 2).

Species / Subfamily	*S. cerevisiae*	*A. thaliana*	*C. elegans*	*D. melano-gaster*	*H. sapiens*	GTPase specificity
DOCK-A	YLR422W	SPIKE1	CED-5	Mbc	Dock180 Dock2 Dock5	Rac Rac ?
DOCK-B				CG31048	Dock3 Dock4	Rac Rap? Rac? Cdc42?
DOCK-C			AAB37023	CG11376	Dock6 Dock7 Dock8	? ? ?
DOCK-D			CAB02974	CG6630	Dock9 Dock10 Dock11	Cdc42 ? ?

Table I: Dock180 superfamily of proteins and their GTPase specificity

DOCK-A, DOCK-B subfamily members share a SH3 domain at their very N-termini and proline-rich region at their C-termini (Figure 2). Since the overall domain structures of DOCK-A and DOCK-B subfamily members are similar to those of *C. elegans* CED-5 and *Drosophila* Myoblast city (Mbc), these proteins have been referred to as CDM family (to denote the members CED-5, Dock180, Myoblast city proteins) (Erickson et al., 1997; Hasegawa et al., 1996; Wu et al., 1998; Yajnik et al., 2003). No obvious domain structure besides the DHR1 and DHR2 domains is detectable in the DOCK-C subfamily. DOCK-D subfamily members contain a PH domain at their N-termini instead of the SH3 domain seen in the DOCK-A and DOCK-B family members (Meller et al., 2002). It is noteworthy that the PH domains in DOCK-D subfamily members are quite distant from the DHR2/CZH2 catalytic domains, making it different from the tandem DH-PH domains seen in conventional Rho GEFs.

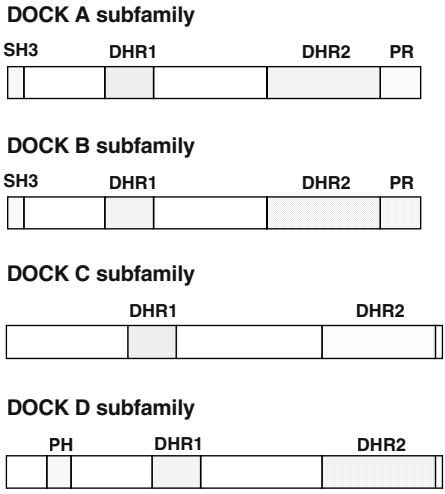

Figure 2. Schematic view of the domain structure of Dock180 subfamilies.

2. DOCK-A SUBFAMILY

DOCK-A subfamily members include Dock180, Dock2 and Dock5 in human and mice, CED-5 in *C. elegans* and Mbc in *Drosophila*. Besides the conserved DHR1 and DHR2 domains, they all have a N-terminal SH3 domain and C-terminal proline-rich region. While the GTPase specificity for Dock5 is unknown, the other four proteins have been demonstrated to function upstream of Rac (Brugnera et al., 2002; Kiyokawa et al., 1998a; Nolan et al., 1998; Sanui et al., 2003b; Wu et al., 1998).

2.1 Dock180 and Dock2

Among the DOCK-A family members in mammals, Dock180 and Dock2 proteins are the best characterized to date and function as upstream activators of Rac. They differ in their tissue specific expression pattern: while Dock180 is widely expressed, Dock2 is mainly expressed in non-adherent hematopoietic cells (Nishihara et al., 1999). Although it was initially thought that Dock180 and Dock2 are reciprocally expressed, recent studies show that Dock180 can be expressed in cells of hematopoietic origin (Akakura et al., 2004)

Dock180 is the prototype member of this family and is the most extensively characterized. It was originally identified as the binding partner of the CrkII adapter protein (Hasegawa et al., 1996). Expression of membrane targeted Dock180 by farnesylation induced 3T3 cells to adopt a flat, polygonal shape, a morphology reminiscent of that induced by constitutively active Rac. Moreover, the change in cell shape induced by Dock180 overexpression was suppressed by dominant negative Rac (Kiyokawa et al., 1998a). These observations implicated that Dock180 might be involved in the Rac signaling pathway and Rac dependent actin cytoskeletal organization.

It was subsequently observed that overexpression of Dock180 in 293T cells increased the level of active GTP-bound Rac. In addition, Dock180 bound to nucleotide-free Rac, but not GDP- or GTP-bound Rac, a feature characteristic of GEFs. The interaction with nucleotide-free Rac was not observed for Rho or Cdc42 (Kiyokawa et al., 1998a). These findings strongly suggested that Dock180 might be a Rac specific GEF. However, there were two fundamental unanswered questions in these initial experiments: first, these experiments were carried out in whole cell lysates and did not distinguish whether the binding to nucleotide-free Rac was mediated directly by Dock180, or through another protein bound to Dock180; second, since Dock180 does not contain a typical DH domain seen in essentially all previously known GEFs for Rho GTPases, how Dock180 might activate Rac remained elusive. Recently, three groups independently identified an evolutionarily conserved domain within Dock180 family proteins, denoted as Docker/DHR2/CZH2 domain (Brugnera et al., 2002; Cote et al., 2002; Meller et al., 2002). The Docker domain of Dock180 is both necessary and sufficient for binding to nucleotide-free Rac and is capable of catalyzing nucleotide exchange for Rac *in vitro* and increasing Rac-GTP level within cells (Brugnera et al., 2002; Cote et al., 2002). When two sets of conserved residues within the Docker domain (either ISP or YI residues) were mutated to alanines, the mutant proteins no longer bound to nucleotide-free Rac (Brugnera et al., 2002). Consequently, the mutants were inactive in exchanging nucleotide for Rac *in vitro* or increasing Rac-GTP level *in vivo* (Brugnera et al., 2002), and failed to mediate phagocytosis or rescue developmental defects *in vivo* (Grimsley et al., 2004).

The biological functions of Dock180 are analogous to that of *C. elegans* CED-5. Dock180 has been shown to be involved in the clearance of apoptotic cells and in cellular migration in mammalian system (Albert et al., 2000; Grimsley et al., 2004; Gumienny et al., 2001). In both events, the ability to activate Rac is essential for Dock180 to be functional (Grimsley et al., 2004). The functional conservation between CED-5 and Dock180 was also demonstrated by the ability of Dock180 to rescue the DTC

mismigration phenotype in CED-5 deficient worms (Grimsley et al., 2004; Wu et al., 1998).

Dock2 is a close relative of Dock180. Dock2 has been shown to activate Rac1 and Rac2 (Nishihara et al., 2002a; Nishihara et al., 2002b; Sanui et al., 2003b). But unlike Dock180, expression of Dock2 protein is mainly restricted to non-adherent hematopoietic cells (Nishihara et al., 1999). This expression pattern of Dock2 is consistent with its physiological functions *in vivo*. Dock2 knockout mice grow normally, indicating that it is not essential for embryonic development. However, lymphocytes in Dock2 deficient mice have defects in migrating to the periphery, mainly due to unresponsiveness to chemokine stimulation. In lymphocytes from these mice, the chemokine-induced Rac activation, as well as the chemokine-induced actin polymerization, was greatly reduced (Fukui et al., 2001). At the level of whole organism, this function of Dock2 is similar to that of CED-5 and Mbc in DTC migration and border cell migration, respectively (Duchek et al., 2001; Wu et al., 1998). It also correlates with the function of Dock180 and Dock2 in promoting cell migration *in vitro* (Grimsley et al., 2004).

In T cells, Dock2 is associated with the TCR• chain and is essential for Rac activation after TCR stimulation (Sanui et al., 2003a). In T cells from Dock2 null mice, the Rac activation induced by TCR stimulation was abolished (Sanui et al., 2003a). Consequently, several Rac dependent processes such as the immune synapse formation, antibody induced TCR clustering and lipid raft clustering were impaired. Overall, the antigen specific T cell response was decreased. Dock2 deficiency also affects the positive and negative selection during T cell development. Interestingly, compared with phenotypes associated with Vav deficiency, the function of Dock2 seems to be specific for Rac activation, as some other defects associated with Vav deficiency such as PI3-K and PKB activation, Pyk2 and PLCγ phosphorylation, Ca mobilization and NF-κB activation were all normal. These data suggest that Dock2 plays an important and very specific role in activating Rac in T cells.

2.2 CED-5

CED-5 is the *C. elegans* orthologue of human Dock180. It was isolated in *C. elegans* as a gene whose loss was manifested by defects in engulfment of apoptotic cells (Wu et al., 1998). During *C. elegans* development, cells undergoing apoptosis are rapidly engulfed by neighboring cells. This process requires actin cytoskeletal reorganization and membrane extension in the engulfing cells (Hengartner, 2001). One signaling pathway linking

upstream receptors to actin cytoskeletal reorganization involves the proteins CED-2/CED-5/CED-12/CED-10 (Fadeel, 2003). CED-10 is the Rac homologue (Reddien et al., 2000), while CED-2 and CED-12 are the homologues of the CrkII and ELMO, respectively (Gumienny et al., 2001; Reddien et al., 2000; Wu et al., 2001; Zhou et al., 2001) (see below). Activation of CED-10 is essential for actin cytoskeletal rearrangement and subsequent cellular events, such as plasma membrane protrusion and cellular migration. Genetic studies suggest that CED-2, CED-5 and CED-12 function upstream of CED-10 (Fadeel, 2003). In CED-5 mutant worms, apoptotic corpses linger around due to the failure of timely clearance by engulfing cells (Wu et al., 1998). Another phenotype associated with CED-5 deficiency is the defects in migration of gonadal distal tip cells (DTC), whose migration pattern defines the gonad shape in adult worms. In CED-5 mutant worms, DTC constantly change their migration path or stop prematurely, resulting in an altered shape of the gonad (Wu et al., 1998).

Besides its major roles in engulfment of apoptotic cells and gonadal DTC migration through its function upstream of CED-10, CED-5 also plays subtle roles in neuronal cell development, including CAN axon pathfinding, P cell migration as well as development of D-type motor neurons (Lundquist et al., 2001; Wu et al., 2002). Intriguingly, during CAN axon pathfinding and migration, CED-5 function is uncoupled from CED-10 activation, instead, CED-5 functions upstream of two other Rac-like GTPases in *C. elegans*, Rac-2 and MIG-2. It is possible that the GTPase specificity of CED-5 is not limited to CED-10/Rac-1 in *C. elegans*, or that CED–5 might function upstream or downstream of these other GTPases under certain conditions. The upstream stimuli and signaling molecules likely determine which particular GTPase is activated and the pathways that are regulated.

2.3 Myoblast city

Myoblast city (Mbc) was identified in *Drosophila* as a gene whose mutation resulted in multiple developmental defects (Erickson et al., 1997). Mbc mutant embryos exhibited myoblast fusion defects characteristic of the absence of differentiated muscle fibers and the presence of a large number of unfused myoblasts (Rushton et al., 1995). Another phenotype observed in Mbc mutant flies is a defect in dorsal closure (Erickson et al., 1997). About 80% of Mbc mutant flies failed to complete dorsal closure in the same time course as seen in wild type flies. At late stages of dorsal closure, cells at the leading edge of migrating epidermis failed to elongate and became rounded. Staining for actin filaments revealed that accumulation of actin filaments was decreased in the epithelial cells of Mbc mutant flies (Erickson et al.,

1997). These phenotypes associated with *mbc* mutation are reminiscent of defects derived from *Drosophila* Rac (dRac) mutation, suggesting that Mbc might function through Rac. This idea is consistent with the role of Mbc in *Drosophila* eye development. Transgenic overexpression of wild type Rac in flies resulted in developmental eye morphology changes. The eye development defects induced by overexpressed Rac, but not those by Rho or Cdc42, were suppressed by dominant Mbc mutants (Nolan et al., 1998).

Similar to CED-5 involvement in DTC migration, Mbc is implicated in border cell migration toward the oocyte (Duchek et al., 2001). Border cell migration requires activation of the cell surface receptor PVR, a PDGF/VEGF type of receptor in *Drosophila*. Engagement of PVR leads to dRac activation and actin filament formation. Cells overexpressing PVR accumulated abnormal amount of actin filaments because of excessive dRac activation. This actin filament accumulation was suppressed by a mutant of Mbc, similar to that of dominant negative Rac. This suggests that Mbc functions as an intermediate between PVR and dRac during border cell migration.

Mbc also plays a role in *Drosophila* neural development (Nolan et al., 1998). Mbc mutant flies exhibit defects in fasciculation of axons in CNS, possibly through improper migration of neurons in the central nervous system, an event dependent on dRac activation.

It is of note that two individual single point mutations within Mbc, G168E and P1579L, rendered Mbc nonfunctional and caused developmental defects similar to that of Mbc null mutants (Erickson et al., 1997). The P1579L mutation is in the center of a stretch of 27 amino acids highly conserved among CDM members. This region is within the conserved DHR2/CZH2 domain that is responsible for binding GTPases and catalyzing the GDP/GTP exchange. It is likely that the P1579L point mutation might have disrupted the interaction between Mbc and dRac and thus resulted in failure to activate dRac. Interestingly, a G171E point mutation in Dock180, analogous to the G168E mutation in Mbc, resulted in ~50% decrease in the GEF activity of Dock180 toward Rac (our unpublished observations). The phenotypes resulting from these point mutations suggest that optimal dRac activation by Mbc is critical during Drosophila development.

3. DOCK-B SUBFAMILY

DOCK-B subfamily consists of Dock3 and Dock4 in mammals. Their overall structures are very similar to DOCK-A subfamily members, with a SH3 domain at N-terminus and proline-rich region at the C-terminus.

Recent studies suggest interesting roles for Dock3 and Dock4 proteins as discussed below.

3.1 Dock3

Dock3 has been previously referred to as presenilin-binding protein (PBP) (Kashiwa et al., 2000) or modifier of cell adhesion (MOCA) (Chen et al., 2002) protein. It shares 40% sequence homology with Dock180. Like Dock2, the expression of Dock3 appears tissue specific and is mainly present in neurons (Kashiwa et al., 2000). It was originally isolated in a yeast two-hybrid screen as a binding partner for the cytoplasmic loop of presenilin (Kashiwa et al., 2000), a protein implicated in the processing of β-amyloid precursor protein (APP) associated with Alzheimer's disease (AD). Dock3 is normally present in the soluble fraction of brain extracts, but becomes insoluble in AD brains and is associated with neurofibrillary tangles (Kashiwa et al., 2000; Namekata et al., 2002). Overexpression of Dock3 appears to decrease the APP and β-amyloid secretion and adhesion to extracellular matrix (Chen et al., 2002). Dock3 is also implicated in neuronal cell migration (Chen et al., 2002). In neuroblastoma cell line SY5Y, endogenous Dock3 is localized to the leading edge of membrane protrusions. In cultured primary cortical neurons, Dock3 co-localizes with actin filaments at the growth cones of elongating axons. These biological functions of Dock3 are likely mediated via Rac activation, as Dock3 specifically binds to Rac but not Cdc42 or Rho. Consistent with this, overexpression of Dock3 increases the level of Rac-GTP in cells, but not that of Cdc42 or Rho (Grimsley et al., 2004; Namekata et al., 2004)

3.2 Dock4

Dock4 is expressed in multiple tissues, with high levels in skeletal muscle, prostate and ovary (Yajnik et al., 2003). The overall domain structure of Dock4 is very similar to other members of the CDM family. The C-terminal proline-rich region is capable of binding to adapter protein CrkII. The biological importance of Dock4 was uniquely revealed when several point mutation mutants of Dock4 were isolated in an elegant screen for genes regulating tumor cell metastasis (Yajnik et al., 2003). The mutations include a P1718L mutation that disrupts the C-terminal motif-1 shared by Dock4 and CED-5; a V1884M point mutation disrupts the motif-2 shared by Dock4 and Dock180; and a third point mutation K1059T affects a conserved residue among all CDM family members.

Yajnik et al. initially reported that transient expression of Dock4 in 293T cells activates Rap (a GTPase in the Ras subfamily), but not Rac, Cdc42 or Rho (belonging to the Rho family). Remarkably, the P1718L mutant version of Dock4 isolated in the screen did not activate Rap, yet it activated Rac and Cdc42 instead! This point mutation also abrogated the ability of Dock4 to rescue the engulfment defects in CED-5 deficient worms. Given that the P1718L mutation is outside the DHR2/CZH2 catalytic domain, how this switch in GTPase specificity is achieved is a mystery. Consistent with Rap activation by wild type Dock4, 3081 osteosarcoma cells reconstituted with wild type Dock4 adopted a flattened morphology rich in stress fibers; in contrast, consistent with the Cdc42 activation by the P1718L mutant, cells reconstituted with this mutant had filopodia. Wild type Dock4 restored adherens junction and contact inhibition in reconstituted 3081 osteosarcoma cells, while the P1718L mutation abolished this activity. The ability of Dock4 to activate Rap and maintain adherens junction correlates its functional role as a tumor suppressor. Whether Dock4 directly activates Rap or acts indirectly through activating another Rho GTPase, remains to be clarified. Recent unpublished studies suggest that wild type Dock4 can bind nucleotide-free Rac and can mediate Rac activation within cells (V. Yajnik, personal communication).

4. DOCK-D SUBFAMILY

DOCK-D family includes Dock9, Dock10 and Dock11. Besides the conserved DHR1 and DHR2 domains, a characteristic PH domain exists at their N-termini. The DHR2 domain localizes to the very C-termini in this family of proteins.

Dock9/Zizimin1 is the only characterized member in this subfamily (Meller et al., 2002). It was purified from NIH-3T3 cells lysates as a 220KD protein that specifically bound nucleotide-depleted Cdc42, but not GTP-bound Cdc42. Wild type Dock9, but not the DHR2/CZH2 deleted Dock9, induced GTP loading on Cdc42 when overexpressed in NIH-3T3 cells. Consistent with Cdc42 activation, both overexpressed and microinjected Dock9 induced filopodia formation in adherent cells. This was inhibited by dominant negative Cdc42, but not by dominant negative Rac or Rho. Deletion of different regions of Dock9 mapped the Docker/DHR2/CZH2 region of Dock9 (amino acids 1512-end) as both necessary and sufficient for direct binding to nucleotide-depleted Cdc42, though the binding was less efficient than an extended truncation mutant (amino acids 923-end). When affinity-purified and tested in an *in vitro* GEF assay, this elongated

DHR2/CZH2 mutant was able to enhance the dissociation of GDP from Cdc42 to the extent achieved by oncogenic Dbl, a DH domain containing GEF for Cdc42. Taken together, these data suggest Dock9 is a specific guanine nucleotide exchange factor for Cdc42. At present, it is unclear precisely how Dock9/Zizimin functions in regulating cell morphology, adhesion or migration. Given that events such as cell migration are complex processes involving both polarized Rac and Cdc42 activation, whether multiple DOCK family members may play a role in different steps of these process remains to be determined.

5. REGULATORS OF THE CDM FAMILY MEMBERS

As many of the Dock180 family members are large proteins with multiple domains and motifs, it would be expected that they would bind to other proteins, which, in turn could regulate their intrinsic GEF activity. In fact, Dock180 was originally identified as a CrkII interacting protein (Hasegawa et al., 1996). In principle, these binding partners of the Dock180 family could regulate the localization, post-translational modifications or the intrinsic GEF activity of the Dock180 family members. Coexpression of several members of the DOCK-A family with another protein ELMO, has been shown to enhance the Rac-GEF activity mediated via the Dock180 proteins (Brugnera et al., 2002; Grimsley et al., 2004; Gumienny et al., 2001; Katoh et al., 2003). Moreover, *C. elegans* CED-5 and Dock180 appear to not function in the absence of another protein CED-12/ELMO (Gumienny et al., 2001; Katoh et al., 2003). This suggested that the intrinsic activity of Dock180 family members would be subject to regulation by other proteins. The section below discusses the role of two proteins, ELMO and CrkII, which have been shown to bind Dock180 and regulate Dock180 function.

5.1 CED-12/ELMO proteins

CED-12 was initially cloned as a novel member of the pathway that also include CED-2/CED-5/CED-10 (which in mammals represent CrkII/Dock180/Rac) pathway controlling engulfment of apoptotic cells and DTC migration in *C. elegans* (Gumienny et al., 2001; Wu et al., 2001; Zhou et al., 2001). Worms deficient in CED-12 exhibit phenotypes similar to those in CED-5 or CED-2 deficient worms, with a failure to clear apoptotic corpses promptly and defects in DTC migration. The only recognizable features within the CED-12 protein were a PH domain followed by a PxxP

motif near the C-terminus (Gumienny et al., 2001; Zhou et al., 2001). It has now been demonstrated that CED-12 physically interacts with CED-5 (Wu et al., 2001), and that at least part of their interaction is mediated by the SH3 domain of CED-5 binding to the proline-rich region of CED-12.

The mammalian homologues of CED-12 were also cloned simultaneously (Gumienny et al., 2001). There are three CED-12 homologues in humans denoted ELMO1, ELMO2, ELMO3. All three proteins possess a PH domain at the C-terminal half followed by a PxxP motif. It has now been demonstrated that this PxxP motif provides a docking site for the SH3 domain of multiple CDM family members (Grimsley et al., 2004) including Dock180, Dock2, Dock3 and Dock4. Recent evidences also suggest that in addition to the PxxP mediated binding, ELMO proteins can interact via a second less well defined motif (immediately adjacent to the PxxP motif) (Lu et al, unpublished observations). Endogenous proteins, as well as overexpressed Dock180 and ELMO appear to form a tight molecular complex, that migrates ~700kDa (Yin et al., 2004). This suggests that the Dock180/ELMO complex may exists as oligomers, although the precise stoichiometry is not yet defined.

A number of studies now suggest that the Dock180/ELMO complex functions as a bi-partite GEF for Rac. Compared to Dock180 alone, the Dock180/ELMO complex exhibits higher activity in the *in vitro* GEF assays and *in vivo* Rac activation assays (Brugnera et al., 2002). Moreover, addition of bacterially expressed and purified ELMO to the Dock180 precipitates enhanced the Rac-GEF activity (Lu et al, unpublished observation). This suggests that the intrinsic GEF activity of Dock180 is regulated by ELMO. Besides the role in regulating the nucleotide exchange activity of Dock180, ELMO appears to regulate Dock180 function at multiple levels. This is consistent with the absolutely critical requirement for CED-12/ELMO proteins for CED–5/Dock180 to function *in vivo*. The role of ELMO in regulating Dock180 functions appears to occur via multiple mechanisms (Figure 3):

(i) *The N-terminal region of ELMO appears facilitates Dock180 targeting to the plasma membrane*. Coexpression of ELMO with Dock180 promotes membrane ruffling and Dock180/ELMO colocalize in membrane ruffles (Brugnera et al., 2002; Grimsley et al., 2004), suggesting that, *in vivo*, ELMO brings DOK180 to plasma membrane where Rac is available for activation. Recently, it was shown that the N-terminal half of ELMO binds to GTP-bound RhoG, a Rac-like GTPase (Katoh et al., 2003). GTP-RhoG, ELMO and Dock180 were able to form a ternary complex. Coexpression of RhoG with Dock180 and ELMO translocated Dock180/ELMO to plasma

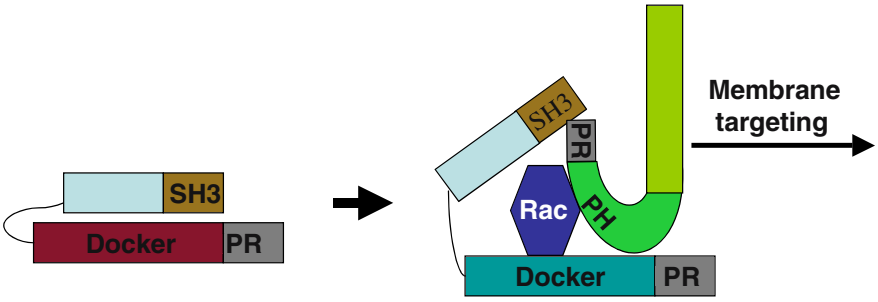

Figure 3. Model for ELMO mediated regulation of Dock180 GEF activity. The proline-rich region of ELMO relieves a self-inhibition of Dock180 through binding to the SH3 domain of Dock180. The PH domain of ELMO helps to stabilize the binding of nucleotide-free Rac to Dock180. The N-terminal region of ELMO facilitates targeting of the Dock180/ELMO complex to plasma membrane.

membrane and further enhanced Dock180/ELMO-mediated Rac activation.

(ii) *ELMO enhances the intrinsic GEF activity of Dock180 toward Rac by a novel interaction via the ELMO-PH domain* (Lu et al., 2004). The PH domain of ELMO appears to bind and stabilize the Dock180/Rac complex, and thereby forms a ternary complex of Dock180, ELMO and nucleotide-free Rac. This, in turn, contributes to the enhanced Rac activation. A series of studies using site-directed mutagenesis within the PH domain, functional assays in cells and rescue studies in C. elegans, suggested that this ternary complex is critical for Dock180/CED-5 mediated Rac activation in vivo. This contribution by the PH domain of ELMO is analogous to that of some DH-PH containing GEFs, where the PH domain makes direct contacts with nucleotide-free GTPase and facilitates the DH domain-mediated nucleotide exchange (Rossman et al., 2002). Interestingly, in the case of ELMO, the PH domain comes from a different protein (Lu et al., 2004), yet functions similarly to the tandem DH-PH cassette in some of the conventional GEFs for Rho GTPases. Whether ELMO makes direct contact with Rac in the Dock180/ELMO/Rac complex remains to be determined. The ternary complex formation also provides a mechanistic basis for the 'bi-partite' GEF activity mediated via the Dock180/ELMO complex. Although the above studies were done primarily with Dock180 and ELMO, it is quite possible that a similar mechanism of action could be envisioned where ELMO regulates the GEF activity of other DOCK-A and DOCK-B family members. In this regard, members of the DOCK-D family do not possess an ELMO interacting SH3 domain yet contain a distant PH domain. Whether this PH

domain functions together with their Docker domain in regulating nucleotide exchange remains to be seen.

(iii) *ELMO contributes to the GEF activity of Dock180 by relieving a basal intramolecular inhibition within Dock180* (Lu et al, manuscript in reparation). Like certain Rho GEFs, the N-terminal region of Dock180 seems to be inhibitory to its GEF activity. Biochemical studies suggest that the N-terminal region of Dock180 (in particular the SH3 domain) binds to the catalytic Docker domain and thus blocks the access of Rac to the Docker domain. The binding by the PxxP motif of ELMO to the SH3 domain of Dock180 results in a conformational change within Dock180, and thereby relieves the inhibitory interaction between the N-terminus of Dock180 and Docker domain. It is likely that this mechanism is operational for many, if not all, of the members of the DOCK-A and DOCK-B family members that have a N-terminal SH3 domain capable of binding of ELMO, but this remains to be examined.

Taken together, the existing data suggest that Dock180 family members may be regulated by ELMO via multiple mechanisms. Since reconstitution studies in *C. elegans* deficient in either CED-12 or CED-5 have confirmed these biochemical studies in mammalian cell lines (Grimsley et al., 2004; Lu et al., 2004), these mechanisms appear to be evolutionarily conserved and relevant at the whole organism level.

5.2 CED-2/CrkII proteins

CrkII and the worm homologue CED-2 are adapter proteins that consist of one SH2 domain and two SH3 domains. CrkII could be considered as an important regulator of Dock180 function for the following reasons. The prototype member Dock180 was cloned as a protein that binds to the N-terminal SH3 domain of CrkII. Subsequent studies also suggested a role for CrkII and Dock180 in regulating focal adhesions (Kiyokawa et al., 1998b), and coexpression of CrkII and Dock180 promoted migration of COS cells (Klemke et al., 1998). CrkII has also been shown to form a trimeric complex with Dock180 and ELMO (Gumienny et al., 2001). It is noteworthy that CrkII does not directly contact ELMO, but rather via Dock180, in that the N-terminal and C-terminal regions of Dock180 bind ELMO and CrkII, respectively. Coexpression of CrkII with Dock180 and ELMO appears to the further enhance the Rac GEF activity, compared with the Dock180/ELMO complex (Brugnera et al., 2002). This also translates into enhanced phagocytosis and abundant membrane ruffles seen when these three proteins are coexpressed.

The function of CrkII and its link to Dock180 and ELMO proteins also seem to be evolutionarily conserved. The worm homologue CED-2 functions in the same genetic pathway with CED-5 during engulfment of apoptotic cells and gonadal DTC migration (Reddien et al., 2000). CED-2 mutant worms exhibited very similar phenotypes as that derived from CED-5 deficiency. The CED-2 protein has also been shown to physically interact with CED-5 (Reddien et al., 2000).

However, the specific function of CED-2/CrkII at the molecular level is less clear. While the notion that CED-2/CrkII functions as an adapter bridging CED-5/Dock180 with upstream signaling molecules is plausible, this is not experimentally demonstrated. Moreover, while numerous studies suggest a role for CrkII in cell migration, cell adhesion to substratum and oncogenesis, the molecular mechanism of CrkII action remain largely unknown. Although CrkII has been shown to bind other molecules such as $p130^{CAS}$, a protein involved in formation of focal adhesion and cell migration down stream of integrins, how this interaction is linked to Rac activation via Dock180 remains largely unknown. Interestingly, Dock2 lacks the CrkII binding site, instead binds the CrkII isoform CrkL, which also has a hematopoietic cells specific expression (Nishihara et al., 2002a). The biological relevance of the Dock2-CrkL interaction remains to be assessed.

6. SUMMARY AND PERSPECTIVES

Dock180 family of proteins has been recently characterized as guanine nucleotide exchange factors for Rho family GTPases. Members of this family are highly conserved through evolution and have been linked to multiple biological processes. All members of this family possess an evolutionarily conserved signature catalytic domain denoted as Docker/DHR2/CZH2. The known GTPase specificity of some of its members is summarized in Table I. The primary amino acid sequence of Docker/DHR2/CZH2 domain is evolutionarily conserved from yeast to mammals. However, it bears no sequence homology to any of the catalytic domains from other family of GEFs. A key outstanding question is how the Docker domain contacts Rho GTPases and stabilizes them in their nucleotide-free transition states. Some data suggest that, when Rac is in complex with Docker domain, the contact sites and possibly the conformation of Rac are different from that of Rac in complex with DH domains (Brugnera et al., 2002). More detailed understanding of the mechanism of action via the Docker/DHR2/CZH2 domain requires structural

information of this domain alone and in complex with the cognate GTPases. Substrate specificity and biological functions for more than half of the human Dock180 family members remain unknown. Characterization of these proteins at a biochemical and structural level should lead to better understanding of the function under various physiological conditions.

The GEF activity mediated via this family of proteins is under regulation at various levels, exemplified by the Dock180/ELMO complex. Current evidences suggest that the Dock180/ELMO complex functions as a bi-partite GEF, wherein ELMO regulates Dock180 mediated GEF activity at multiple levels. A relevant question is whether ELMO indeed contacts nucleotide-free Rac in the Dock180/ELMO/nucleotide-free Rac ternary complex. The crystal structure of this ternary complex should help to unequivocally resolve this issue. Whether members of the DOCK-C and DOCK-D family are also regulated by ELMO or ELMO-like proteins remains to be answered. Moreover, additional regulatory features of the members of this family are likely to yield novel insights and interesting future investigations.

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Chapter 5

RHO GAPS – REGULATORS OF RHO GTPASES AND MORE

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Abstract: The cycling of Rho proteins between GTP bound 'on' and GDP- bound 'off' states is essential for their transient signalling to downstream effector proteins, which include kinases, cytoskeletal regulatory proteins and enzymes. Rho GTPase-activating proteins (RhoGAPs) can terminate Rho GTPases by stimulating their intrinsic rate of GTP hydrolysis. This rapidly converts Rhos to their GDP-bound state, and these membrane-bound proteins are then thought to be sequestered by guanine nucleotide dissociation inhibitors, allowing subsequent transport between membranes. RhoGAPs are multi-domain proteins with various protein and lipid interactive domains capable of precise targeting and regulation in signalling complexes. Some have more than one catalytic activity and potentially signal to other pathways in addition to terminating Rho signals. In this respect Rho GTPases may provide spatial and temporal information to GAPs that in turn serve as protein adaptors in a variety of intracellular compartments. Certainly much has yet to be learnt about this diverse family of proteins.

1. INTRODUCTION – GAPS IN THE BEGINNING

The cycling of Rho proteins is proposed to be essential for their transient signaling through associated effector proteins, such as kinases, ROK and PAK, actin regulatory proteins N-WASP and IRSp53 and many others. RhoGAPs, GTPase-activating proteins, down regulate RhoGTPases by stimulating their intrinsic rate of GTP hydrolysis (Figure 1). Initial purification of RhoGAPs using biochemical assays (Garrett et al., 1991; Hart et al., 1991; Tsai et al., 1989) lead to the identification of chimaerin and

E. Manser (ed.), RHO Family GTPases, 93-112.

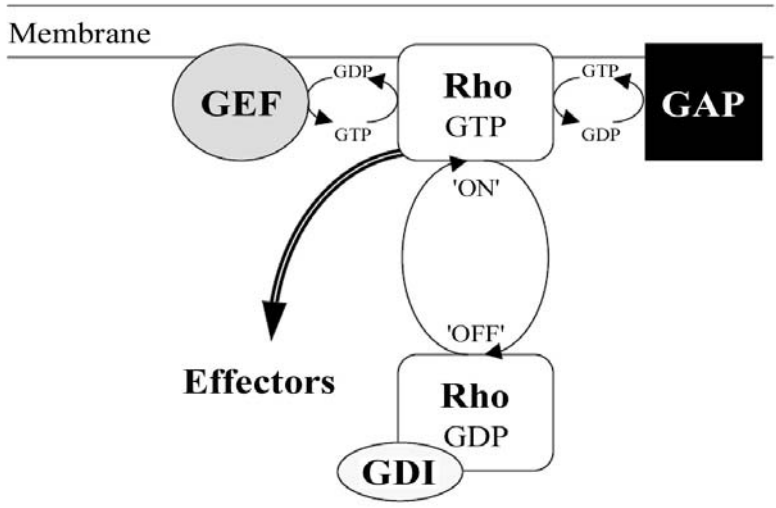

Figure 1. The Rho GTPase cycle. Active Rho GTPases reside on cellular membranes. The transition from inactive state to active state is promoted by guanine nucleotide exchange factors (GEFs) and inactivation is accelerated by GTPase-activating proteins (GAPs). GDP dissociation inhibitor (GDI) negatively regulates Rho GPTase by sequestering GDP-bound Rhos in the cytosol and allowing movement between different cell compartments.

BCR as proteins sequence-related to p50 RhoGAP with GAP activity (Diekmann et al., 1991). This set the precedent for the identification of many other Rho GAPs from sequence information alone. At the same time the analysis of tissue extracts identified an unexpected diversity of Rho GAP proteins (Manser et al., 1992) and up to 80 RhoGAPs are now known to be encoded in the human genome (Lander et al 2001), out-numbering the Rho GTPases approximately 4-fold. Additional details of RhoGAPs can be found in reviews that are published elsewhere (Moon and Zheng, 2003; Bernards and Settleman, 2004). Here we focus on some of the more extensively studied mammalian RhoGAPs, particularly those with an established role in neuronal development; for some others only cDNA sequence information is available (see Table 1).

A conserved GAP domain of approximately 140 amino acids defines the Rho GAP family. Although the primary sequences of Rho GAPs differ from other classes of GAPs, their tertiary structure somewhat resembles RasGAPs (Bax 1998) (see chapter 3 for further details). A conserved GAP domain arginine ‘finger’ is inserted into active site to stabilize charges formed during the transition state of GTP hydrolysis, and positions the catalytic glutamine residue of the GTPase to coordinate with a nucleophilic water

TABLE 1 The HUMAN RhoGAP FAMILY

NAME	GeneID	Specificity	Alternate name	Chr	designation
p50RhoGAP	392	Rh,Cd,Ra	Cdc42GAP	11p12-q12	ARHGAP1
BCR	613	Cd,Ra		22q11.23	
ABR	29	Cd,Ra		17p13.3	
α chimaerin	1123	Ra	n-chimaerin ARHGAP2	2q31.1	CHN1
β chimaerin	1124	Ra	ARHGAP3	7p15.1	CHN2
p115 RhoGAP	393	Rh	C1, RGC1	Xq28	ARHGAP4
p190A RhoGAP	2909	Rh	p190-A	19q13.32	GRLF1
p190B RhoGAP	394	Rh,Cd,Ra	p190-B	14q12	ARHGAP5
RhoGAPX-1	395			Xp22.3	ARHGAP6
p122 RhoGAP	10395	Rh	DLC1 STARD12	8p22	ARHGAP7
DLC2	90627		STARD13	13q12-q13	
BPGAP1	23779		PP610	22q13.31	ARHGAP8
RGL1	64333		10C	12q14	ARHGAP9
GRAF2	79658	Rh, Cd	FLJ20896	4q31.22	ARHGAP10
GRAF	23092	Cd(Rh)	OPHNL	5q31.1	ARHGAP26
OPHN1	4983	Rh,Cd,Ra	oligophrenin	Xq13.1	OPHN1
SRGAP1	57522	Cd	slit-robo GAP	12q14.2	ARHGAP13
SRGAP2	9901	Cd	WRP,MEGAP	3p25.3	ARHGAP14
FNBP2	23380		formin binding	1q32.1	
ARHGAP12	94134			10p11	ARHGAP12
ARHGAP15	55834	Ra		2q22.3	ARHGAP15
RICH1	55114	Ra,Cd	Nadrin	16p12.1	ARHGAP17
RICH2	9912		KIAA0672	17p12	
MgcRacGAP	29127	Ra(+Rh)	RACGAP1	12q13.12	
RALBP1	10928	Cd,Ra	RLIP76, RIP1	18p11.22	
PARG1	9411	Rh, Ra,Cd	PTPL-associated	1p22.1	
GMIP	51291			19p11-	
MYO9B	4650	Rh	myr5	19p13.12	
MYO9A	4649	Rh	myr7	15q22-q23	
3BP1	3616		SH3BP1	22q13.1	
p85α	5295	none	PI3 kinase	5q13.1	PIK3R1
p85β	5296	none		19p13.11	PIK3R2
OCRL-1	4952	Ra	INPP5F	Xq25-26	OCRL -1
INPP5B	3633		inositol p-5-phosphatase	1p34	
CdGAP	57514	Cd	KIAA1204	3q13.33	
TCGAP	115703	Cd,TC10	SNX26 Sorting nexin26	19q13.13	
ARAP1	116985	Rh,Cd,Ra	centaurin,delta2	11q13.4	
ARAP2	116984		centaurin delta1	4p14	
ARAP3	64411			5q31.3	
RICS	9743	Rh,Cd,Ra	p250GAP, GRIT	11q24-25	
ARHGAP18	93663		MacGAP	6q22.23	ARHGAP18
ARHGAP19	84986			10q24.1	ARHGAP19
RAGAP	57569		KIAA1391	11q22.3-	ARGHAP20
ARHGAP21	57584		ARHGAP10	10p12.1	ARHGAP21
ARHGAP22	58504		Rhogap2	10q11.22	ARHGAP22
ARHGAP23	57636		KIAA1501	17q12	ARHGAP23
ARHGAP24	83478		DKFZP564B1162	4q21.23	ARHGAP24
ARHGAP25	2358		KIAA0053	2p13.3	ARHGAP25
CAMGAP1	201176		CIN85-associated	17q21-31	ARHGAP27

GTPase abbreviation Rh = RhoA, Cd = Cdc42, Ra = Rac1 (see also Bernards and Settleman, 2004)

Table 1: The human RhoGAPs are identified by their chromosomal location and individual gene ID http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Gene. Alternative names or orthologous sequence are also listed. See also Bernards and Settleman (2004).

molecule (Rittinger et al., 1997). GAPs also serve to stabilize the switch regions, an essential feature of their activity (Fidyk and Cerione, 2002). The inositol polyphosphate 5-phosphatase GAP domain, which lacks the conserved arginine is devoid of activity (Jefferson et al., 1995). Deletion of a number of other conserved residues also abolishes GAP activity, without significantly interfering with GTPase binding (Ahmed et al., 1994). Curiously the p85 subunit of PI-3-kinase has the critical arginine, yet is inactive toward the GTPases so far tested. This p85 associates with Rac1 and Cdc42, thereby activating PI3K by localizing the catalytic subunit to the membrane (Zheng et al., 1994, Bokoch et al., 1996), indicating that the GAP domain can also serve as a GTPase 'effector' motif.

The substrate specificity of RhoGAPs has been determined experimentally for fewer than half of the known RhoGAPs (see Table 1). Many are active on more than one Rho GTPase however p190 RhoGAP is specific for Rho (Ridley et al, 1993), as is RhoGAP6 (Prakash et al., 2000) while ARHGAP15 is specific for Rac *in vivo* and *in vitro* (Seoh et al, 2003). The multi-domain BCR stimulates Rac1 and Cdc42 but not RhoA (Diekeman et al.,1991), however it should be emphasized that most Rho GTPases have not been tested with any GAP. The first RhoGAP protein to be purified, p50RhoGAP, was found to act on Cdc42, Rac and Rho *in vitro* (Barfod et al., 1993; Lancaster et al.,1994). As discussed later srGAP2 acts on Cdc42 while the chimaerins and the related MgcRacGAP are Rac GAPs, although the latter is thought to demonstrate a switchable GAP activity (Minoshima et al., 2003). The GAP activities determined *in vitro* may be of limited value, in view of the spectrum of Rho GTPase family members, and the observation that their specificity can differ in vivo. There is also evidence that specificity can be altered by lipids (Ligeti et al., 2004).

2. PROTEIN DOMAINS FOUND IN RHO GAPS

Many domains are apparent in RhoGAPs (Figure 2), which are thought to promote interactions particularly with lipid membranes where their Rho GTPase targets are active. Phospholipids also serve as major regulators of RhoGAPs, to activate GAP activity or even selectively influence their substrate preference (Ligeti et al., 2004).

2.1 Cysteine-rich C1 domains

PKC-like cysteine rich domains (C1) mediate binding to diacyglycerol and mimics termed ‘phorbol esters’, and are located immediately N-terminal of

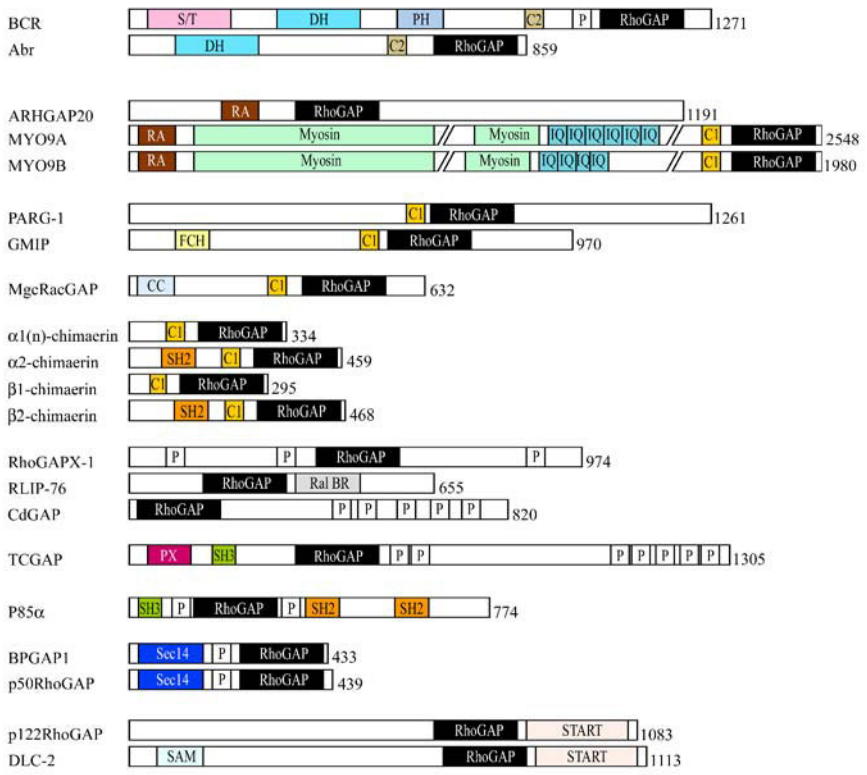

Figure 2. (part1) The domain structures of Rho GAP are illustrated approximately to scale. The total number of residues in each protein is shown on the right side. Abbreviations used for various domains are as follows: Ank, Ankrin Repeat; BAR, Bin/amphiphylin/Rvs; ArfGAP GTPase Region for Arf GTPases; C1 Cysteine-rich zinc finger; C2 Calcium-dependent Lipid Binding; CC, predicted coiled-coil oligomerization region; DH, Dbl Homology; EH, Eps15 Homology; FBH, FNBP2-FNBP1 Homology; FCH, Fes/CIP4 Homology; IQ, Calmodulin Binding; P, Proline-rich SH3/WW target; PH Pleckstrin Homology; PPase, PX, Phox Homology; Inositol 5-phosphatase catalytic; RDH, Rab 13 Homology; Ral GTPase binding; RalBR, RA, Ras-associating; SH3, Src Homology 3; SH2, Src Homology 2; Sec14, Homology to Yeast PI-transfer Protein Sec14p; S/T, serine/threonine kinase; SAM, Sterile α Motif; START, Star-related Lipid Transport; W, two signature Tryptophan (WW), proline-rich binding.

the RhoGAP domain in α and β chimaerins, (Hall et al, 1990; Leung et al, 1993), Gmip (Rho GAP; Aresta et al., 2002), PARG1 (RhoGAP; Saras et al 1997) MgcRacGAP (Rac/Cdc42; Toure et al., 1998), Myosin 1Xa and Myosin1Xb (Wirth et al, 1996). α1chimaerin was the first non-PKC phorbol ester/ diacylglycerol receptor identified and shares the concensus $HX_{12}CX_2CX_{(13\text{-}14)}CX_2CX_4HX_2CX_7C$ with PKC (Hall et al, 1990; Ahmed et

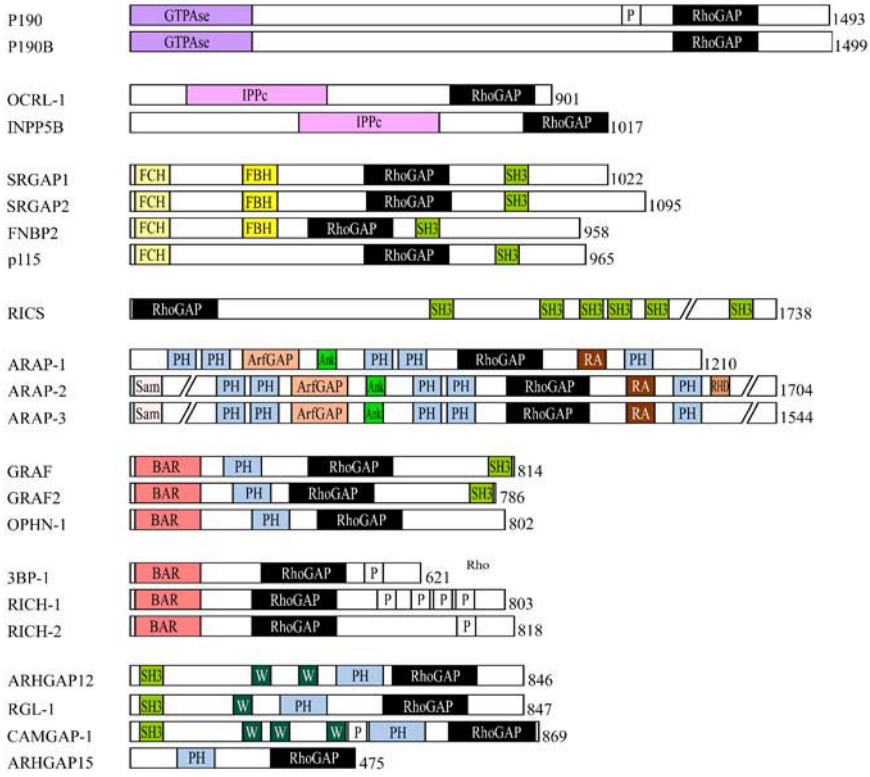

Figure 2. (part2)

al., 1990). The C1 domain mediates phorbol ester induced membrane translocation (Caloca et al 1999; Caloca et al 2001; Caloca et al 2003; Brown et al 2004). Lipid association with the C1 domain regulates chimaerin RacGAP activity in vitro and in vivo (Ahmed et al., 1993, Caloca et al.,2003, Brown et al., 2004). The C1 domain of β2-chimaerin also associates with TMP21-I, a Golgi associated protein (Wang and Kazanietz, 2002). The precise function of C1 domains in other GAPs is unclear, but because of sequence variances in this domain it is doubtful whether Gmip or MgcRacGAP are regulated by diacylglycerol.

2.2 Pleckstrin homology, Sec and Start domains

Pleckstrin homology (PH) domains, are almost invariably associated with RhoGEF (DH) domains, but are also present in a number of RhoGAPs; these are likely to mediate association with phosphoinositides. Such domains are present in the related GRAF, GRAF2/PSGAP (Hildebrand et al., 1996; Ren et al., 2001) Oligophrenin (OPHN1), and in ARHGAP9 (Furukawa et al.,

2001), ARHGAP10/21(Basseras et al., 2002), and CAMGAP1 (ARHGAP 27; Sakakibara et al., 2004) as well as ARHGAP15 (Seoh et al., 2003). There are an astonishing 5 copies in ARAP1-ARAP3 (Miura et al., 2002; Krugmann et al., 2002). The Sec14 domain of p50RhoGAP also mediates phospho-inositide binding (Krugmann et al., 2002) while START (StAR-related lipid transfer) domains in p122RhoGAP (Homma et al., 1995) and other RhoGAPs serve as alternative lipid binding domains (Ponting et al., 1999).

2.3 The Bar domain

The bar domain, a region of structural., similarity to BIN, Amphiphysin and Rvsp has recently been identified in oligophrenin-like and Nadrin RhoGAPs (Bernards and Settleman 2004). This domain is thought to induce membrane curvature and play a role in membrane invagination and endocytosis (Peter et al, 2004; Haberman 2004) and may allow Rac or Arf GTPase association (Haberman, 2004). RICH1B contains a BAR domain with homology to endophilins, which functions as an LPA acyl transferase causing invagination of the plasma membrane (Schmidt et al., 1999). This domain in RICH-1 is responsible for tubulation of liposomes (Richnau et al, 2004).

2.4 Protein interactions via SH2, SH3, WW and PDZ domains

Protein binding motifs in RhoGAPs also provide functional links to a variety of signaling molecules. SH3 domains and their partner proline-rich sequences are well represented amongst RhoGAPs (Figure 2). Because these interactions are somewhat promiscuous in vitro care must be taken in assessing the in vivo significance of the partnerships. The SH3 domain of GRAF (Hildebrand et al., 1996) interacts with both Focal adhesion kinase (FAK) and the Rho effector kinase PKNβ (Shibata et al., 2001). Similarly, PSGAP SH3 can interact with FAK and the related PYK2 kinase (Ren et al., 2001). A RacGAP termed 3BP-1 binds c-Abl (Cichetti et al., 1992), and Rich-1 (Cdc42/Rac GAP) associates with the CIP-4 SH3 domain (Richnau et al., 2001). A Rho GAP variously designated Grit, p200RhoGAP, p250RhoGAP, RICS, GC-GAP interacts with TrkA receptor and adaptor proteins N-Shc and CrkL/Crk (Nakamura et al., 2002) and this protein can interact with SH3 domains of Src, and PLCγ and with scaffolding proteins GAB1/2 which are thought to localise GAP activity (Moon et al., 2003; Zhao et al., 2003). RICS has been also shown to bind β-catenin (Okabe et al., 2003).

The PDZ (PSD-95/DlgA/ZO1 like) domains of ARHGAP10 (also known as ARHGAP21) (Basseres et al., 2002) are probably important for post-synaptic density localization, while PARG1 has been shown to bind a PDZ of protein tyrosine phosphatases PTPL1 (Saras et al., 1997). Nadrin (a Rho/Rac/Cdc42 GAP) binds the PDZ of EBP50 (Reczeck and Bretscher, 2001).

Although p120 RasGAP has two SH2 domains through which it binds p190RhoGAP and is also linked to receptor tyrosine kinases, SH2 domains are rare in RhoGAPs. The products of the α and β chimaerin genes, located respectively on chromosomes 2q32 and 7p15, are alternatively spliced at equivalent positions generating isoforms which are expressed in a tissue and developmentally specific manner as •α1/2-chimaerin (Hall et al., 1993) or β 1/2 chimaerin (Leung et al., 1993, Leung et al., 1994). Both α2 and β2 chimaerin contain an N-terminal SH2 domain (Figure 1). α2 chimaerin is capable of binding phosphotyrosine residues, despite a sequence variance in the normally invarient N-terminal tryptophan of the SH2 domain; other key phosphotyrosine-binding residues are however conserved (Hall et al., 2001). Although CRMP-2 (collapsin response mediator protein) is a target of α2-chimaerin SH2 domain it is not clear that this involves a phosphotyrosine interaction (Brown et al., 2004).

3. INTEGRATION OF GTPASE SIGNALS

Several RhoGAPs possess dual or multiple catalytic domains such as GEF domains or GAP domains towards non-Rho GTPases indicative of tight co-ordination of signaling pathways. Some RhoGAPs contain an effector-like GTPase binding region: the ARHGAP15 contains a Rac binding region that is also found in ARHGAP9 and ARHGAP12 (Seoh et al, 2003). Lipid phosphatase and protein kinase domains are present in various RhoGAP family members. ABR and BCR contain both Rho GEF and GAP domains (Heisterkamp et al, 1989; Tan et al, 1993) while ARAPS have Rho and Arf GAP domains, the latter implicated in the assembly of vesicle coats. OCRL1 is a RhoGAP and a phosphatidylinositol 4,5 biphosphate 5-phosphatase providing a direct link between Rho GTPases, PI(4,5)P2 metabolism and the actin cytoskeleton.

4. REGULATION OF GAPS

There is evidence that associated proteins can directly regulate GAP activity. CdGAP (Cdc42/Rac) interacts via its SH3 domain with endocytic protein

intersectin, a Cdc42 GEF, thus inhibiting GAP activity (Jenna et al., 2002). The Ras superfamily member Gem interacts with the RhoGAP Gmip and may upregulate its activity (Aresta et al., 2002). P35/Cdk5 has also been found to associate directly with the GAP domain of α chimaerin, although no regulation of GAP activity has been shown (Qi et al., 2004, Brown et al., 2004). Alternatively, Rho GAPs may regulate their associated proteins for example p122RhoGAP (Rho) binds and activate PLCγ (Homma et al., 1995). .

4.1 Regulation by Phosphorylation

The association of GAPs with kinases/phosphatases is not unexpected since phosphorylation likely regulates aspects of RhoGAP activity, location or complex formation. Various RhoGAPs are tyrosine phosphorylated; thus p190 RhoGAP is a major Src family kinase substrate and is phosphorylated at residues close to the GAP domain, through which it associates with p120 RasGAP SH2 domain (Roof et al., 1998). Such recruitment of the RasGAP stimulates p190RhoGAP activity, and provides cross-talk between Ras and Rho pathways. Fyn phosphorylates both p190RhoGAP (Wolf et al., 2001) and p250RhoGAP (Taniguchi et al., 2003) during oligodendrocyte maturation. PSGAP also associates with both FAK and PKY2. Co-expression of PSGAP with PKY2, but not FAK, in cells leads to tyrosine phosphorylation and inhibition of its Cdc42GAP activity. P200RhoGAP was also tyrosine phosphorylated upon association with Src in cells (Moon et al., 2003).

RhoGAPs regulation by Ser/Thr phosphorylation is known: PKC has been shown to phosphorylate p190RhoGAP leading to translocation to membrane ruffles (Brouns et al., 2000). GRAF is phosphorylated by mitogen activated protein kinase, MAPK (Taylor et al., 1998) and RICS phosphorylation by Ca/Calmodulin dependent protein kinase II directly inhibited GAP activity. Phosphorylation of MgcRacGAP by Aurora B serine kinase during cytokinesis, reportedly regulates substrate specificity in vitro (Minoshima et al., 2003)..

4.2 Auto-regulation of GAPs

As well as the wide potential for inter-molecular interactions, an auto-inhibited conformation achieved through intra-molecular interactions appears to tightly regulate GAP activity in some RhoGAPs. The N-terminus of OPHN-1 (Fauchereau et al., 2003) and α2 chimaerin (Brown et al., 2004) was found to negatively regulate GAP activity. α2 chimaerin appears to reside inactive in the cytosol. Mutation of the N-terminal SH2 domain, or

treatment with phorbol ester, results in translocation of α2 chimaerin to the membrane and upregulated Rac GAP activity (Hall et al., 2001; Brown et al, 2004). An N-terminal GTP binding domain in p190RhoGAP regulates its GAP activity (Tatsis et al, 1998) with tyrosine phosphorylation by Src conversely reducing GTP affinity (Roof et al., 2000).

5. GAPS WITH EFFECTOR FUNCTIONS?

In a few cases GAPs appear to potentiate GTPase signaling. Whilst the isolated GAP domain of α1 chimaerin inhibits Rac stimulated lamellipodia formation, full length protein, cooperates with Rac1 and Cdc42 to induce lamellipodia and filopodia structures, requiring Rac binding but not GAP activity, suggestive of an effector function (Kozma et al., 1996). GAPs could conceivably promote GTPase signalling through increased cycling rates in coordination with GEF. TCGAP translocation to the plasma membrane upon insulin stimulation allows it to interact with Cdc42 and TC10•• (Chiang et al., 2003)••. GAP activity has only been demonstrated in vitro and is undetectable in cells implying possible effector function or repression of GAP activity in vivo. The lack of GAP activity of some RhoGAP has suggested this domain may serve as a RhoGTPase binding motif for possible effector function. Some heterotrimeric G-protein GAPs are well established as effectors, for example p115RhoGAP which contains the Gα12/13 GAP (or RGS) domain (see chapter 1).

6. NEURONAL DEVELOPMENT AND AXONAL GUIDANCE

Rho GTPases clearly play key roles in the regulation of the actin cytoskeleton during neuronal migration, axonal growth and guidance, and in formation of synapses. Thus it is only to be expected that regulators of Rho GTPases such as GAPs would be found in a number of contexts to play key roles in neuronal morphogenesis. A number of findings have implicated the involvement of RhoGAPs in the proper development of the nervous system. Firstly sevaral RhoGAPs are brain specific or brain enriched and mutations in such RhoGAPs, for example OPHN-1 and OCRL, are associated with X-linked mental retardation (Billuart et al., 1998, Lin et al., 1997). OPHN-1 acts on multiple Rho GTPases as a GAP, and has recently been shown to play role in controlling dendritic spine length by functioning in tandem with the adaptor protein Homer (Govek et al., 2004). Other RhoGAPs which are

brain specific or enriched include ARHGAP4 (Foletta et al., 2002) and Nadrin - neuronal associated developmentally regulated protein (Harada et al., 2000). The srGAPs (Slit/Robo GAPs) are involved in Cdc42 regulation (Wong et al., 2001), as discussed in the next section. The p190RhoGAP is a well characterised Rho GAP required for axon outgrowth and guidance, mediating Src/Fyn dependent cell adhesion. A major substrate for Fyn in the brain is indeed p190 RhoGAP (Wolf et al., 2001) and the p250 RhoGAP (Taniguchi et al., 2003) which participates in oligodendrocyte maturation.

RhoGAP are also expected to be involved in neuronal synaptic transmission since synaptic vesicles are tethered to the actin cytoskeleton. Nadrin, a neuron-specific RhoGAP, is involved in the regulation of Ca2+-dependent exocytosis. In this case the protein is suspected to act on the cortical filament network under the presynaptic membrane where Nadrin co-localizes with actin (Harada et al., 2000).

6.1 Slit-Robo GAPs.

Growing neurites possess at the axon tip a growth cone that is responsive to both positive and negative molecular guidance cues. Growth cones guidance mechanisms enable correct connections to be established in the central and peripheral nervous systems during development. Intracellular signalling pathways connect with the array of extracellular guidance cues through their specific receptors, as exemplified by the Slit (extracellular) protein binding to the Robo receptor, which controls the migration of neurons at the midline (Wong et al., 2001). The Robo cytoplasmic domain interacts with 'Slit–Robo GAPs' (srGAPs), which are predominantly active toward Cdc42. Binding of Slit to Robo causes the activation/recruitment of srGAP, and down-regulation of Cdc42. The differential cellular localization of srGAP and/or recruitment to the Slit-activated Robo receptor thus generates a gradient of Cdc42 activity that probably in turn signals to Rac1. Polarization of actin structures at the growth cone provides a mechanism of Slit-initiated repulsive effects in neuronal migration (Wong et al., 2001). This pathway is conserved in flies where the srGAP termed Vilse has been genetically identified as a component in Slit-Robo repulsive signalling (Lundstrom et al., 2004). Defects in srGAP function in humans is associated with severe mental retardation (Endris et al., 2002).

6.2 Chimaerins

α Chimaerins are diacylglycerol (phorbol ester) targets with GTPase activity towards Rac (Diekeman et al., 1991, Manser et al., 1992). Two N-terminal splice variants of αchimaerin are referred to as the α1 and α2 isoforms (Hall

et al., 1993). The mRNA for α1chimaerin is abundant in areas of the adult brain associated with learning and memory, while α2 chimaerin is highly expressed in postmitotic neurones of the foetal brain (Hall et al., 2001). α2 chimaerin, which contains an SH2 domain rather than an amphipathic sequence, adopts a more cytosolic location compared to the predominantly membrane-bound α1 chimaerin. The SH2 domain has been shown capable of binding phospho-tyrosine residues, although specificity and in vivo receptors remain elusive. α1 chimaerin unexpectedly cooperates with Rac/Cdc42 to induce lamellipodia and filopodia in growth cones of N1E-115 cells (Kozma et al., 1996), indicative of Rac/Cdc42 effector functions. Long-term expression of α2 chimaerin also induces neuritogenesis in N1E-115 neuroblastomas (Hall et al., 2001) which is opposite of the effect expected for a RacGAP, possibly a consequence of increased Rac cycling. Recent evidence implicates α2-chimaerin as a component in growth cone collapse induced by Semaphorin 3A, a repulsive guidance signal (Brown et al, 2004). α2-chimaerin associates with two other components of this signalling pathway, Cdk5/p35 kinase and its substrate CRMP-2 (Brown et al, 2004).

6.3 p190 RhoGAP

Studies of the abundant p190 RhoGAP by targeted gene disruption in mice show that the RhoGAP is required for axon outgrowth, guidance and fasciculation, processes that require correct neuronal morphogenesis (Brouns et al., 2000; Brouns et al., 2001). Cells of the neural tube floor plate in p190 knockout mice exhibit excessive accumulation of polymerized actin, suggesting a negative role of p190 in the regulation of Rho-mediated actin assembly within the neuroepithelium (Brouns et al., 2000). The p190 is enriched with F-actin at the distal end of the axon and is associated with abnormal neurite formation (Brouns et al., 2001) indicating that p190 RhoGAP is one critical regulator of Rho-mediated actin reorganization at the neuronal growth cones. Interestingly deletion of neuronal adhesion molecules leads to defects similar to those seen in p190RhoGAP-null mice (Brouns et al., 2001). It is suggested that p190 RhoGAP effects are mediating by Src-dependent adhesion through balancing the RhoA activity. Such a role parallels RNA interference effects of p190 RhoGAP in Drosophila, where blockage of p190 RhoGAP leads to the retraction of axonal branches by upregulating RhoA activity and affecting a signalling pathway from RhoA to effector Drok (Billuart et al., 2001). Interestingly p190 RhoGAP, which can act as a GAP for both Rho and Rac1, is differentially sensitive to certain phospholipids in terms of its RhoGAP activity versus its RacGAP activity (Ligeti et al., 2004). The RhoA-related

Rnd proteins, which are GTPase-deficient, appear to utilize p190 RhoGAPs causing loss of cellular actin stress fibres and a round (Rnd) phenotype. This occurs because Rnd proteins increase the GAP activity of p190 towards RhoA (Wennerberg et al., 2003). The exact mechanism underlying these observations needs to be assessed.

7. CELL DIVISION REQUIRES A GAP

The conserved MgcRacGAP is one example of a RhoGAP playing a critical role in the cytokinesis step of cell division. The RhoGAP member MgcRacGAP and its *Caenorhabditis elegans* orthologue CYK-4 localize to the central spindle during cytokinesis (Hirose et al., 2001; Mishima and Glotzer, 2003), and MgcRacGAP can be co-precipitated with microtubules through its N-terminal region (Hirose et al., 2001). In *C. elegans*, a CYK-4 mutant initiates but does not complete the formation of a central spindle. Moreover, the localization of CYK-4 to microtubules of the central spindle is dependent upon the kinesin-like protein ZEN-4; indeed CYK-4 and ZEN-4 cooperate in central spindle assembly (Jantsch-Plunger et al., 2000). The mammalian orthologues of CYK-4 and ZEN-4 form a heterotetramer, named centraspindlin, that is involved in microtubule bundling critical for cell separation (Mishima and Glotzer, 2003). Unfortunately it is not clear whether the RhoGAP activity of MgcRacGAP is required for the function of the complex. It is suggested that central spindle localization of CYK-4 could accelerate RhoA.GTP hydrolysis locally, which may be required for cytokinesis (Lee et al., 2004). These events are coupled to myosin-dependent contractile ring assembly/disassembly and completion of cytokinesis. Phosphorylation of MgcRacGAP is regulated by the cell cycle, and some evidence has been presented for activation of a latent RhoGAP activity of MgcRacGAP during cytokinesis. As MgcRacGAP localizes to the midbody region, it becomes phosphorylated on Ser387 by Aurora-B. This event may switch the substrate specificity of MgcRacGAP from Rac1 to RhoA (Minoshima et al., 2003) although other mechanisms are also proposed (Ban et al., 2004). A different regulatory mechanism controls the timing of central spindle complex assembly in which Cdk1/cyclin B phosphorylates the motor domain of ZEN-4 on a conserved site (Mishima et al., 2004). Phosphorylation by Cdk1 diminishes the motor activity of ZEN-4 by reducing its affinity for microtubules. Experimentally Cdk1 inhibition leads to enhanced localization of ZEN-4 to the metaphase spindle and resulting defects in chromosome segregation.

8. SUMMARY

Considering the number of Rho GAPs encoded by the human genome, and the diversity of structures associated with various functional domains, it seems likely that a detailed understanding of GTPase regulation will remain a challenge. Here we have described the regulatory schemes that have been reported to influence the activity of some such GAPs. Many studies have shown that protein–protein interactions, phosphorylation, lipid interactions, and subcellular translocation all have important roles in RhoGAP modulation; no doubt exciting additional mechanisms remain to be uncovered.

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Chapter 6

RHOA/C AND THE ACTIN CYTOSKELETON

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Abstract: Rho belongs to the Rho family guanosine triphosphatases (GTPases) including Rho, Rac, Cdc42, TC10, and so on. Rho is categorized into RhoA, B, and C. The Rho family GTPases exhibit guanine nucleotide-binding activity and function as molecular switches by cycling between an inactive guanosine diphosphate (GDP)-bound form and an active GTP-bound form. Rho participates in the regulation of actin cytoskeletons, cell adhesions, cytokinesis, smooth muscle contraction, cell morphology, cell motility, neurite retraction, and polarity formation through their specific effectors. The characterization of these effectors has begun to clarify how Rho regulates some phenotypes. This article focuses on the roles of RhoA/C and their effectors.

1. INTRODUCTION

The rearrangements of cytoskeletons regulate various cellular processes such as cell morphology, migration, and cytokinesis (Fishkind and Wang, 1995; Mitchison and Cramer, 1996; Stossel, 1993; Zigmond, 1996). The spatial-temporal reorganization of cytoskeletons is induced by the extracellular signals, growth factors, hormones, and biologically active substances (Mitchison and Cramer, 1996; Stossel, 1993; Zigmond, 1996). Cytoskeleton consists of three filaments: actin filaments, microtubules, and intermediate filaments (Fukata et al., 2003; Gundersen, 2002; Mitchison and Cramer, 1996; Small et al., 2002; Zigmond, 1996).

Rho participates in the regulation of actin cytoskeletons and cell adhesions (Etienne-Manneville and Hall, 2002; Hall, 1998; Kaibuchi et al., 1999; Van Aelst and D'Souza-Schorey, 1997). Rho regulates the formation

E. Manser (ed.), RHO Family GTPases, 113-136.

of stress fibers and focal adhesions (Ridley and Hall, 1992), cell morphology (Paterson et al., 1990), cell aggregation (Tominaga et al., 1993), motility (Takaishi et al., 1994), membrane ruffling (Nishiyama et al., 1994), smooth muscle contraction (Gong et al., 1996; Hirata et al., 1992), neurite retraction (Jalink et al., 1994; Nishiki et al., 1990), and cytokinesis (Kishi et al., 1993; Mabuchi et al., 1993). However, these molecular mechanisms have not been clear until recently. This review outlines the molecular mechanisms of RhoA/C and their functions in the regulation of actin cytoskeletons.

2. PROPERTIES OF RHO A/C PROTEINS

The Rho family guanosine triphosphatases (GTPases) belong to the Ras superfamily of GTPases. RhoA differs from RhoB and RhoC at 29 and 16 amino acids, respectively. RhoA/C contain the five conserved regions required for GTP/guanosine diphosphate (GDP) binding and GTP hydrolysis, and CAAL box at C-terminal. RhoA-C have the same amino acid sequence in the effector domain. Thus, it appears that RhoA-C share their effectors. The C-terminal CAAL box (RhoA:-CLVL, RhoB:-CKVL, RhoC:-CPIL) is the signal for posttranslational modification. The leucine residues of CAAL box in RhoA/C are substrates for a geranylgeranylation but not for farnesylation, (Adamson et al., 1992; Katayama et al., 1991), whereas RhoB is a substrate for both geranylgeranyl and farnesyl transferase (Adamson et al., 1992). RhoA/C contain a stretch of basic residues, four arginine and two lysine, close to its CAAL box. Unlike RhoA or RhoC, RhoB does not contain a stretch of basic residues close to its CAAL box; instead, it has two cysteine residues. Both of these are sites for palmitoylation. When cells are fractionated biochemically, RhoA/C are predominantly cytosolic and RhoB is on endosomes. It seems that the posttranslational modification is important for their localizations.

3. PARTNERS OF RHO A/C

3.1 GDP/GTP exchange proteins

Rho works as a molecular switch by cycling between GDP-bound inactive (GDP·Rho) and GTP-bound active (GTP·Rho) forms (Figure 1). The ratio of these two forms of Rho depends on the activity of regulating factors.

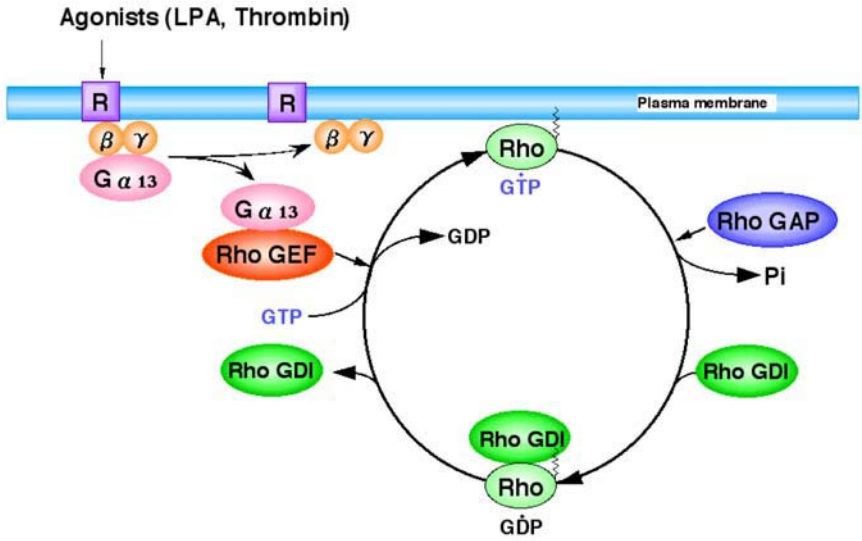

Figure 1. Mode of activation of the Rho family GTPases.

GTPase-activating protein (GAP) acts as a negative regulator by accelerating the intrinsic GTPase activity of Rho and reconverting it to the inactive GDP·Rho. Guanine nucleotide dissociation inhibitor (GDI) inhibits the exchange of GDP for GTP. Guanine nucleotide exchange factor (GEF) facilitates the release of GDP from Rho, thereby promoting the binding of GTP.Rho interacts with effectors and then triggers various cellular responses.

3.2 Guanine nucleotide exchange factors (GEF)

GEF stimulates the dissociation of GDP from Rho and thereby increases the GTP form of Rho. GEFs for the Rho family GTPases share a common motif, which is the Dbl-homology (DH) domain. In addition to the DH domain, most GEFs for the Rho family GTPases contain the pleckstrin homology (PH) domain in tandem. Both DH and PH domains are essential for the GEF activity (Cerione and Zheng, 1996; Schmidt and Hall, 2002).

Studies on heterotrimeric G proteins first clarified how Rho GEF is regulated. Examination of the sequence of p115 Rho GEF revealed that its NH_2-terminal region has a "regulator of G protein signaling" (RGS) domain. The RGS domain of p115 Rho GEF specifically stimulates the intrinsic GTPase activity of $G\alpha_{12}$ or $G\alpha_{13}$ (Hart et al., 1998; Kozasa et al., 1998).

Conversely, activated $G\alpha_{13}$ directly binds to the RGS domain of p115 Rho GEF and stimulates its ability to catalyze nucleotide exchange on Rho. Subsequent analyses have revealed that some of the Rho GEFs directly interact with receptor and are activated by agonist. Leukemia-associated Rho GEF (LARG) and Ephexin are associated with insulin-like growth factor 1 (IGF-1) and Eph receptors, respectively (Shamah et al., 2001; Taya et al., 2001). The association of these GEFs with receptors constitutes a molecular link between receptors and Rho, and provides the mechanism for achieving highly localized regulation of actin cytoskeletons.

3.3 Guanine nucleotide dissociation inhibitor (GDI)

GDI was originally isolated as a molecule that interacts with GDP·Rho and thereby inhibits the dissociation of GDP from Rho (Fukumoto et al., 1990; Takai et al., 1995; Ueda, 1990). Rho GDI was subsequently found to be active on Cdc42 and Rac, as Rho (Leonard et al., 1992). Rho GDI prevents the binding of GDP·Rho, but not GTP·Rho, to the membrane and extracts GDP·Rho from the membranes (Isomura et al., 1991). Thus, Rho GDI regulates the translocation of the Rho family GTPases between the membranes and cytosol. In resting cells, the Rho family GTPases exist mostly as GDP-bound forms and complexes with Rho GDI in the cytosol (Takai et al., 1995). Because Rho GDI counteracts the action of GEFs such as Dbl, it is thought that Rho GDI releases from Rho when Rho becomes susceptible to the GEFs.

3.4 RhoGAPs

RhoGAPs were identified as proteins that stimulate the intrinsic GTPase activity of G-proteins, leading to their inactivation (Moon and Zheng, 2003; Takai et al., 1995). GAPs share the related GAP domain, which consists of about 150 amino acids and shares 20% sequence identity. p50Rho GAP was identified as the first GAP directed towards Rho family GTPases (Garrett et al., 1989). Subsequently, p50 Rho GAP was found to be more active on Cdc42 than were Rho and Rac (Lancaster et al., 1994). A number of GAPs for Rho have been identified. They include p190 Rho GAP (Rho>Rac, Cdc42) (Settleman et al., 1992), p122 Rho GAP/DLC-1 (Rho) (Homma and Emori, 1995), Myr5 (Rho>Cdc42) (Reinhard et al., 1995), and so on. RhoGAP is involved in various physiological phenomena. p190 Rho GAP is required for axon outgrowth, guidance and fasciculation, and neuronal morphogenesis (Brouns et al., 2000; Brouns et al., 2001). Mice lacking the p190 Rho GAP show that p190 Rho GAP is essential for cell differentiation of thymus and brain (Sordella et al., 2002). Oligophernin-1 is

associated with X-linked mental retardation (Billuart et al., 1998). Rho GAP is involved in not only neuronal morphogenesis but also cytokinesis. MgcRacGAP and its *Caenorhabditis elegans* ortholog CYK-4 are localized in the central spindle during cytokinesis (Hirose et al., 2001). MgcRacGAP appears to accelerate GTP hydrolysis of Rho, thereby allowing disassembly of the contractile ring and completion of cytokinesis.

3.5 Rho Effectors

A number of molecules have been identified as the effectors of RhoA/C. They include Rho-associated kinase (Rho-kinase)/ROK/ROCK, the myosin-binding subunit (MBS) of myosin phosphatase, protein kinase N (PKN), rhophilin, rhotekin, citron, citron-kinase, mDia, phospholipase D, and phosphatidylinositol 5 kinase (PI5K) (Etienne-Manneville and Hall, 2002; Hall, 1998; Kaibuchi et al., 1999)(Figure 2).

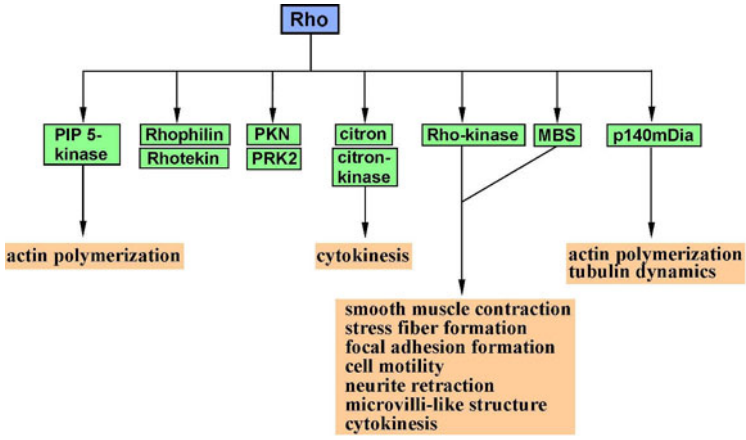

Figure 2. Mammalian targets of Rho.

1) Rho-kinase

The first identified effector for RhoA is known as Rho-kinase/ROKα/ROCK2 (Leung et al., 1995; Matsui et al., 1996; Nakagawa et al., 1996). ROKβ/ROCK1 is an isoform of Rho-kinase (Ishizaki et al., 1996; Leung et al., 1996). Rho-kinase has a kinase domain in the NH_2-terminal portion. This kinase domain has a 72% sequence homology with the kinase domain of myotonic dystrophy kinase. Rho-kinase has a putative

coiled-coil domain in the middle portion and a PH domain in the COOH-terminal portion. Activated Rho interacts with the COOH-terminal portion of the coiled-coil domain and activates the kinase activity of Rho-kinase. Rho-kinase regulates the smooth muscle contraction (Amano et al., 1996a; Kimura et al., 1996; Kureishi et al., 1997), formation of stress fibers and focal adhesions (Amano et al., 1997; Ishizaki et al., 1997; Leung et al., 1996), neurite retraction (Amano et al., 1998; Hirose et al., 1998; Katoh et al., 1998), and cytokinesis (Yasui et al., 1998) through the various substrates. The several molecules were identified as the substrates of Rho-kinase (Kaibuchi et al., 1999; Riento and Ridley, 2003).

2) Myosin binding subunit of PP1

The phosphorylation of myosin light chain (MLC) of myosin II plays a crucial role in regulating the contractile activity of smooth muscle. Myosin phosphatase, which is physiologically responsible for the dephosphorylation of MLC, is composed of three subunits: a 37-kDa type 1 phosphatase catalytic subunit (PP1c); a 130-kDa myosin-binding subunit (MBS); and a 20-kDa subunit (M20; its function remains unknown) (Hartshorne et al., 1998). MBS has a series of ankyrin repeats at the N-terminal end and interacts with both PP1c and a number of substrates, such as MLC and adducin (Hartshorne et al., 1998; Kimura et al., 1998). MBS is thought to target the enzyme to the particular location and to control the phosphatase activity. MBS interacts with the activated Rho through its COOH-terminal domain (Kimura et al., 1996). Rho-kinase phosphorylates MLC and MBS, which leads to the inactivation of myosin phosphatase (Amano et al., 1996b; Kawano et al., 1999; Kimura et al., 1996). Rho-kinase and MBS regulate the phosphorylation levels of MLC cooperatively, as described later(Fukata et al., 2001).

3) Protein kinase novel (PKN)

The kinase PKN (also known as PRK1) consists of an NH2-terminal regulatory domain and a COOH-terminal catalytic domain. The catalytic domain is highly homologous to that of protein kinase C (PKC), whereas the NH2-terminal domain shows no obvious homology with other protein kinases, including PKC (Mukai and Ono, 1994). The regulatory domain contains three leucine zipper-like motifs. Activated Rho interacts with the NH2-terminal portion containing the first leucine zipper-like motif and activates the kinase activity of PKN (Amano et al., 1996c; Watanabe et al., 1996). The physiological functions of PKN/PRK1 are unknown. PRK2 is an isoform of PKN/PRK1 and appears to associate with GTP·Rac as well as Rho in a GDP/GTP-independent manner (Vincent and Settleman, 1997). The expression of a kinase-deficient form of PRK2 disrupts actin stress

fibers, which suggests that PRK2 may be implicated in the actin cytoskeleton reorganization (Vincent and Settleman, 1997).

4) Rhophilin and Rhotekin.
Rhophilin contains three distinct regions, which are Rho-binding, Bro1, and PDZ domain. Rhophilin interacts with both GDP- and GTP-bound Rho in vitro. Rhophilin and Rhotekin have the sequence similarity to both MBS and PKN/PRK1 within the Rho-binding domain and interact with activated Rho (Reid et al., 1996; Watanabe et al., 1996). This domain may be one of the consensus motifs for Rho-interacting interfaces. Little is known about the functions of Rhophilin and Rhotekin.

5) Citron and Citron kinase.
Citron was originally isolated as the Rho-binding protein in a yeast two-hybrid system (Madaule et al., 1995). Citron has the sequence similarity with Rho-kinase in its overall domain structure but lacks a kinase domain. Citron kinase (CRIK) was identified as a splicing variant of citron with a kinase domain homologous to that of Rho-kinase in the NH_2-terminal portion. Citron kinase is localized at the cleavage furrow and mid-body during cytokinesis and is implicated in cytokinesis (Madaule et al., 1998). Recently, Camera and colleagues reported that the brain-specific protein citron-N is enriched at and associates with the Golgi apparatus of hippocampal neurons in culture (Camera et al., 2003). Suppression of the whole proteins or expression of the mutant form lacking the Rho-binding activity results in dispersion of their Golgi apparatus. Citron-N controls the actin local assembly together with Rho-kinase and profilin-IIa, a neuron-specific actin-binding protein.

6) Mammial Diaphenous
mDia is a mammalian homologue of *Drosophila diaphanous* that is required for cytokinesis and belongs to a formin-related protein family. mDia also has the sequence similarity to yeast Bni1p (Kohno et al., 1996). mDia interacts with Rho in a GTP-dependent manner in the NH2-terminal portion and with profilin in the repetitive polyproline stretches (Watanabe et al., 1997). mDia is involved in actin polymerization. Rho-kinase and mDia cooperatively act in the Rho-induced reorganization of actin cytoskeletons (Watanabe et al., 1999). Recent studies show that mDia is engaged in cell morphology, motility, and polarity through the regulation of not only actin cytoskeletons but also the dynamics of microtubule(Gundersen, 2002; Gundersen et al., 2004; Yasuda et al., 2004).

7) Phospholipase D

Rho is thought to regulate the phospholipase D (PLD) (Singer et al., 1997). PLD hydrolyzes phospholipids into phosphatidic acid (PA) and their head groups. PA is claimed to be a direct second messenger, but it can also be metabolized to other second messengers including diacylglycerol and lysophosphatidic acid (LPA) (English et al., 1996; Singer et al., 1997). The activity of PLD is stimulated by a variety of extracellular signals such as growth factors and hormones. Biological and biochemical studies have indicated that Rho regulates the PLD activity cooperatively with Arf, a member of the Ras superfamily (Bowman et al., 1993; Kuribara et al., 1995; Malcolm et al., 1994; Singer et al., 1995).

8) Phosphatidylinositol 5' kinase

PI5K has been implicated in Rho signaling (Chong et al., 1994; Ren et al., 1996). Previous work has shown that phosphatidylinositol (4,5)-biphosphate (PIP_2), the product of PI5K, can regulate the interaction of a number of actin-binding proteins including profilin, α-actinin, gelsolin, and p39CapZ in vitro (Janmey, 1994). Thus, it is possible that increased PIP_2 synthesis results in increasing actin polymerization and the association of actin filaments with focal adhesion. However, the role of PIP_2 in controlling the cytoskeletons during the action of Rho in intact cells remains controversial.

4. BIOLOGICAL FUNCTIONS OF RHO A/C

4.1 Smooth muscle contraction

Specific agonists such as carbacol and endothelin cause Ca^{2+} mobilization and the activation of myosin light chain kinase (MLCK) in smooth muscle. MLCK phosphorylates MLC and activates myosin adenosine triphosphatase (ATPase), and thereby induces the contraction of smooth muscles (Hartshorne, 1987; Kamm and Stull, 1985; Sellers and Adelstein, 1987). However, because the cytosolic Ca^{2+} level is not always proportional to the levels of the MLC phosphorylation and contraction, an additional mechanism to regulate the Ca^{2+} sensitivity of the levels of the MLC phosphorylation and contraction has been proposed (Bradley and Morgan, 1987).

Rho has been identified as a molecule implicated in Ca^{2+} sensitization by agonists in smooth muscle. The Ca^{2+} sensitizing effect of agonists or GTP•S is completely blocked by inhibitory toxins specific for Rho (Hirata et al., 1992; Noda et al., 1995). The introduction of GTPγS·Rho or the

constitutively active form of Rho into permeabilized smooth muscle cells induces Ca^{2+} sensitization(Gong et al., 1996). Furthermore, it has been reported that the increment in the phosphorylation of MLC by the activated Rho at a constant Ca^{2+} concentration is due to a reduction in the dephosphorylation rate and not to an increment in the phosphorylation rate (Noda et al., 1995). Thus, Rho regulates Ca^{2+} sensitivity via the regulation of myosin phosphatase activity. Rho regulates MLC phosphorylation through its effectors, Rho-kinase and MBS (Kaibuchi et al., 1999). Agonists activate Rho through the activation of certain heterotrimeric G-protein-coupled receptors, and the activated Rho interacts with Rho-kinase, leading to the activation of Rho-kinase. Activated Rho-kinase subsequently phosphorylates MBS and inhibits the activity of myosin phosphatase (Kawano et al., 1999; Kimura et al., 1996). Concomitantly, Rho-kinase directly phosphorylates MLC at the same site that is phosphorylated by MLCK (Amano et al., 1996a). Thus, Rho-kinase may regulate smooth muscle contraction via two processes, the inactivation of myosin phosphatase and direct MLC phosphorylation(Fukata et al., 2001) (Figure 3). In nonmuscle cells, MLC phosphorylation is thought to play a role in the interaction of myosin with actin to form stress fibers and to induce their contraction and cell motility (Giuliano and Taylor, 1995). Several studies have established the roles of Rho-Rho-kinase pathway in MLC phosphorylation in nonmuscle cells. MBS is phosphorylated and the myosin phosphatase activity is inactivated during the action of thromboxane A_2 in platelets, and both reactions are reversed by prior treatment of platelets with botulinum C3 toxin (Nakai et al., 1997). Similar observations are obtained in endothelial cells during the action of thrombin (Essler et al., 1998). The expression of dominant inhibitory forms of Rho or Rho-kinase induces MLC phosphorylation in COS7 cells (Chihara et al., 1997) and NIH3T3 cells(Amano et al., 1998), whereas the expression of the dominant negative form of Rho-kinase inhibits the serum-induced MLC phosphorylation. Thus, Rho-kinase appears to regulate MLC phosphorylation downstream of Rho in nonmuscle cells as well as in smooth muscle.

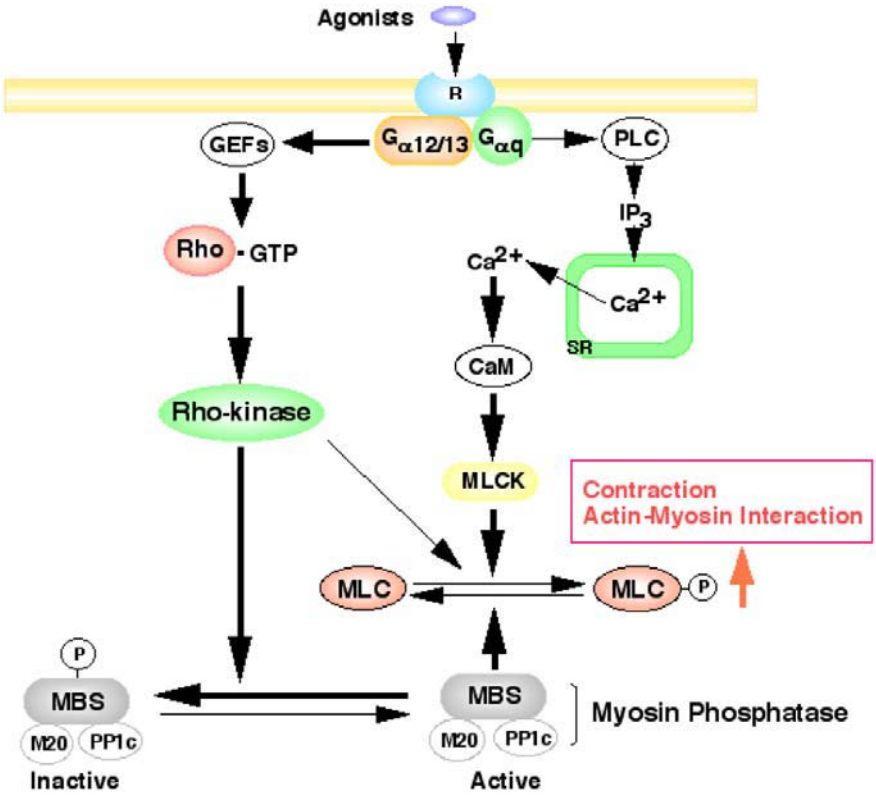

Figure 3. Regulation of smooth muscle contraction by Rho-kinase and myosin phosphatase downstream of Rho. R, receptor for agonists; G, heterotrimeric G protein; PLC, phospholipase C; IP3, inositol 1,4,5-triphosphate; SR, sarcoplasmic reticulum; CaM, calmodulin; MLCK, myosin light chain kinase; MLC, myosin light chain; PP1c, catalytic subunit of myosin phosphatase; M20, 20-kDa subunit of myosin phosphatase.

4.2 Stress fibers and focal adhesions

Rho is thought to regulate the formation of actin stress fibers and focal adhesions on the basis of the following observations. A prior treatment of the cells with botulinum C3 ADP-ribosyltransferase (C3), which interferes with Rho functions, inhibits the LPA-induced stress fiber formation (Paterson et al., 1990; Ridley and Hall, 1992). Microinjection of dominant active Rho into the cells also induces stress fiber formation, indicating that Rho plays central roles in stress fiber formation. Rho is also required for

the LPA-induced formation of focal adhesions (Ridley and Hall, 1992), the site at which stress fibers are linked via integrin to the extracellular matrix such as fibronectin (Huttenlocher et al., 1995; Yamada and Miyamoto, 1995). The expression of the dominant active Rho-kinase induces the formation of stress fibers, whereas the expression of the dominant negative Rho-kinase inhibits the LPA- or RhoV14-induced formation of stress fibers (Amano et al., 1997; Ishizaki et al., 1997; Leung et al., 1996). The phosphorylation of MLC by Rho-kinase leads to the activation of myosin ATPase, thereby generating the contractility of stress fibers (Amano et al., 1996b). Taken together, these observations indicate that the Rho-Rho-kinase pathway plays a critical role in the formation of stress fibers and focal adhesions through activation of myosin II. Moreover, Rho-kinase directly phosphorylates LIM-kinase, which in turn is activated to phosphorylate actin depolymerizing protein, cofilin/ADF (Maekawa et al., 1999). The phosphorylation of cofilin by LIM-kinase inhibits its functions. The inhibition of cofilin results in the stabilization of actin filaments such as stress fiber. In addition to Rho-kinase, mDia also participates in the formation of stress fiber (Watanabe et al., 1999). The coexpression of the dominant active form of mDia1 and Rho-kinase induces the condensed actin fibers, which is reminiscent of Rho-induced stress fibers. These results indicate that Rho-kinase and mDia cooperatively form stress fibers downstream of Rho.

4.3 Cell motility and migration

The reorganization of actin filaments, cell-substratum adhesion, and lamellipodial protrusion are involved in cell motility. The activity of Cdc42 is required for cell polarity (Etienne-Manneville, 2004). Rac appears to drive a force arising from actin polymerization in lamellipodial protrusion (Hall, 1998). Actin at the membrane ruffling area continuously depolymerizes and then repolymerizes during cell movement (Mitchison and Cramer, 1996). Rho regulates a force derived from myosin II driven by MLC phosphorylation (Amano et al., 1996a; Kimura et al., 1996), in the membrane ruffling area and the posterior region of motile cells (Matsumura et al., 1998; Mitchison and Cramer, 1996)(Figure 4). Indeed, the involvement of phosphorylation of MLC in cell migration is confirmed by the injection of anti-MLCK antibody into macrophages (Wilson et al., 1991). The early studies indicate that Rho regulates cell motility (Ridley et al., 1995; Takaishi et al., 1994). The microinjection of Rho GDI or botulinum

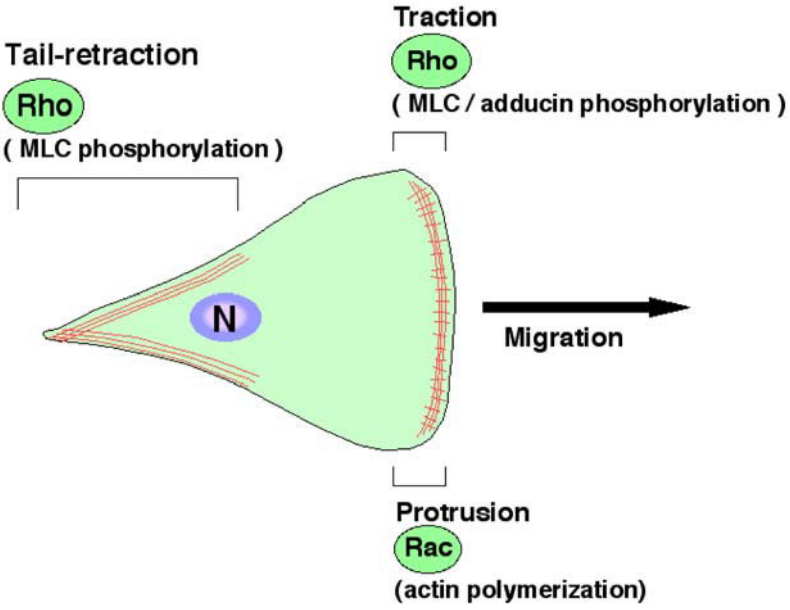

Figure 4. Roles of Rho in cell motility.

C3 toxin, but not that of dominant negative form of Rac, inhibited cell motility induced by hepatocyte growth factor (HGF) in keratinocytes (308R cells). Moreover, the dominant negative form of Rho inhibits the HGF-induced membrane ruffling of MDCK cells (Fukata et al., 1999).

Thus, Rho plays crucial roles in the cell motility of epithelial cells, cycling between GDP-bound and GTP-bound forms. The expression of the dominant negative form of Rho-kinase also inhibits the HGF-induced membrane ruffling and wound-induced cell migration, indicating that Rho-kinase is necessary for cell motility (Fukata et al., 1999). Taken together, these observations suggest that MLC phosphorylation regulated by the Rho-Rho-kinase pathway is necessary for cell motility. In addition to MLC, Rho-kinase regulates cell motility through the phosphorylation of substrates, such as adducin, by the direct phosphorylation and by the inactivation of myosin phosphatase (Fukata et al., 1999; Kaibuchi et al., 1999).

Like neutrophilic leukocytes, differentiated HL-60 cells respond to chemoattractant by adopting a polarized morphology, with F-actin in a protruding pseudopod at the leading edge and contractile actin-myosin complexes at the back and sides. Recently, Xu et al., have shown that

this polarity depends on divergent, opposing "frontness" and "backness" signals generated by different receptor-activated trimeric G proteins (Xu et al., 2003). "Frontness" depends on G_i-mediated production of 3'-phosphoinositol lipids (PI3Ps), the activated form of Rac, a small GTPase, and F-actin. G_{12} and G_{13} trigger "backness" signals, including activation of Rho, a Rho-kinase, and myosin II. Within the cell, the high Rac activity regulates behavior at front, whereas the Rho activity is critical at rear. The antagonism between these signaling pathways determines the directional responsiveness to chemotactic signals.

4.4 Neurite retraction and extension

Chemoattractants and chemorepellants can exert significant effects on neural architecture, ranging from the stimulation of neurite outgrowth to the induction of growth cone collapse and neurite retraction (Tanaka and Sabry, 1995; Tessier-Lavigne and Goodman, 1996). The growth cone is a dynamic structure at a tip of a neurite and consists of filopodia and lamellipodia. Neurite retraction and the extension of developing neurites evoked by extracellular molecules are fundamental to nervous system development and neural plasticity. Rho is implicated in thrombin-, serum-, and LPA-induced neurite retraction and cell rounding in N1E-115 neuroblastoma and PC12 cells (Jalink and Moolenaar, 1992; Nishiki et al., 1990), whereas Rac, Cdc42, or both are implicated in the promotion of filopodia and lamellipodia formation in growth cones and therefore in neurite extension. Competition appears to exist between chemoattractant- and chemorepellant-induced morphological pathways mediated by Rac, Cdc42, or both and by Rho, leading to either neurite development or collapse (Kozma et al., 1997). The Rho-induced neurite retraction is presumed to be driven by a contraction of the cortical actin-myosin system (Jalink et al., 1994), which is regulated by Rho. Rho-kinase induces neurite retraction through the increment of actomyosin contractility downstream of Rho (Amano et al., 1998; Hirose et al., 1998; Katoh et al., 1998). Consistently, the inhibition of Rho-kinase induces the outgrowth of axonal processes in cerebellar granule neurons (Bito et al., 2000).

Accumulating evidence indicates that the activation of Rho evokes growth cone collapse downstream of guidance cues such as Semaphorin and Ephrin (Jin and Strittmatter, 1997; Wahl et al., 2000). The plexins are the signal transduction component of most Semaphorin receptor complexes (Tamagnone and Comoglio, 2000). Recently, PDZ-Rho-GEF and LARG, which are Rho specific GEFs, were shown to link mammalian plexin B proteins to Rho activation (Swiercz et al., 2002). In addition, Ephexin has

been shown to provide a direct link between Rho and Eph receptors in response to ephrin-A1 stimulation (Shamah et al., 2001). Wahl and his colleagues reported that ephrin-5A-induced collapse is mediated by activation of Rho and Rho-kinase in chick retinal ganglion neurons (Wahl et al., 2000). Rho-kinase appears to be involved in LPA-induced growth cone collapse via the phosphorylation of CRMP-2 (Arimura et al., 2000).

4.5 Cytokinesis

Cells undergo cytokinesis in the mitotic phase through the formation of a contractile ring beneath the plasma membrane. The contractile ring is composed mainly of actin filaments and myosin and is thought to divide the cells into two daughter cells by pulling the membrane inward (cytoplasmic division) through its contractility (Fishkind and Wang, 1995; Theriot and Satterwhite, 1997). The cytoplasmic division of *Xenopus* fertilized embryos is inhibited by the microinjection of Rho GDI or botulinum C3 toxin, whereas nuclear division occurs normally (Drechsel et al., 1997; Kishi et al., 1993; Mabuchi et al., 1993). Coinjection of Rho GDI with the activated RhoA prevents the Rho GDI action. Thus, Rho appears to control the cytoplasmic division through regulation of the contractility of the contractile ring.

Rho and Rho-kinase accumulate at the cleavage furrow (Kosako et al., 1997; Takaishi et al., 1995), where MLC phosphorylation occurs (Matsumura et al., 1998). The expression of dominant negative Rho-kinase inhibits the cytokinesis of Xenopus embryo and mammalian cells, resulting in multiple nuclei (Yasui et al., 1998). Thus, MLC phosphorylation by the Rho-Rho-kinase pathway provides contractility to the contractile ring and plays a critical role in cytokinesis. Rho-kinase also phosphorylates intermediate filament proteins such as glial fibrillary acidic protein (GFAP) and vimentin, exclusively at the cleavage furrow during cytokinesis (Goto et al., 1998; Kosako et al., 1997). The expression of GFAP mutated at Rho-kinase phosphorylation sites induces impaired glial filament segregation into postmitotic daughter cells (Yasui et al., 1998). These results suggest that Rho-kinase is essential not only for cytokinesis but also for the segregation of GFAP filaments into daughter cells, which in turn ensures efficient cellular separation.

Citron kinase is also localized at the cleavage furrow and mid-body (Madaule et al., 1998). The overexpression of Citron kinase mutants results in multinucleate cells, whereas that of a kinase-active mutant causes abnormal contraction during cytokinesis, suggesting that Citron kinase is involved in cytokinesis. Citron kinase knockout mice grow at slower rates, are severely ataxic, and die before adulthood as a consequence of fatal

seizures (Di Cunto et al., 2000). Their brains display defective neurogenesis, with depletion of specific neuronal populations. These abnormalities arise during development of the central nervous system due to altered cytokinesis and massive apoptosis. Recently, the substrate of Citron kinase was identified (Yamashiro et al., 2003). Citron kinase phosphorylates MLC of myosin II at both Ser-19 and Thr-18 in vitro. Citron kinase may be involved in regulating diphosphorylation of MLC during cytokinesis downstream of Rho.

4.6 Microvilli

The ERM (ezrin, radixin, and moesin) family proteins function as cross-linking proteins between the plasma membranes and actin filaments (Algrain et al., 1993; Arpin et al., 1994; Tsukita et al., 1989). The NH_2-terminal and COOH-terminal domains of the ERM family proteins are thought to directly bind to some integral membrane proteins such as CD44 (Tsukita et al., 1994) and actin filaments, respectively (Pestonjamasp et al., 1995; Turunen et al., 1994). The NH_2- and COOH-terminal domains appear to mask each other, presumably through the intramolecular head-to-tail association between the NH_2- and COOH-terminal domains, thereby inhibiting the interaction of the ERM family proteins with their partner proteins (Gary and Bretscher, 1995; Martin et al., 1995). Once the intramolecular association is released and thereby the ERM family proteins are activated, they appear to be translocated from the cytosol to the plasma membranes, where they serve as cross-linkers (Bretscher et al., 1997; Tsukita et al., 1997). Moreover, the ERM family proteins are essential for Rho- and Rac-induced cytoskeletal reorganization with a permeable cell reconstitution assay (Mackay et al., 1997).

Biochemical analysis has shown that Rho-kinase phosphorylates moesin at Thr-558, thereby inhibiting the intramolecular association between the NH_2- and COOH-terminal domain of radixin (Fukata et al., 1998; Matsui et al., 1998). Thus, the Rho-Rho-kinase pathway can activate the ERM family proteins through their phosphorylation. Other studies have shown that LPA stimulation of serum-starved NIH3T3 cells results in relocalization of radixin into microvilli-like structures, which is blocked by botulinum C3 toxin (Shaw et al., 1998). The expression of the dominant active form of Rho in COS7 cells induces moesin phosphorylation and the formation of microvilli-like structures at apical membranes where the Thr-558-phosphorylated moesin accumulates (Oshiro et al., 1998). The expression of dominant active Rho-kinase also induces moesin phosphorylation. Thus, Rho-kinase appears to regulate moesin phosphorylation downstream of Rho, and the phosphorylation of moesin by Rho-kinase may play a crucial role in

the formation of microvilli-like structures. However, another laboratory has reported that PI5K is also involved in ERM-dependent microvilli formation downstream of Rho (Matsui et al., 1999), but Rho-kinase is not. Further study is necessary for determining which kinase is engaged in ERM-dependent microvilli formation downstream of Rho. Recently, Rho-kinase-dependent phosphorylation of ERM family proteins was found in the coronary artery, central nervous system, lungs, and heart. Interestingly, this phosphorylation is closely associated with the pathogenesis of hypertension, vasospasm, and arteriosclerosis (Abe et al., 2004; Hattori et al., 2004a; Hattori et al., 2004b; Ito et al., 2003). It is hoped that the physiologic meaning of this phosphorylation will be elucidated.

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Chapter 7

CDC42 AND RAC CONTROL OF THE ACTIN CYTOSKELETON

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Abstract: Cell shape changes are critical to cell differentiation, movement and motility. Reorganization of the actin cytoskeleton is crucial to these changes, which are elicited in response to extracellular stimuli. This reorganization is achieved by the action of kinases, phosphatases and effectors of Rho GTPases on actin-containing filaments. In this review, we shall discuss how the different proteins such as WASP, WAVE, PAK and IQGAP, which are downstream of Cdc42 and Rac affect the actin cytoskeleton. Guanine nucleotide exchange factors that activate different Rho GTPases also contribute towards the regulation of the cytoskeleton. Many of the Rho GTPase targets are kinases or are regulated by phosphorylation. Thus phosphatases are essential in the control of cell adhesion and spreading as well. The integration and modulation of the different signalling pathways downstream of the Rho proteins are key to the final cellular responses. How the different downstream proteins are shuttled and shuffled is also of interest because the same molecules may participate in pathways that are regulated by Cdc42, Rac and the antagonistic RhoA.

1. INTRODUCTION

The small GTPases of the Rho family play key roles in transducing extracellular stimuli into distinct responses including cell motility, adhesion, cell division and phagocytosis. The GTPases cycle between GTP-bound and GDP-bound forms where activation requires the action of guanine nucleotide exchange factors (GEFs) to promote conversion of GDP to GTP states. Individual members of the Rho GTPases are known to cause specific changes to the actin cytoskeleton of the cells. Active RhoA promotes actin

E. Manser (ed.), RHO Family GTPases, 137-156.

stress fibre formation, while expression of dominant active Cdc42 and Rac1 induces the formation of filopodia and lamellipodia respectively. The rearrangement of the cytoskeletal structures is pivotal to the outcome of the signal transduction events downstream of the Rho GTPases (reviewed by Lim et al., 1996; Van Aelst and D'Souza-Schorey, 1997).

Some of the downstream effectors of the Rho GTPases and the pathways they regulate have been well studied. In particular, Rho-kinase/ROK (Amano et al., 1997; Leung et al., 1995) and mDia (Watanabe et al., 1997), which are downstream of RhoA have been shown to promote the formation of stress fibres. ROK can phosphorylate and inactivate the myosin binding subunit of the light chain (MLC) phosphatase (Kimura et al., 1996). This results in an increase in phosphorylated MLC, its enhancement of actin binding/bundling activity and consequently of stress fibre formation. The effector proteins downstream of Rac1 in lamellipodia formation were not as well characterized, until the recent discovery of the WAVE subfamily of the WASP proteins. WAVE promotes the formation of lamellipodia downstream of Rac1 (Miki et al., 1998b; Miki et al., 2000). POR1 may also be involved in this process (Van Aelst et al., 1996). N-WASP mediates the link between Cdc42 and the Arp2/3 proteins in actin polymerization, and participates in the formation of filopodia (Miki et al., 1998a; Rohatgi et al., 1999). The ROK-related target MRCK is involved in the formation of focal complexes and filopodia as a kinase-inactive mutant can block these processes downstream of Cdc42 (Leung et al., 1998).

In this review, we discuss the control of cytoskeleton by Rac and Cdc42 as well as their effectors, activators and other proteins that interact with the downstream targets of these GTPases. The effect of RhoA shall not be described extensively.

2. THE ACTIN CYTOSKELETON AND CELL SHAPE CHANGES

Cell shape changes are crucial to cellular movement and cell motility. The rearrangement of the cortical actin filaments is essential for the changes in cell shape, elicited in response to upstream signals. Hematopoietic cells need to migrate through the blood vessels walls and tissue spaces to reach the site of infection. Metastatic cancer cells move and invade other tissues to cause the spread of the disease. Neuronal cells send out neurites and axons and also migrate from the site of proliferation to other sites in response to developmental cues. Actin polymerization at the leading edge of the cell is responsible for driving the cell membrane forward, involving peripheral cell structures such as lamellipodia and filopodia. Lamellipodia are made up of

short branches of actin filaments forming a network. Each actin filament has branches at an angle of 70°, with the barbed end towards the cell membrane (Svitkina and Borisy, 1999). On the other hand, filopodia do not contain a meshwork of actin branches and are made up of long actin bundles (Figure 1). The spatial-temporal modulation of actin polymerization, branching and depolymerization is important to the maintenance of lamellipodia and filopodia at the leading edge of the cells. However, the formation of the trimeric nucleus for actin filament assembly is very slow although monomeric globular actin protein (G-actin) spontaneously forms trimeric nuclei. This nucleation process was shown to be initiated and promoted by the Arp 2/3 complex (Machesky et al., 1994) formed by the two actin-related proteins, Arp2 and Arp3, and five novel subunits. Increasing biochemical purification of the Arp2/3 complex led to a decline in its nucleation activity. This is because the Arp 2/3 complex needs to be activated first. One of the earliest activators found was ActA (Welch et al., 1998), a protein from the

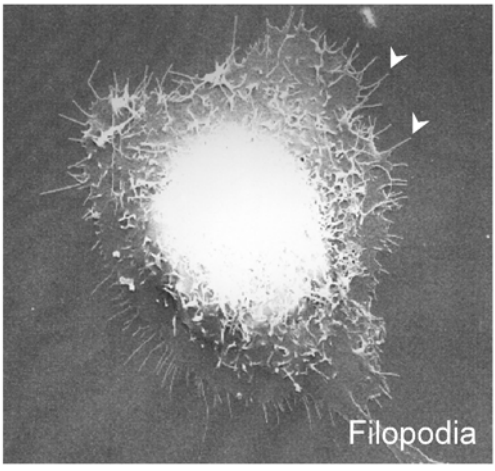

Figure 1. Electron micrographs of filopodia, ruffles and lamellipodia.

bacterium Listeria. Others have found WASP, myosin-I and cortactin to bind and activate the Arp2/3 complex (Lechler et al., 2000; Lee et al., 2000; Machesky et al., 1999; Rohatgi et al., 1999; Weaver et al., 2001).

2.1 The WASP family and Cdc42

The WASP (Wiskott-Aldrich syndrome) proteins are divided into two subfamilies: WASP and WAVE proteins. The WASP group consists of WASP and N-WASP, and the WAVE group has three very similar members (WAVE1-3). The expression of N-WASP is ubiquitous whereas WASP is expressed mainly in hematopoietic cells. These proteins contain many functional domains. The WH1 (WASP homology) domain binds phosphatidylinositol 4,5-bisphosphate (PIP2; Miki et al., 1996). It is thought that WASP/N-WASP anchors itself to the cell membrane by interacting with phospholipids. The GBD/CRIB domain is the binding site of Cdc42. It is this binding which regulates the activity of the WASP proteins. The proline-rich region binds several SH3-containing proteins. The VCA (Verpolin-Connecting/Cofilin-Acidic) domain at the C-terminus is important for binding to actin and the Arp2/3 complex. WASP proteins appear to adopt an auto-inhibitory conformation and need to be activated before they can bind to the Arp2/3 complex and actin. Co-expression of Cdc42 and N-WASP induces the formation of long filopodia (Miki et al., 1998a). A mutant N-WASP that cannot bind Cdc42 does not induce filopodia formation. The interaction of active Cdc42 and N-WASP causes a conformational change to expose the VCA domain and allow N-WASP to bind both actin and the Arp2/3 complex (Figure 2A).

However, N-WASP (-/-) knockout cells still form filopodia when microinjected with active Cdc42 (Snapper et al., 2001). There then must exist other proteins that can stimulate actin nucleation and polymerization, apart from the N-WASP-Arp2/3 complex. It has been argued that the Arp2/3 complex nucleates actin to form branches rather than long bundles of actin filament as in the case with filopodia. Perhaps the Arp2/3 complex does not regulate the extension of filopodia. On the other hand, IRSp53 could provide the link between Cdc42 and Ena/VASP (Mena) protein to direct actin nucleation and filament assembly of filopodia (Bear et al., 2002). IRSp53 was originally identified as a substrate of the insulin receptor kinase (Yeh et al., 1996) and there have been other reports that Cdc42 uses IRSp53 as an intermediary to form complexes which induce filopodia and neurite outgrowth (Govind et al., 2001; Krugmann et al., 2001). It has also been reported that Diaphanous-related formin, p134mDia2 (Drf3), could be the effector downstream of Cdc42 that facilitates filopodia formation (Peng et

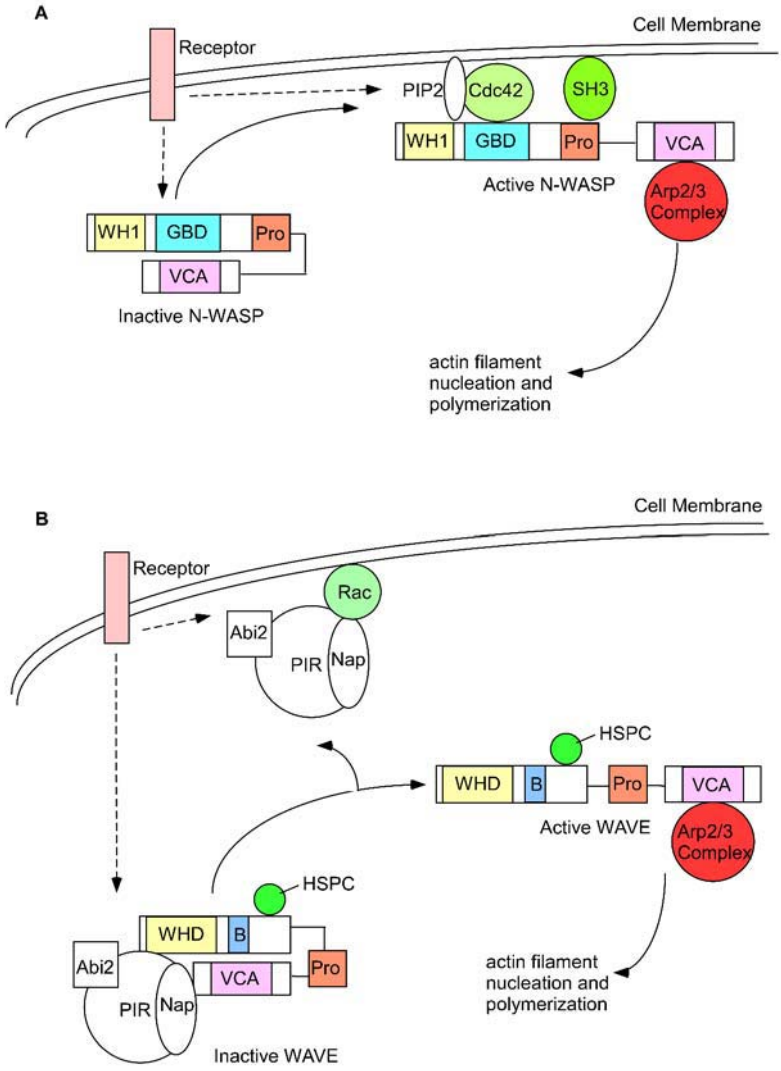

Figure 2. The activation of WASP and WAVE proteins. (A) When the cell receives activating signals from the receptor, WASP is targeted to the membrane. Active Cdc42 can then bind to WASP at the GTPase binding domain (GBD). Similarly, PIP2 and SH3- containing proteins such as Nck can also bind to their respective binding sites on WASP to activate WASP. Upon binding to Cdc42, PIP2, or Nck, the auto-inhibition of WASP is relieved and the VCA domain thus exposed is able to interact with and activate the Arp2/3 complex to stimulate actin nucleation. (B) WAVE1 is kept inactive by associating with four other proteins: Nap125, PIR121, Abi2 and HSPC300. When active Rac is present, the complex dissociates into NAP125-PIR121-Abi2 sub complex and WAVE1-HSPC300 sub complex. WAVE1 is now free to activate the Arp2/3 complex.

al., 2003). The formin homology domains, FH1-FH2 regions, of the yeast protein Bni1p have been proposed to nucleate actin filaments in vitro and promote the growth of actin filament at the barbed end. A similar and more potent actin nucleating activity was also observed in constructs containing the FH2 domain of mDia1 (Li and Higgs, 2003). mDia1 has also been reported to adopt an auto-inhibitory mode of regulation much like that of N-WASP. The N terminus of mDia1 inhibits the actin nucleation activity of the C-terminus. RhoA binding to the N terminus can only partially relieve the inhibition, implying the involvement of other regulatory molecules.

2.2 The WAVE Proteins and Rac

The WAVE proteins act down stream of Rac to induce actin polymerization responsible for lamellipodia formation. As with WASP, all three members of the WAVE proteins contain multiple functional domains. They have a proline-rich domain followed by the VCA domain at the C-terminal. However, the N terminal WAVE/SCAR homology domain (WHD/SHD) is different from that of WASP and its function is not known (Bear et al., 1998). There is no GTPase-binding domain present in WAVE. How the WAVE proteins are regulated by Rac remained a puzzle until recently, although it has been reported that Rac binds to IRSp53, which in turn binds to WAVE2 to activate it (Miki et al., 2000). However, WAVE1 and 3 do not bind IRSp53. Purified WAVE1 also appears to be constitutively active. There must exist other mechanisms to activate Arp2/3 through WAVE. The puzzle was apparently solved with the discovery of an inhibitory complex that renders WAVE1 inactive (Eden et al., 2002). WAVE1 is kept inactive by associating with four other proteins: Nap125, PIR121, Abi2 and HSPC300. When active Rac is present, the complex dissociates into sub complexes of Nap125-PIR121-Abi2 and of WAVE1-HSPC300. WAVE1 is now free to active the Arp2/3 complex (see Figure 2B).

There is a further twist to the story. More recently, there have been findings which are contradictory to the inhibitory function of the Wave-Abi complex (Innocenti et al., 2004). It was reported that the WAVE2-Abi1-Nap1-PIR121 complex binds to Rac and was targeted to the lamellipodia. Contrary to the earlier report, the binding to Rac did not result in the dissociation of the complex and that the recruitment of the WAVE2-Abi1-Nap1-PIR121 complex to the lamellipodia actually resulted in site-directed nucleation of actin filaments. It was also shown that the undissociated complex was active in stimulating Arp2/3. Further work is required to reconcile the differences in the findings.

Cdc42 and Rac are not the only regulators of WASP and WAVE. Acidic lipids such as phosphatidylinositol 4,5-bisphosphate can also bind to and

activate N-WASP (Miki et al., 1996; Rohatgi et al., 2000). The adaptor protein Nck also participates through one or more of its SH3 domains in binding N-WASP and the WAVE1-complex (Eden et al., 2002; Rohatgi et al., 2001). Other SH3-containing proteins such as WISH (Fukuoka et al., 2001), Ash/Grb2 (Carlier et al., 2000) and Profilin (Suetsugu et al., 1998; Yang et al., 2000) bind to the proline-rich domain of N-WASP to relieve its auto-inhibition. Tyrosine phosphorylation of N-WASP at residue 291 has also been shown to activate N-WASP independently of Cdc42 (Cory et al., 2002).

2.3 IQGAP and Cdc42/Rac

IQGAP1 is another multi-domain protein that interacts with Cdc42 and Rac. Starting from the N-terminal, it has a Calponin homology domain that binds actin, a WW domain and four tandem repeats of the IQ motif which binds calmodulin, myosin light chain and S100B, a Zn2+- and Ca^{2+}-binding protein (Briggs and Sacks, 2003). There is also a GAP-related domain (GRD), which interacts with Cdc42 and Rac but not RhoA and Ras. This is followed by a RasGAP-like domain at the C-terminal. However, this domain does not have any GAP activity and instead it interacts with microtubule-binding protein CLIP170 and is necessary for binding E-cadherin. Mammalian IQGAP1 colocalizes with actin in lamellipodia (Hart et al., 1996) and induces filopodia and microspike formation when over-expressed (Swart-Mataraza et al., 2002). The precise mechanism of how IQGAP induces the formation of filopodia is not known but it has been shown that IQGAP inhibits the intrinsic GTPase activity of Cdc42 in vitro (Hart et al., 1996). This will stabilize GTP-bound Cdc42 and thus increase the pool of active Cdc42. There are also reports on the actin cross-linking activities of IQGAP1 (Bashour et al., 1997; Fukata et al., 1997).

2.4 PAK and Cdc42/Rac

PAK (p21-activated kinase, Manser et al., 1994) was one of the first effectors identified for Cdc42 and Rac. Thus far, three members of the conventional group one PAK have been identified (reviewed by Jaffer and Chernoff, 2002). The N-terminal regulatory region of PAK contains three proline-rich sequences, a GTPase binding (PBD/CRIB) and a kinase-inhibitory domain. The C-terminal kinase domain is similar to that of the Ste20 protein of the budding yeast. PAK normally exists as a dimer with the inhibitory domain of one binding to the kinase domain of another PAK molecule. Upon binding to active Cdc42 or Rac, the inhibition is relieved and PAK becomes activated and self-phosphorylates (Lei et al., 2000). PAK

is an important regulator of cytoskeleton dynamics and cell motility. It has been shown that activated PAK caused the disassembly of stress fibres and the dissolution of focal adhesion complexes (Manser et al., 1997). Others have also reported that PAK is recruited to the leading edge of cells (Dharmawardhane et al., 1999; Harden et al., 1996; Li et al., 2003) and that it is involved in axon guidance(Newsome et al., 2000). A recent and fuller account of PAK and its function can be found in the review of Bokoch (2003).

2.5 PAK, its substrates and the regulation of cytoskeleton

2.5.1 LIM kinases

The numbers of protein found to be phosphorylated by PAK have increased steadily following its discovery. Many of these substrates participate in the regulation of the cytoskeleton and cell motility. LIM kinases 1 and 2 are such substrates that have been implicated in actin cytoskeletal regulation (Edwards et al., 1999). They are serine/threonine kinases which phosphorylate and down-regulate the cofilin/actin depolymerising factor (ADF) family of proteins under the control of Rac GTPases (Arber et al., 1998; Yang et al., 1998). One of the main functions of cofilin/ADF in the cell is to depolymerize actin filaments. Cofilin/ADF increases the off-rate at the pointed ends of filaments without changing the off-rate at the barbed ends (Carlier et al., 1997). Inhibition of cofilin/ADF will result in increased cellular levels of F-actin. In short, increased Rac activity will lead to the activation of PAK and its phosphorylation of LIM kinase. The activated LIM kinase in turn will phosphorylate and inactivate cofilin/ADF. The final outcome is more actin polymerization. Interestingly, the critical Thr508 regulatory site in LIM kinase-1 can also be phosphorylated by the myotonic dystrophy-related Cdc42-binding kinase (MRCK) and by Rho-kinase (Maekawa et al., 1999; Sumi et al., 2001). This implies that the LIM kinases could be regulated by all members of the Rho GTPase family. Cross talk between the different Rho GTPases is likely to determine the outcome and the response.

2.5.2 Regulatory myosin light chain and myosin light chain kinase

It has long been known that Cdc42 and Rac induce protrusions at the leading edge of cells whereas RhoA promotes retraction. Myosin II contractility is

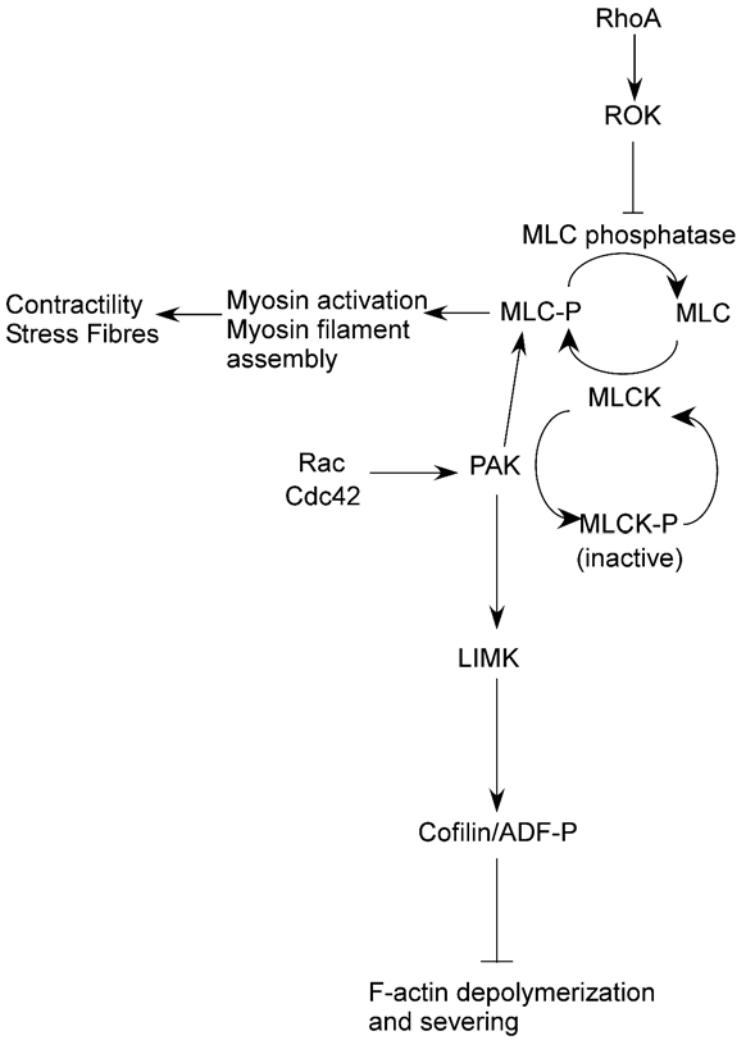

Figure 3. The regulation of stress fibres and myosin/actin contractility in the cell. Members of the Rho GTPase family regulate the actin cytoskeleton through activities of their effectors. ROK/ROCK is activated by binding to RhoA and PAK is activated by active Cdc42 and Rac. ROK/ROCK phosphorylates myosin light chain (MLC) phosphatase and inhibits the phosphatase activity. Thus MLC remains phosphorylated and can promote myosin and myosin filament assembly, which leads to contractility of stress fibres. MLC is phosphorylated by MLC Kinase (MLCK) which itself can be phosphorylated by PAK and becomes inactive. However, PAK can also phosphorylate MLC directly, resulting in myosin activation. Another substrate of PAK is LIM kinase (LIM-K). Active LIM-K phosphorylates and inactivates cofilin/ADF, thus inhibiting the F-actin depolymerization and severing activity of cofilin. The balance of the two activities of PAK is crucial in the maintenance of stress fibres.

crucial to such cell shape changes. Myosin forms filaments that consist of two heavy chains and two light chains. Myosin filaments interact with actin to generate the contractile forces required for cell retraction or spreading. The phosphorylation of the regulatory myosin light chain (R-MLC) at Thr18 and Ser19 by Ca^{2+}-dependent myosin light chain kinase (MLCK) has been reported to be responsible for the modulation of myosin contractility. Phosphorylation at these two residues induces conformational changes to allow the myosin molecules to form filaments. PAK has been shown to phosphorylate and inhibit MLCK (Goeckeler et al., 2000; Sanders et al., 1999). Phosphorylated/inhibited MLCK can no longer phosphorylate the regulatory myosin light chain (R-MLC), which results in a decrease in actin-myosin filament assembly. RhoA has the opposite effect on R-MLC. RhoA acting via Rho- kinase, ROK (Kimura et al., 1996) promotes the phosphorylation and subsequent inactivation of myosin light chain phosphatase. The resultant increase in phosphorylated R-MLC enhances myosin filament assembly and thus generates tension on the actin filaments and bundles them into stress fibres. Interestingly, ROK can also directly phosphorylate R-MLC, bypassing the MLCK pathway (Amano et al., 1996). On the other hand, there are also reports of PAK phosphorylation of the R-MLC at the critical Ser19 that could result in increasing contractility (Chew et al., 1998; Zeng et al., 2000). Here again, cross talk between Cdc42, Rac and Rho is clearly shown to be important in coordinating the complex events regulating the actin-myosin contractility affecting cell protrusion or retraction (see Figure 3).

Another Cdc42 effector, MRCK, can also phosphorylate MLC (Leung et al., 1998). In addition, over-expression of MRCKα promoted filopodia formation while that of a MRCK kinase-deficient mutant inhibited the induction of filopodia by Cdc42. The mechanisms are not known.

3. GUANINE NUCLEOTIDE EXCHANGE FACTORS AND THE ACTIN CYTOSKELETON

Cycling of Rho GTPases between the GTP-bound and GDP-bound states serves as a molecular switch to turn on and turn off the downstream pathways. Rac, Cdc42 and Rho are the best studied of the Rho GTPases. Although the Rho family proteins have intrinsic GTPase activities, the rate of hydrolysis of the GTP to GDP is slow. This rate and thus down regulation of the Rho proteins are enhanced by GAPs. The GDP-bound GTPase is kept in the cytosol by RhoGDI (Guanine nucleotide dissociation inhibitor). Rho GTPases are prenylated at their C-terminal, enabling them to anchor to the membrane. The binding of RhoGDI leads to the masking of the prenylated

tail so that the complex remains in the cytosol and interaction with the membrane is inhibited (Olofsson, 1999). The GTPase becomes active again when a GEF (Guanine nucleotide exchange factor) comes along and catalyses the exchange of GDP for GTP and also dissociates the GTPase from its complex with RhoGDI. The active GTP-bound GTPase can now translocate to the membrane where its activity is required.

Over-expression of many GEFs can lead to diverse changes in cell morphology such as increased formation of lamellipodia (Tiam1, Vav) and filopodia (hPem2) as well as of stress fibres (Dbl) (Ava and Aaronson, 1985; Bustelo, 2000; Bustelo, 2001; Habets et al., 1994; Hart et al., 1991; Reid et al., 1999). These changes can be attributed to the global activation of the relevant Rho family proteins. However, under physiological conditions, activation of the Rho GTPases is likely to be restricted to different cellular compartments. In migrating cells, the establishment and maintenance of cell polarity is fundamental to cell movement. Thus spatial and temporal activation of the different Rho GTPases is important. This could be achieved by specific localization of GEF or GAP to activate or inactive Rho GTPases at different sub-cellular locales. For example, Cdc42 is thought to control cell polarity through its interaction with the Par6-Par3-PKC□ complex (Etienne-Manneville and Hall, 2003). The final outcome of this interaction is the localization and reorganization of the microtubule-organizing centre (MTOC). The Cdc42-induced MTOC orientation appears to facilitate microtubule growth into the lamella and the microtubule-mediated delivery of membrane proteins via the Golgi vesicles to the leading edge. Active Cdc42 at the leading edge also leads to the exclusion of the Pten lipid phosphatase at the front, and Pten accumulation at the rear end of the cell. Phosphotidylinositol-3 kinase (PI3K) is also found to accumulate at the leading edge of the cell. The products of PI3K are PIP3 and PIP2, which have been reported to activate a number of Rac GEFs such as Vav and Tiam1 (Bustelo, 2000; Bustelo, 2001; Mertens et al., 2003) besides recruiting these GEFs through their PH domains. Once Rac is activated, several positive feedback loops are available to maintain continual activation of Rac and its downstream pathways to generate lamellipodia and other cell protrusions.

The localization of GEF is important to its activity. In the case of Tiam1, translocation to the membrane is crucial for its activation of Rac to induce membrane ruffles. The mechanism responsible for the translocation and activation of Tiam1 is still not clear. However, it has been observed that the N-terminal of Tiam 1 is important for membrane localization, presumably through the interaction of the N-terminal PH domain with phosphoinositides (Stam et al., 1997; Michiels et al., 1997). There are also reports that Tiam 1 is phosphorylated, possibly activated and translocated by Ca^{2+}/calmodulin

kinase II (CaMK-II) and protein kinase C (PKC) when Swiss 3T3 cells are treated with lysophosphatidic acid or PDGF (Fleming et al., 1998; Fleming et al., 2000). Other GEFs may contain domains that are responsible for their cellular localization. Peripheral localization of the Rac/Cdc42 GEF, β1PIX, is crucial for its activation of Cdc42 and Rac to induce formation of microvilli and ruffles, respectively (Koh et al., 2001). Deletion of the membrane targeting sequences in β1PIX prevented such localized activation of the GTPases. From these various reports, it is clear that the sub-cellular location of the GEFs is critical for its downstream activities. A number of GEFs were found to form dimers or multimers. β1PIX exists as dimers and Dbl can form oligomers (Kim et al., 2001; Koh et al., 2001; Zhu et al., 2001). Dimerization of PIX provides the prospect of forming multimeric complexes with PAK, GIT1/p95PKL and other associated proteins (Bagrodia et al., 1999; Turner et al., 1999; Zhao et al., 2000). This prospect allows for the generation of distinct functional outcomes, depending on the composition of the complexes. With Dbl, oligomerization resulted in the formation of a protein complex that could co-ordinate and further augment its GEF activities (Zhu et al., 2001).

In summary, the localization and activation of these RhoGEFs play crucial roles in the organization of the cytoskeleton and cell morphology though their activation of the Rho GTPases dictated by different spatial and temporal cues.

4. PHOSPHATASES AND THE ACTIN CYTOSKELETON

Cell adhesion and migration are dynamic processes that depend on cellular systems, whose protein components can be rapidly and precisely regulated. An example of this regulation is the modification of the proteins by phosphorylation and dephosphorylation. The balance between the activities of the kinases and phosphatases is crucial to achieve the desirable outcome. Many of the effectors of Rho GTPases are kinases. Thus regulation of the phosphorylation status of the substrates and even of the kinases themselves becomes critical for the modulation of signaling pathways. Earlier studies have indicated that phosphatases participate in the regulation of cell attachment and migration. It has been shown that the spreading of BHK21 cells on fibronectin could be inhibited by vanadate, which is a tyrosine phosphatase inhibitor (Edwards et al., 1991). Similarly Okadaic acid, which is a PP2A inhibitor, has been used to inhibit the migration of macrophages (Wilson et al., 1991).

Protein phosphatases can be classified as protein tyrosine phosphatases (PTPs) or serine/threonine phosphatases (PPs). As the name implies, PTPs dephosphorylate the phospho-tyrosine residues. Members of this family include the soluble PTPs, the receptor PTPs (RPTPs), dual specificity PTPs and low-molecular weight PTPs. The catalytic domains of all the PTPs are very conserved. The dual specificity PTPs can dephosphorylate phosphorylated tyrosine, serine and threonine residues. Pten which is a phosphatidylinositol 3,4,5-triphosphate lipid phosphatase can also be considered as a dual specificity phosphatase because it has tyrosine phosphatase activity as well.

The PPs dephosphorylate phospho-serine and phospho-threonine residues. Most of the PPs are holoenzymes and require one or two other regulatory subunits for activity. The PPs can further classified into phosphoprotein phosphatase (PPP) and metal ion-dependent protein phosphatase (PPM) subfamilies. PPMs require Mg2+ or Mn2+ ions for activities. PP2C phosphatases belong to this class. Members of the PPP subfamilies include PP1, PP2A, PP2B (calcineurin), PP4 and PP5.

Phosphatases such as Pten and PP2A directly act on proteins that are known to participate in cell adhesion or migration such as FAK, Src, and Paxillin. The activities of Rho GTPases can also be regulated through the inactivation or activation of their GAPs and GEFs via dephosphorylation. The Rac GEF Vav is activated by tyrosine phosphorylation (Crespo et al., 1997). It has been speculated that tyrosine phosphatase PTP-PEST (PTP-proline, glutamate, serine and threonine-rich domain) could inhibit Rac activation by dephosphorylating and down-regulating Vav (Sastry et al., 2002). There are also other reports showing that PTP-PEST can suppress Rac activity by acting through p130Cas (Garton and Tonks, 1999) and Paxillin (Shen et al., 1998).

Another PTP which could affect the activity of Rho GTPases is SHP-2 (Src homology region 2 containing PTP-2). It has been reported that SHP-2 levels and activity affect cytoskeleton organization, cell adhesion and migration (Inagaki et al., 2000; Oh et al., 1999; Saxton et al., 1997). The exact mechanisms are not known, with groups reporting SHP-2 to activate or to inhibit the activity of RhoA. It could well be that SHP-2 can function as both a positive and negative regulator of RhoA (Lacalle et al., 2002). SHP-2 could act as an activator of RhoA via the dephosphorylation of p190BRhoGAP, thereby decreasing the GAP activity. At the same time, SHP-2 might also reduce the activities of GEFs of the Vav family.

Pten can also suppress the activity of Rac1 and Cdc42. The inhibition is dependent on the lipid phosphatase activity of Pten (Liliental et al., 2000). Fibroblast cell lines from Pten (-/-) knockout mouse show increased cell motility and also significant increase in Rac1 and Cdc42 activities.

Reintroduction of wild type Pten, but not the catalytically inactive or lipid phosphatase-deficient mutants, reduced the enhanced cell motility of these Pten (-/-) cells.

Phosphatases may also have an effect on the actin cytoskeleton by regulating the targets of Rho GTPases. Serine/threonine phosphatases of the PP2C family, POPX1 and POPX2 (Partner of PIX, the PAK interacting exchange factor), have been found to dephosphorylate and down-regulate PAK (Koh et al., 2002). Co-expression with POPX prevents activated PAK from promoting the loss of stress fibres. PP2A has also been found in the same complex as PAK (Westphal et al., 1999) and to dephosphorylate PAK. Interestingly, PP2A can also dephosphorylate ADF/cofilin resulting in an increase in cell motility.

5. CONCLUSION

Although many proteins have been identified to participate in the organization of actin cytoskeleton downstream of Cdc42 and Rac, how the different complexes and pathways are integrated is still unclear. It is apparent that the same molecule can lie downstream of antagonistic signals such as RhoA versus Rac and Cdc42. Much work is needed to determine the mechanisms for achieving the right balancing of different signals through cross talk.

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Chapter 8

REGULATION OF CELL-CELL ADHESION BY RHO FAMILY GTPASES

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Abstract: Cell-cell adhesion is a fundamental determinant of tissue organization. Adhesive interactions between cells help model body plan and histoarchitecture during development, while disorders of cell adhesion contribute to common diseases, including cancer and inflammation. Given their wide-ranging ramifications, it is not surprising that these interactions are subject to strict cellular regulation. In particular, classical cadherins, major mediators of cell-cell adhesion in many tissues, are key targets of Rho GTPase signalling. In this chapter we review recent developments in understanding the interrelationship between cadherin function and Rho family members. It is increasingly apparent that cadherin function is tightly regulated by membrane-local GTPase signals localized to cell-cell contacts. These may be activated both by cadherins themselves or by cadherin-dependent juxtacrine signalling receptors. These GTPases exert profound, but often pleiotropic effects on cadherin function, through their ability to regulate both cadherin-actin cooperativity and cadherin trafficking.

1. INTRODUCTION

Adhesive cell-cell interactions are fundamental determinants of tissue organization. In the embryo such adhesive interactions participate in the cellular rearrangements that establish body plan during morphogenesis and mould specialized histological architectures during organogenesis (Gumbiner, 1996). In post-developmental life cell-cell adhesion also exerts a ubiquitous influence (Yap et al., 1997), both through its well-recognized capacity to support cellular cohesion and also through dynamic cell-cell interactions that persist in the adult organism. Conversely, abnormal cell-cell

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adhesion often occurs in a range of pathologic states where tissue architecture is disturbed, notably cancer and epithelial inflammation (Birchmeier and Behrens, 1994; Hermiston and Gordon, 1995b). Given its profound influence, both during health and disease, it is not surprising that cell-cell adhesion is subject to stringent cellular regulation.

In this chapter we will focus on the role that Rho family GTPases play in regulating cell-cell interactions mediated by the classical cadherin family of cell adhesion receptors. These are, to date, the best-understood, though by no means the only (Mizoguchi et al., 2002), molecular determinants of cell-cell adhesion. A series of recent studies have clearly established that Rho and Rac critically influence the integrity and adhesive function of cadherin-based cell-cell contacts. We will develop the notion that these key influences reflect substantially the action of membrane-bound signalling pathways located at cell-cell contacts. These signalling pathways coordinate the surface binding activity of cadherins with the actin cytoskeleton and cellular trafficking. However, the multiple ways in which Rho family GTPases can intersect with the morphogenetic activity of cadherins have the capacity to generate a great deal of phenotypic complexity.

2. CLASSICAL CADHERIN ADHESION RECEPTORS AS MORPHOGENETIC DETERMINANTS

The cadherin superfamily consists of a large and diverse collection of cell surface glycoproteins defined by sequence homology to an ~ 120 amino acid domain first identified in the so-called classical cadherins (Nollet et al., 2000). The classical cadherin subfamily contains some of the best-known members of this superfamily, including E-, N-, C- and P-cadherin. The classical cadherins were the first to be cloned and remain the best-understood branch of the cadherin superfamily. We will, accordingly, confine our discussion to these proteins and for convenience will often refer to them simply as cadherins.

2.1 The cadherin-catenin complex

Classical cadherins are single-pass Type 1 membrane glycoproteins that function as core proteins in membrane-spanning macromolecular complexes (Nollet et al., 2000; Takeichi, 1991; Yap et al., 1997). The primary sequences of cadherins diverge most notably in the extracellular regions (ectodomains) that are responsible for adhesive binding (Nollet et al., 2000).

In contrast, the cytoplasmic tails are highly conserved between classical cadherins and bind a number of cytoplasmic proteins, the best characterized being the catenins (Aberle et al., 1994; Ozawa et al., 1989; Ozawa and Kemler, 1992). β-catenin binds with high affinity to the distal C-terminus of the cytoplasmic tail where it serves as an anchor for α-catenin (Aberle et al., 1994; Jou et al., 1995). (Under some circumstances, plakoglobin may substitute for β-catenin, although its principal physiological role is likely to occur in desmosomes.) p120-catenin (p120-ctn) forms a more labile direct interaction with the membrane-proximal region of the cytoplasmic tail (Thoreson et al., 2000). The assemblage of these four proteins forms what is commonly called the "cadherin-catenin" complex.

However, it is important to realize that many other proteins can also interact with the cadherin-catenin complex in ways that are influenced by cell type and physiological context. These include proteins that can link cadherins to the actin cytoskeleton, such as α-actinin (Knudsen et al., 1995), as well as α-catenin itself, which binds actin filaments directly (Rimm et al., 1995). (This issue is discussed further below). Classical cadherins are also reported to associate, directly or indirectly, with a range of signalling molecules (reviewed in [Wheelock and Johnson, 2003; Yap and Kovacs, 2003]). These include tyrosine kinases and phosphatases (of both receptor- and non-receptor classes), PI3-kinase, the presenilin transmembrane protease, heterotrimeric G proteins of the G•• subclass, and adaptor proteins, such as Shc. The cadherin-catenin complex thus possesses the capacity to interact in many ways with the actin cytoskeleton and cell signalling pathways.

2.2 Cohesive and dynamic cell adhesion

Cadherins were first identified by their ability to support homophilic cell-cell adhesion. Experimentally, this was defined by the tendency of cells expressing identical cadherins to cohere together and segregate away from cells expressing different cadherins. This phenomenon is commonly displayed during development: for example, neural tube precursors change their repertoire to express N-cadherin as they segregate and migrate away from the E-cadherin-expressing ectoderm (Takeichi, 1991). Conversely, ectopic expression of N-cadherin disturbs the integrity of the ectoderm (Chihara et al., 2003). Importantly, cultured cells engineered to express different cadherins can be readily shown to sort out, forming cohesive groups of cells expressing the same cadherin (Nose et al., 1988). Homophilic adhesion has commonly been attributed to distinct binding specificities conferred by the cadherin ectodomains, although this may be an over-simplification (Duguay et al., 2003), since functional purified ectodomains

from different cadherins can cross-bind robustly (Niessen and Gumbiner, 2002). Nonetheless, it is clear that cadherins are key mediators of cell-cell recognition.

Morphologically, productive cadherin adhesion has generally been associated by the ability of cells to establish cohesive contacts with one another. This is exemplified by the organization of epithelial sheets into mechanically integrated monolayers, a process that requires E-cadherin. However, cadherin adhesion participates in diverse other forms of intercellular cohesion, such the linkage of neurons together at synapses (Takai et al., 2003). Such morphological cohesion has often been taken as indicating stable adhesion, but it should be noted that these structures are not necessarily static. Indeed, remodelling of cell-cell contacts occurs continually in tissues such as the gut epithelium (Hermiston and Gordon, 1995a) as well as in synapses (Murase et al., 2002). We therefore prefer to use the term "cohesive adhesion" to describe this morphologic expression of cadherin activity.

As well as supporting cohesive adhesion, it is important to recognize that cadherins also participate in a range of overtly dynamic intercellular interactions. This occurs most commonly during development where cells engage in morphogenetic movements upon one another (Gumbiner, 1992), such as the processes of convergent-extension movements in vertebrate gastrulation (Brieher and Gumbiner, 1994) and border cell migration in the Drosophila egg chamber (Montell, 2003). Here cadherins are used to provide traction for cells to move upon one another, a process that must entail rapid remodelling of adhesive interactions (Gumbiner, 1992). In post-developmental life, dynamic adhesive interactions also preserve tissue organization in organs such as the gastrointestinal epithelium (Hermiston and Gordon, 1995a), that undergo continual and rapid cellular turnover, and participate in re-establishing tissue form during wound healing and repair.

Dynamic forms of cadherin adhesion are also identifiable in cultured cells, most evidently when migrating cells first establish productive cadherin-based contacts with one another. This involves a process of contact zone extension, where limited initial points of cell-cell contact are laterally extended into broader zones of adhesion (Ehrlich et al., 2002; Vasioukhin et al., 2000). Again, the movement of cell surfaces upon one another, associated with remodelling of cadherin contacts, is common to this process, which for convenience we will call "dynamic" adhesion. There is increasing evidence that these morphogenetic processes are regulated by signals that include Rho family GTPases. So, while cohesive and dynamic forms of cadherin adhesion may coexist in tissues, any comprehensive understanding of cadherin regulation must encompass mechanisms to support both expressions of cadherin activity.

3. RHO GTPASES REGULATE CADHERIN FUNCTION

A large number of studies have established that Rho GTPase signalling can regulate the integrity and morphology of cadherin-based cell-cell contacts. Much of this work was conducted in cell culture models, although in recent years developmental systems, especially Drosophila, have begun to be used more commonly. Generally, GTPase activity has been manipulated by expression of mutant GTPases or by treating cells with inhibitors of GTPase activity (e.g. C3-transferase). The effects of depleting GTP signalling by loss of function mutants or RNAi-mediated knock-down have only recently begun to be reported, again principally in Drosophila.

These studies examined effects on both cohesive cell contacts (notably cell-cell adhesion in confluent epithelial monolayers) and, to a lesser extent, dynamic cadherin adhesion. The functional outcomes have not always been simple, at least in part because the consequences of GTPase signalling are significantly influenced by cellular context. Nonetheless, reasonably clear requirements for GTPase signalling were identified, most particularly for Rac and Rho.

3.1 Roles for Rho

To date, the most consistent effects on cadherin function have been observed when Rho signalling was manipulated. Most studies have measured patterns of cadherin localization and the morphology of cell-cell contacts. In cultured keratinocytes (Braga et al., 1997) and simple polarized epithelial cells (Sahai and Marshall, 2002; Takaishi et al., 1997), Rho signalling appears to be necessary to maintain the concentration of cadherin at cell-cell junctions. Thus inhibition of Rho signalling by ADP-ribosylation with C3 transferase caused E-cadherin to be lost from cell-cell contacts in established epithelial monolayers (Braga et al., 1997; Takaishi et al., 1997). Total cellular levels of cadherin were not affected by dominant-negative (DN) Rho mutants (Jou and Nelson, 1998), suggesting that inhibition of Rho caused the cadherin to redistribute away from the cell-cell contacts. This loss of junctional cadherin was sometimes (Sahai and Marshall, 2002), but not always (Braga et al., 1997), accompanied by disruption of cell-cell cohesion. Similarly, junctional concentration of cadherin was disrupted in *RhoA* mutant Drosophila embryos, where DE-cadherin instead appeared to become diffusely distributed across the cells (Magie et al., 2002). Loss of cadherins from cell-cell contacts was also often accompanied by changes in the actin cytoskeleton (notably reductions in stress fibres) (Jou and Nelson, 1998),

suggesting that Rho influences the functional relationship between surface cadherins and the actin cytoskeleton (discussed further below).

In addition to a general requirement for Rho signalling, changes in the degree of Rho activity may affect the integrity of cohesive cadherin contacts. Thus expression of constitutively-active (CA) Rho mutants also perturbed the localization of E-cadherin in epithelial cells, accompanied by changes in cell-cell cohesion and morphology (Sahai and Marshall, 2002). Taken together, these findings indicate that precise control of Rho signalling is necessary for the formation and maintenance of cadherin-based cell-cell contacts. The extent to which this manifests as changes in cadherin adhesive activity has not, however, been directly addressed.

3.2 Roles for Rac

Manipulation of Rac signalling also affects cadherin localization and junctional organization, but with much more pleiotropic effects than reported for Rho. In a number of cultured cell lines, including keratinocytes (Braga et al., 1997) and MDCK renal epithelial cells (Takaishi et al., 1997), expression of DN Rac mutants reduced the amount of E-cadherin that accumulated at cell-cell contacts, albeit to varying degrees. Conversely, expression of CA Rac increased E-cadherin staining in contacts between MDCK cells (Takaishi et al., 1997). Interestingly, rather than being principally concentrated in subapical regions (the classic site of the zonula adherens), the increased cadherin expression was distributed homogenously throughout the lateral cell surfaces (Takaishi et al., 1997). Electron microscopy revealed that, compared with control cells, cells expressing CA Rac also showed much more extensive regions of cell-cell apposition, which encompassed large proportions of the lateral cell surfaces. This suggested that Rac might affect the extent of adhesive contact that cells were able to make with one another. Indeed, similar effects were seen when contact zone extension was tested directly, by examining the dynamics with which cells formed contacts with one another (Ehrlich et al., 2002), or with substrata coated with a cadherin ligand (Kovacs et al., 2002a). Taken together, these observations suggested that Rac may promote dynamic cell-cell contacts where cells extend contacts upon one another. Consistent with this, expression of DN Rac blocked the migration of border cells in the Drosophila egg chamber (Murphy and Montell, 1996), a form of morphogenetic cell-on-cell locomotion that requires dynamic DE-cadherin-based adhesion (Montell, 2003).

In addition to contact formation and dynamics, the effect of Rac signalling on cadherin adhesion has been directly tested, yielding results consistent with a positive contribution to cadherin function. Adhesion in

aggregates of MDCK cells was promoted by CA Rac (Ehrlich et al., 2002; Hordijk et al., 1997) and reduced by DN Rac (Ehrlich et al., 2002). Similarly, expression of CA Rac restored the adhesiveness of a cadherin mutant incapable of activating Rac signalling (Goodwin et al., 2003).

In other studies, however, manipulation of Rac signalling yielded results consistent with a negative effect of Rac on cadherin contact formation. Microinjection of CA Rac in keratinocytes caused the breakdown of cell-cell contacts (Akhtar and Hotchin, 2001; Braga et al., 2000) and accumulation of E-cadherin in large cytoplasmic vesicles (Akhtar and Hotchin, 2001). Similarly, Drosophila embryos zygotically null for Rac 1 and Rac 2 showed increased DE-cadherin staining at cell-cell contacts in the trachea (Chihara et al., 2003), often extending beyond the subapical junctions to be found throughout lateral contact zones. Expression of CA Rac, on the other hand, disrupted cell-cell cohesion.

These discrepancies between studies cannot be readily resolved at this time. They are likely to reflect differences in experimental models used, in the approaches taken to manipulate Rac signalling, and in the assays used to measure cadherin function. Perhaps, most importantly, there are a range of points at which Rac signalling may impinge on cadherin function (see below). Thus pleiotropy may not be so surprising should the balance between these processes vary depending on cell context. Nonetheless, these findings clearly implicate Rac as a key determinant of cadherin-based cell contacts.

3.3 Roles for Cdc42

In contrast to Rho and Rac, a definite contribution of Cdc42 signaling to cell-cell adhesion has yet to emerge in the literature. Manipulations of Cdc42 signaling, by expression of either dominantly-inhibitory or constitutively-active mutants, had little reported effect on the morphology of cell contacts or on the accumulation of cadherins at those contacts (Takaishi et al., 1997). Cdc42 signaling is clearly implicated in the generation of matrix-based filopodial protrusions (Nobes and Hall, 1995), which are implicated in the initial contacts between migrating cells that serve as the precursors to contact zone extension (Raich et al., 1999; Vasioukhin et al., 2000). Cdc42 signaling may thus support this precursor stage for contact formation. However, Cdc42 signaling has also been identified in established cell-cell contacts (Kim et al., 2000; discussed below). Whether Cdc42 signaling at this later stage also contributes to cell-cell adhesion or contact formation is less evident. It is possible that Cdc42 signaling in contacts principally mediates signalling events from cell contact, rather than the stabilization or maintenance of adhesive contacts themselves

4. HOW MIGHT GTPASE SIGNALLING AFFECT CADHERIN FUNCTION?

Taken together, the data reviewed above identify Rac and Rho GTPase signalling as critical determinants of cadherin function. Note that to date Rac and Rho signalling have been principally implicated in controlling the junctional distribution of cadherins during contact formation and morphogenesis (though this may not exhaust the extent of their influence). It is therefore necessary for us to understand how Rho family GTPase signalling regulates the surface distribution of cadherins. Such surface expression of cadherin molecules is influenced by several mechanisms, including the binding activity of the cadherin ectodomains (Yap et al., 1997); the association of cadherins with the actin cytoskeleton (Adams and Nelson, 1998); and the transport of cadherins to the surface by both biosynthetic and recycling pathways (Peifer and Yap, 2003). Of these factors, both cytoskeletal association and cadherin trafficking have been identified as potential targets for regulation by Rac and Rho.

4.1 Interactions with the actin cytoskeleton

It has long been recognized that cadherin function in close cooperation with the actin cytoskeleton. Disruption of actin integrity with drugs such as cytochalasin D profoundly perturbs cell-cell adhesion (Angres et al., 1996; Jaffe et al., 1990). Conversely, productive assembly of cadherin-based cell-cell contacts is accompanied by actin reorganization (Adams et al., 1996; Vaezi et al., 2002). Given the central role that Rho GTPases play in regulating the actin cytoskeleton, it was immediately attractive to postulate that their impact on cadherin function might arise from changes in cadherin-actin cooperativity. Early studies suggested that this was, indeed, the case (Braga et al., 1997), while more recent work has begun to elucidate the complex biochemical and functional connections that cadherins can make with the actin cytoskeleton.

At the molecular level, cadherins can interact with the actin cytoskeleton via several molecules that allow classical cadherins to indirectly associate with actin filaments (figure 1). These include α-catenin, which couples to the cadherin complex via β-catenin, and can bind F-actin directly (Rimm et al., 1995). Additionally, α-catenin itself can recruit both α-actinin (Knudsen et al., 1995) and ZO-1 (Fanning et al., 2002; Itoh et al., 1997), proteins that

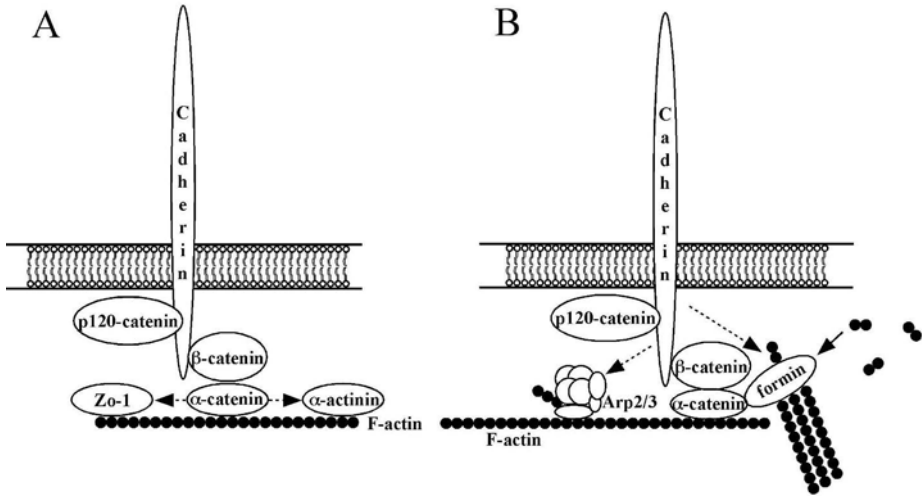

Figure 1. Interactions of the cadherin-catenin complex with the actin cytoskeleton. ***A.*** Coupling of cadherin complexes to preformed actin filaments. The cadherin-catenin complex binds to actin filaments via α-catenin. This interaction may be further reinforced by recruitment of the actin-binding proteins ZO-1 and α-actinin to the complex. ***B.*** Cadherin ligation induces actin assembly. The cadherin-catenin complex can direct actin assembly at adhesive contacts by recruiting and signalling to the Arp2/3 complex and Formin-1. Arp2/3 supports the formation of branched networks of actin filaments whereas Formin-1 supports actin assembly at the ends of actin bundles. Note that the minimal unit of cadherin adhesion is likely to be a lateral dimer. However, for simplicity we have drawn the cadherin as monomer.

possess independent F-actin-binding capacities. These modes of interaction are commonly envisaged to passively couple the cadherin onto pre-formed actin filaments. However, there is also increasing evidence that cadherins interact with molecular machinery that initiates actin filament assembly. Thus E-cadherin can co-immunoprecipitate with the Arp2/3 actin nucleator complex (Kovacs et al., 2002b) and α-catenin can recruit formin-1 (Kobielak et al., 2004), a protein capable of supporting actin assembly at the ends of actin bundles. While much remains to be elucidated, it is increasingly clear that cadherins can interact with multiple biochemically distinct states of the actin cytoskeleton.

Moreover, cadherins can also interact with at least three functionally distinct states of the subcortical actin cytoskeleton: actin bundles, actin filaments undergoing retrograde flow, and with actin filaments at cell surface protrusions. Of these, the capacity of cadherins to interact with actin bundles is the best documented. In epithelial cells, prominent actin bundles are commonly located adjacent to cadherin-rich adherens junctions (Boller et al., 1985; Hirano et al., 1987) and circumferential perijunctional actin bundles are characteristic of fully-confluent epithelial monolayers (Madara, 1987; Madara and Dharmsathaphorn, 1985; Rodriguez-Boulan and Nelson,

1989). Moreover, during the biogenesis of epithelial sheets, cadherin junctions and actin bundles assemble together in an apparently coordinated fashion (Adams et al., 1996; Vaezi et al., 2002). The specialized proteins responsible for actin bundling at cadherin contacts remain to be thoroughly characterized, but may include both α-catenin (which has the capacity to self-oligomerize as well as bind F-actin; [Rimm et al., 1995]) and α-actinin (Knudsen et al., 1995), a well characterised actin-bundling protein which is reported to associate with α-catenin.

Cadherins are also often localized in regions of the cell surface distinguished by more diffuse actin filament organization. This is particularly evident in those dynamic regions of the plasma membrane where cells are engaged in making contact with one another. One form of cadherin-actin cooperativity in these areas involves the coupling of cadherins to cortical actin filaments that display retrograde flow (Lambert et al., 2002). This is an active process, described for a range of adhesion molecules, where transmembrane proteins are moved laterally in the plane of the membrane by coupling to subcortical actin filaments. Retrograde flow is commonly envisaged to participate in cell motility (Lin and Forscher, 1995; Lin et al., 1996), and thus might participate in the cadherin-based cell-upon-cell locomotion associated with tissue morphogenesis (Gumbiner, 1992; Montell, 2003).

Dynamic regions of cell contact are also the sites where cadherins interact with proteins that mediate actin assembly, notably the Arp2/3 actin nucleator complex (Kovacs et al., 2002b). This is a stable macromolecular complex of seven proteins that mediates actin nucleation, catalysing the energetically unfavourable step of converting actin dimers to trimers (Pollard et al., 2000). Characteristically, Arp2/3-mediated actin nucleation generates branched meshworks of growing filaments, rather than bundles, which are orientated outwards towards the plasma membrane. Arp2/3 activity has therefore commonly been implicated as a force-generating mechanism for outward protrusion of the plasma membrane. Interestingly, E-cadherin homophilic ligation is sufficient to recruit Arp2/3 to the cell surface, suggesting that cadherin adhesion may act as an instructive signal to direct the force-generating capacity of Arp2/3 (Kovacs et al., 2002b). The recruitment of Arp2/3 has the potential to concentrate the protrusive activity of this actin nucleator, thereby providing forces for cadherin contacts to be extended upon one another. Indeed, expression of a dominant inhibitor of Arp2/3 activity significantly decreases the efficiency with which tissue culture cells extend contacts with one another (Verma, S. et al., submitted).

How, then, might Rho GTPases influence these forms of cadherin-actin cooperativity? One mode of regulation is likely to involve differential control of the distinct functional states of the actin cytoskeleton. Thus, Rho

has commonly been implicated in actin bundling (Hall, 1998). It likely exerts this effect by several mechanisms, including stimulating forming-mediated actin assembly (Kobielak et al., 2004) and promoting myosin-based contractility (Chrzanowska-Wodnicka and Burridge, 1996). Indeed, Rho kinase, a downstream component of the Rho signalling pathway, is necessary for cadherin-associated actin bundles to assemble during the biogenesis of keratinocyte sheets (Vaezi et al., 2002). In contrast, both Rac and Cdc42 are implicated in controlling actin assembly through Arp2/3 (Machesky and Insall, 1999). Of note, actin assembly at cadherin contacts was blocked by inhibiting Rac (Lambert et al., 2002), suggesting that Rac may regulate Arp2/3 activity at cadherin contacts. Rac and Cdc42 stimulate Arp2/3 via intermediary proteins such as cortactin and WASP/WAVE family proteins, which are also found in cadherin-based adhesions (R. Ali and A.S. Yap, unpublished; (Helwani et al., 2004)). Rac has also been specifically implicated in coupling N-cadherin to retrograde cortical flow (Lambert et al., 2002). Thus one possibility is that Rho and Rac signalling specify the distinct activity states of actin to which cadherins locally couple.

Rho family GTPases may also control the biochemical association between cadherins and the actin cytoskeleton. Inhibition of Rho by C3-transferase was reported to uncouple α-catenin from the E-cadherin molecular complex (Sahai and Marshall, 2002), which would be predicted to weaken molecular linkages between cadherins and actin filaments. IQGAP, which binds the GTP-loaded forms of both Rac and Cdc42, may also compete α-catenin away from β-catenin (Fukata et al., 1999; Kuroda et al., 1998), suggesting an alternative mechanism to uncouple cadherins from actin filaments. The physiological significance of these potential changes, however, remains to be elucidated. It is likely that the identification of different biochemical interactions between cadherins and the actin cytoskeleton will provide alternative ways for Rho GTPase signalling to regulate cadherin-actin cooperativity.

Taken together, these findings indicate that Rho GTPases can affect cadherin-actin cooperativity both by controlling the functional state of the actin cytoskeleton and the biochemical linkages between cadherins and the cytosketon. How then might these changes affect the surface expression of cadherins? While this issue has yet to be definitively tested, we can suggest several possible scenarios. During the biogenesis of cell-cell contacts, Rho and Rac may cooperate to support the initial formation and subsequent maturation of cell-cell adhesion. For example, recent studies demonstrate that after cells first touch one another, contacts form by a process of lateral contact zone extension, where cell surfaces spread upon one another in a Rac-dependent fashion (Ehrlich et al., 2002). Such a notion of Rac-driven contact extension is consistent with the morphological evidence that

sustained Rac signalling increases zones of contact between epithelial cells. One source of force in these dynamic contacts is Arp2/3-mediated actin assembly (Kovacs et al., 2002b); Verma et al., submitted) which is potentially regulated by Rac. By linking adhesion to actin assembly, Rac may thus support the initial extension of cell contacts that is essential for junctions to assemble.

Rho signalling, on the other hand, is likely to be critical for other processes that work in concert with contact zone extension during the biogenesis of cohesive cell-cell contacts. Of note, signaling via Rho kinase is important to mechanical couple cells together during epithelial biogenesis, a process associated with the formation of cadherin-linked actin bundles (Vaezi et al., 2002). Such actin bundles likely mediate contractile forces that reinforce cell-cell contacts and mechanically integrate cells together into cohesive populations. Thus the concerted action of Rac and Rho may serve to coordinate the extension and subsequent mechanical coupling of adhesive contacts that are necessary for productive junctional assembly.

4.2 Cadherin trafficking

Rho GTPases can also influence cadherin function is by modulating cellular trafficking. Like other transmembrane proteins, the synthesis, cellular localization, and degradation of classical cadherins are linked by a complex set of intracellular trafficking pathways (Peifer and Yap, 2003). Such trafficking influences the post-translational fate of cadherins, and thus their expression and activity at the cell surface. Following synthesis, cadherins are transported from the Golgi apparatus to the plasma membrane via a vesicular pathway. Efficient transport in this biosynthetic pathway requires association with β-catenin (Chen et al., 1999) and, in polarized cells, is also influenced by targeting information residing in the cadherin cytoplasmic tail (Miranda et al., 2001). Surface cadherins are eventually endocytosed, from which they may be targeted for lysosomal degradation. Even in confluent monolayers, E-cadherin has a half-life of ~ 5 hours (Shore and Nelson 1991), implying that constant metabolic turnover is a prominent feature in the cadherin life cycle.

However, upon internalisation, cadherins are not obligately targeted for degradation. Instead, cadherins can also enter a rapid, post-Golgi recycling pathway for transport back to the cell surface (Le et al., 1999). Such recycling may provide a mechanism for cellular cadherins to be redistributed when cells are remodelling adhesive contacts, such as during morphogenetic cell movement (Jarrett et al., 2002; Miller and McClay, 1997). While, many of the details of this recycling process remain to be elucidated, the initial step of internalisation can occur through both clathrin-dependent (Palacios et

al., 2001) and clathrin-independent mechanisms (Paterson et al., 2003). Furthermore, it is probable that this trafficking pathway intersects with other trafficking pathways (Mukherjee et al., 1997), notably those for lysosomal degradation.

It is increasingly apparent that the route that cadherins take in this network of trafficking pathways influences critically their surface expression and metabolic fate. For example, control of endocytic uptake can potentially influence surface expression: inhibition of endocytosis would be predicted to promote surface expression of cadherin, whereas increased endocytosis might favour reduced surface expression. Indeed, there is increasing evidence that regulation of endocytosis constitutes such a regulatory point in the life-cycle of cadherins. Thus cell-cell adhesion appears to withdraw cadherins from endocytic recycling (Le et al., 1999). Moreover, p120-ctn plays a critical role in preventing cadherin endocytosis: loss of p120-ctn binding promotes cadherin internalisation and degradation, ultimately leading to reduced steady-state surface and cellular levels of classical cadherins (Davis et al., 2003; Xiao et al., 2003). Conversely, blocks in the recycling of cadherins back to the cell surface would be predicted to reduce surface cadherin expression, and potentially promote degradation if internalised material becomes shunted towards lysosomes.

Like other networks of trafficking pathways, the itinerary of internalised cadherin towards recycling or degradation is likely to be tightly regulated. Importantly, many steps in cell trafficking are affected by Rho GTPase activity (Symons and Rusk, 2003). These include post-Golgi exit and transport of vesicles in the biosynthetic pathway,which are regulated by both Rac and Cdc42, at least in part by controlling interactions with the actin cytoskeleton (Stamnes, 2002). Similarly endocytic internalisation via both clathrin-dependent and clathrin-independent uptake processes are affected by Rho, Rac and Cdc42 activity (Symons and Rusk, 2003). Other steps in vesicular transport, including recycling through the endosomal pathway, are also likely to be influenced by Rho family GTPase signalling (Figure 2).

Emerging evidence suggests that cadherin trafficking is also influenced by these GTPases. Over-expression of CA Rac in keratinocytes caused E-cadherin to accumulate in a prominent large intracellular compartment (Akhtar and Hotchin, 2001), suggesting that transport of protein to the surface from either the biosynthetic and/or recycling compartments was

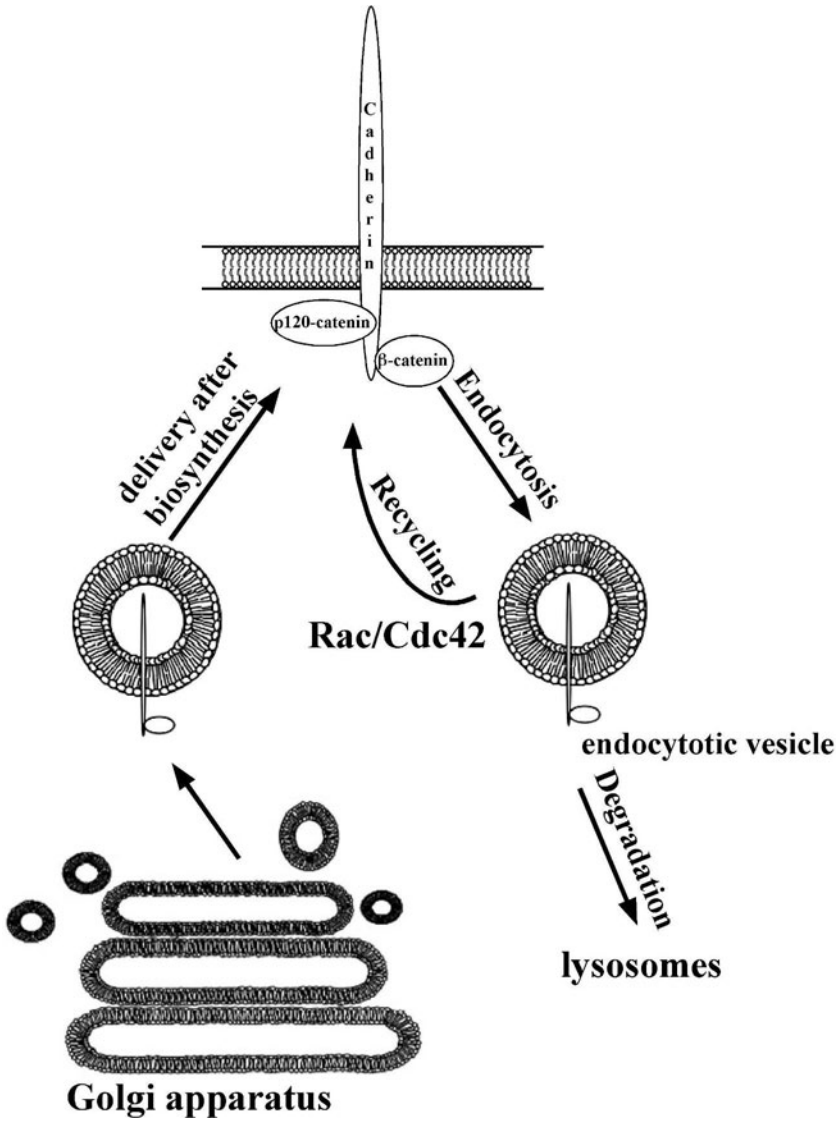

Figure 2. Rho family GTPases and cadherin trafficking. Newly synthesized cadherin molecules are transported from the Golgi apparatus to the plasma membrane via a vesicular pathway. Following endocytosis cadherins can either be targeted for lysosomal degradation or recycled to the plasma membrane. Rac and Cdc42 are potentially regulate several of these trafficking pathways.

being blocked. The GTPase ARF6 can also regulate E-cadherin trafficking, both during the earliest phases of internalisation and also to promote the net

internalisation, and potential degradation, of cadherin (Palacios et al., 2001). Some downstream actions of ARF6 are mediated via Rac (Donaldson and Jackson, 2000), but whether this contributes to its regulation of cadherin trafficking remains to be elucidated. Thus there are several steps at which GTPase signalling may affect cadherin trafficking and it is likely that more will be identified as the regulation of cadherin trafficking is explored more extensively.

The multiple sites at which GTPase signalling may influence cadherin trafficking is one likely explanation for the pleotropic effects reported when GTPase signalling is globally manipulated in different experimental systems. For example, the nett effect of globally stimulating Rac signalling will depend on the balance of Rac effects at different stages in cadherin trafficking (biosynthetic transport, endocytic internalization, endosomal trafficking). Given their disparate physiological functions, such a balance is likely to vary between different cell types and tissues. The likely coordination of cadherin trafficking with cadherin-actin cooperativity adds an additional layer of complexity. This multiplicity carries an important implication: It suggests that identifying the physiologically significant effects of these signals requires identifying the relevant sites where they work to regulate cadherin trafficking and activity. Elucidating the physiology of cadherin regulation by Rho GTPases will therefore require a detailed understanding of how these signals are controlled and localized at specific regions of the cell. Therefore, we will now turn our attention to reviewing evidence that Rho GTPases signal at cell-cell contacts.

5. RHO GTPASE SIGNALLING AT CELL-CELL CONTACTS: CENTRES OF GTPASE SIGNALING

Where, then, are the GTPase signals that exert such potentially significant effects on cadherin function? Formally, Rho family GTPases might affect cadherin function locally, at the adhesive contacts themselves, or at a distance, by affecting processes such as intracellular trafficking. In all likelihood, both possibilities exist and it is important to note that these alternatives cannot be distinguished by maneuvers which manipulate GTPase signalling globally within cells (as applies for most approaches used to date, including use of mutant GTPases, and genetic manipulation of GTPase expression). Instead, it is increasingly apparent that the biological action of many small GTPases depends fundamentally on the subcellular location where these molecules act. Therefore it would be both attractive and parsimonious to postulate that Rho family GTPases signal at cell-cell contacts. Indeed, several lines of evidence point to this being the case. These

include 1) the identification of GTPases at cell-cell contacts; 2) the demonstration that assembly of cell-cell contact modulates GTPase activity; and 3) the identification of receptors that modulate GTPase signalling at cell-cell contacts, including the capacity for cadherins themselves to act as upstream receptors for GTPase signalling. We will review these three lines of evidence in the following section.

5.1 Identification of Rho family GTPases in cell-cell contacts

The subcellular localization of Rho family GTPases to cell-cell contacts has been most commonly reported in studies using epitope-tagged mutant GTPases. When expressed in polarized epithelial cells, constitutively-active forms of Rac, Rho, and Cdc42 have been localized to cell-cell contacts by immunofluorescence microscopy (Charrasse et al., 2002; Jou and Nelson, 1998; Kuroda et al., 1997; Takaishi et al., 1997). Dominant negative mutants have also been found in contacts, more consistently for Rac than for either Rho or Cdc42 (Takaishi et al., 1997). Junctional localization of wild-type Rho (Magie et al., 2002) and Rac (Ehrlich et al., 2002) has also been reported, but less commonly than in studies utilizing mutant GTPases. One possible reason is that GTPase recruitment to cellular contacts is a very dynamic process that is difficult to capture, particularly in fixed specimens. The use of mutant molecules may promote membrane localization by increasing the affinity of the GTPase for membrane-associated scaffolding or effector molecules (that recognize the GTP-bound form) or for exchange factors (in the case of dominant-negative mutants).

While these findings indicate the capacity for Rho GTPases to localize at cell-cell contacts the spatio-temporal dynamics of GTPase recruitment is an important issue that is only now beginning to be addressed (Kraynov et al., 2000). Indeed, the use of mutant GTPases may have obscured the potential importance that subcellular or regional localization plays in GTPase regulation of cadherin contacts. Thus, it is increasingly apparent that Rho GTPase activation in response to growth factors, chemotactic agents, and integrin adhesion molecules is subject to strict spatio-temporal regulation within cells (Del Pozo et al., 2002; Symons, 2000). Rather than necessarily being diffusely activated throughout cells, these signals may principally arise from specific subcellular regions, such as the leading edges of migrating cells (Del Pozo et al., 2002; Kraynov et al., 2000). The recruitment of Rho family GTPases to cadherin-based cell-cell contacts is likely to show similar spatio-temporal regulation.

Indeed, it was recently demonstrated that as migrating MDCK epithelial cells make contacts with one another, Rac is recruited to nascent adhesive

contacts (Ehrlich et al., 2002). Assembly of such contacts entails a process where the margins of the contacts progressively sweep outwards, thereby extending the zones of adhesion between cells. Strikingly, Rac preferentially localized to the outward-moving margins of the contacts, being progressively lost from older regions of adhesion. A similar pattern of localization for Rac was observed when cells adhered to, and spread upon, cadherin-coated substrata (Kovacs et al., 2002a). This suggests Rac, at least, may be preferentially recruited to newly-forming cell-cell adhesive interactions, even within the single zone of adhesion, such has also been reported for integrin-based adhesion. It seems probable that further studies will emphasize and clarify the manner in which Rho GTPase signalling is spatio-temporally localized in cell-cell contacts.

An even greater challenge is to identify the subcellular localization of the active, GTP-loaded form of Rho proteins. A number of approaches are available, including the use of GFP-tagged proteins that bind to GTP-loaded proteins, as was used to identify active Cdc42 in cadherin-based contacts of aggregating cells (Kim et al., 2000). Other approaches employing various forms of fluorescence resonance energy transfer (FRET) technology have been used to identify signalling by Ras, Rap, Rac and Rho in cells (Chiu et al., 2002; Itoh et al., 2002; Mochizuki et al., 2001). These have not yet been successfully adapted to identify Rho GTPase signalling at cell-cell contacts, but will undoubtedly provide a major analytic tool when available.

5.2 Cell-cell contact modulates Rho GTPase signalling

Several studies have now documented changes in Rho GTPase activity when cells make contact with one another. Generally, these experiments assessed the amount of active molecules by using isolated fragments of effector proteins (PAK, rhotektin, WASP) to affinity-purify GTP-loaded Rho proteins from cell lysates. However, the published studies have used a range of cell types and manipulations of cell-cell contact, which has complicated comparison and interpretation of the findings.

The clearest patterns have emerged for Rac signalling. Steady-state GTP.Rac levels were increased in monolayers of endothelial cells expressing VE-cadherin, compared with cells where cadherin was deleted by homologous recombination (Lampugnani et al., 2002). Moreover, GTP.Rac levels also rise within minutes when monolayer cultures are allowed to re-establish cohesion after cadherin-based contacts are broken by chelation of extracellular calcium (which abrogates the adhesive binding activity of the cadherin ectodomain) (Betson et al., 2002; Lampugnani et al., 2002; Nakagawa et al., 2001; Noren et al., 2001). The effects of cell contact on other GTPases have been less consistent. In one study employing MDCK

epithelial cells Rho activity progressively fell after cells reassembled contacts with one another (Noren et al., 2001), but did not change in another report (Nakagawa et al., 2001). In contrast, in myogenic C2C12 cells, cell-cell contact was found to stimulate GTP.Rho levels over a time scale of several hours (Charrasse et al., 2002). In the case of Cdc42, increased GTP-loading was found in some (Kim et al., 2000), but not all (Nakagawa et al., 2001), reports.

These discrepancies likely reflect differences in cell types, endogenous levels of GTPases, growth conditions, and the maneuvers used to manipulate cell-cell contacts. In addition, it should be noted that the affinity purification techniques used to identify changes in GTP-loading of Rho proteins measure changes in the total cellular concentrations of GTP-loaded proteins. These assays do not detect changes in GTP-GTPase levels that are confined to specific regions within cells, such are increasingly recognised as functionally significant. It is possible that cell-cell contact induces significant regional subcellular changes in GTPase activation that are not readily detected against the background of the total cellular pool.

Despite these complexities, taken together, the current data identify assembly of cadherin-based cell-cell contacts as capable of regulating Rho family GTPase signalling. Notably, they identify Rac and Rho activation as consequences of contact formation, perhaps with different time profiles that reflect distinct cellular functions. Most of the current data identify Rac activation as an early-immediate response to contact formation, while Rho may be activated at a later stage. Furthermore, reduction in Rho activity may accompany the early activation of Rac, thus coordinately altering the balance of Rho and Rac signaling.

5.3 Receptors for GTPase signalling at cadherin adhesive contacts

How, then, might the assembly of cell-cell contacts modulate GTPase signalling? From first principles, GTPases might be activated as direct downstream consequences of cadherin adhesion, where the cadherin itself serves as the receptor for the signalling pathway; or through other surface-bound (juxtacrine) signalling receptors that are activated when cell surfaces are brought together (Yap and Kovacs, 2003). There is now increasing evidence for both scenarios (see Figure 3).

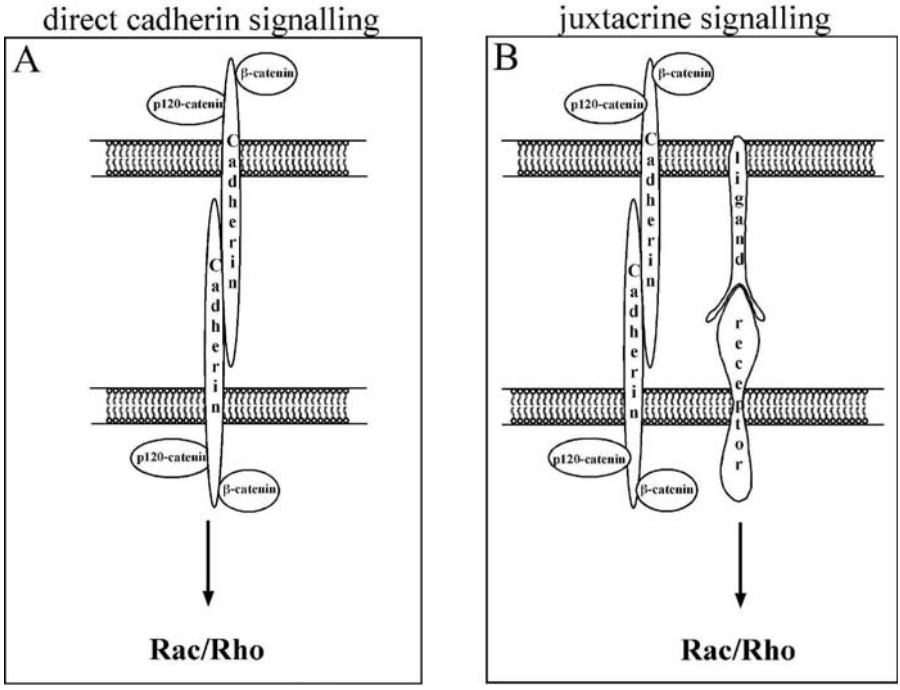

Figure 3. Models for cadherin-dependent signalling: Direct cadherin-activated signalling versus juxtacrine signalling. A. Direct cadherin-activated signalling. The cadherin-catenin complex itself is able to signal to the small GTPases Rac and Rho. B. Juxtacrine (indirect) signalling model. Cadherin-mediated adhesion brings the plama membrane of two neighbouring cells into close proximity. This enables other receptors to be activated by surface-bound ligands on the opposing membrane. The activated receptor signals to Rac and Rho.

1) Classical cadherins as "direct" activators of Rho GTPase signaling. In characterizing the upstream events that activate Rho family signalling, a good deal of recent attention has been directed to testing the possibility that classical cadherins themselves act as receptors to trigger GTPase signalling. In this scenario, the cadherin molecule itself would serve as an adhesion-activated signalling receptor, whereby GTPase activity is stimulated as an early-immediate response to adhesive ligation of the cadherin ectodomain. Although, this is conveniently called "direct" cadherin-activated signalling, we emphasize that this does not necessarily mean that GTPase activation arises from a direct molecular interaction between the two proteins. Instead, and akin to the paradigms established for peptide hormone receptors and

growth factor receptors (Schmidt and Hall, 2002), it is more plausible if other cytoplasmic molecules mediated GTPase activation in response to adhesive ligation.

However, whilst it is clear that GTPase signalling is activated in a cadherin-dependent fashion as cells form contacts with one another, it has been more challenging to establish whether these are direct responses to cadherin ligation itself. This is because in studying native cell-cell interactions it is analytically difficult, if not impossible, to determine whether downstream signals are activated as direct responses to the cadherin itself, or due to juxtacrine signalling (i.e. contact-dependent signals that require adhesion to appose the cell surfaces but which are not themselves direct early-immediate responses to cadherin ligation).

An important advance has been the recent development of several recombinant cadherin-specific adhesive ligands that provide reductionist assays to identify cadherin-activated cell signaling. Generally, these utilize the complete ectodomain of a classical cadherin synthesized as a secreted protein in mammalian cells. Such ligands have now been reported for a variety of classical cadherins (Yap and Kovacs, 2003). When presented immobilized on planar substrata or coated on beads, these reagents support cadherin-specific cell adhesion without apparent involvement of integrins, recruit catenins, and induce interaction with the actin cytoskeleton. They therefore present the opportunity to identify specific consequences of cadherin adhesive ligation alone, independent of any secondary effects that may occur when native cell surfaces are brought into contact with one another.

Using this approach, GTP.Rac levels increased within 15-30 minutes as cells attached to substrata coated with ligands for C- or E-cadherin (Kovacs et al., 2002a; Noren et al., 2001). This is a time frame comparable to the acute activation of Rac signalling that occurs in response to cell-cell contact, growth factor and integrin stimulation. Importantly, GTP.Rac levels were not stimulated by adhesion to the non-specific ligand, poly-l-lysine (Kovacs et al., 2002a), indicating that the rapid change in Rac activity was a specific response to cadherin ligation, rather than a non-specific consequence of adhesion or cell spreading. No comparable acute changes in Rho or Cdc42 activity were observed on adhesion to C-cadherin (Noren et al., 2001), suggesting that Rac may be principally activated as an early-immediate response to cadherin ligation. Interestingly, even within individual adhesive contacts Rac appears to preferentially recruit to regions where cadherin molecules are engaged in forming new adhesive connections (Kovacs et al., 2002a). Therefore, Rac may not be activated continuously by all ligand-bound cadherins, but primarily those involved in making new contacts.

If to date Rac activation has been identified as the principal early-immediate response to cadherin ligation, over longer time frames adhesive ligation to purified ligands appears to have more diverse effects on Rho family signalling. Whereas, C-cadherin ligation in fibroblastic cells reduced GTP.Rho levels (Noren et al., 2001), adhesion of mouse C2C12 myoblast cells to chick N-cadherin ligands activated Rho signalling, associated with reductions in GTP.Rac and GTP.Cdc42 levels (Charrasse et al., 2002). Clearly, the range of cellular responses to cadherin ligation have yet to be systematically characterized. It is likely that the patterns of GTPase regulation vary depending both on cell-type and perhaps also the cadherin involved.

2) Juxtacrine signalling at cell-cell contacts. In an alternative model, GTPase signalling might be activated in cell-cell contacts via juxtacrine signalling receptors. In the simplest form of this scenario, GTPase signalling would be stimulated in response to receptors that are activated by surface-bound ligands. Ligand and receptor binding would then occur when cell surfaces are into contact with one another by cadherin-based cell-cell adhesion. Therefore in this model, cadherins would be necessary for signalling to occur (by bringing together surface-bound receptors and their ligands) but not serve as direct activators of GTPase pathways.

One candidate for such juxtacrine signalling is the EGF receptor. This receptor tyrosine kinase is capable of activating Rac signalling and in keratinocytes EGF receptor activity was necessary for the E-cadherin dependent activation of Rac that occurred as cell surfaces came into contact with one another (Betson et al., 2002). The putative ligand in this signalling was not identified, but a variety of surface-bound EGF receptor ligands have been reported, including TGFα•(Massague, 1990). Additionally, it has been suggested that E-cadherin may itself be capable of activating the EGF receptor in an EGF-independent fashion (Pece and Gutkind, 2000), perhaps by inducing co-clustering of the EGF receptor through cis-interactions. Other contact-dependent receptors can localize to sites of cell-cell contact and signal to Rho family GTPases. These include Eph receptors and their Ephrin ligands (Schmucker and Zipursky, 2001), CD151, a member of the tetraspannin protein family (Shigeta et al., 2003), as well as other cell-cell adhesion molecules, such as nectins (Kawakatsu et al., 2002). Thus it is likely that a range of proteins will be found to participate in juxtacrine signaling. Clearly, the capacity of cells to engage in juxtacrine signalling introduces a new level of complexity to analysing the pathways for Rho family GTPases to be regulated at cell-cell contacts. It is at least formally possible that such receptors may indirectly affect cell-cell adhesion by processes such as altering activity of the actin cytoskeleton.

6. MOLECULAR REGULATORS OF GTPASE SIGNALLING AT CELL-CELL CONTACTS

By either direct cadherin-activated or juxtacrine signalling pathways, alterations in GTPase signalling at cell-cell contacts must ultimately be mediated by the proteins that determine the nucleotide status of the GTPase and its spatial localization.

6.1 Regulation of GTP-status: GEFs

The nucleotide status of Rho family GTPases is determined by the interplay between Guanine nucleotide Exchange Factors (GEFs) which promote GTP-loading (and hence competence to interact with effector molecules), GTPase-activating proteins (GAPs) that stimulate the conversion of GTP to GDP, and Rho-family Guanine nucleotide dissociation inhibitors (GDIs) which can extract GTPases from the membrane and retain them in an inactive, GDP-loaded state. Of these factors, most attention has focused on identifying the GEFs that activate Rho family GTPases in cell-cell contacts. To date, two candidates have emerged: VAV2 and Tiam-1.

VAV2 is a ubiquitously-expressed GEF with the capacity to activate Rho, Rac and Cdc42. It can associate biochemically with p120-ctn (Chauvet et al., 2003; Noren et al., 2000) providing a potential mechanism for this exchange factor to be recruited to the cadherin-catenin complex. However, to date an association between VAV2 and p120-ctn has only been identified in cadherin-deficient fibroblastic cell lines (Noren et al., 2000), leading to speculation that this interaction may occur exclusively in the cytoplasm. The precise role that VAV2 may play in cell-cell adhesion thus remains to be resolved.

In contrast, the case for Tiam-1 is more extensive, if still somewhat circumstantial. Originally identified in a screen for genes conferring invasive phenotypes upon T-cell lymphomas, Tiam-1 is a widely-expressed multidomain protein with Rac-specific exchange activity (Mertens et al., 2003). It has the tandem pairing of Dbl-homology (DH) and pleckstrin-homology (PH) domains that marks it as a member of the Dbl family of exchange factors. Of note, Tiam-1 also contains a second PH-domain that binds to phosphoinositides in the inner leaflet of the plasma membrane and can be recruited to the membrane in response to PI3-kinase signalling (see below).

Several lines of evidence suggest that Tiam-1 can participate in the assembly and/or maintenance of cell-cell junctions. Firstly, Tiam-1 localizes with E-cadherin in cell-cell contacts between MDCK cells, a polarized kidney epithelial cell line (Hordijk et al., 1997). Furthermore, expression of

VE-cadherin in VE-cadherin-null endothelial cells sufficed to stably relocate Tiam-1 from the cytoplasm to cell-cell contacts (Lampugnani et al., 2002), suggesting that this GEF is recruited to junctions as a direct or indirect consequence of cadherin adhesion. Secondly, GTP.Rac levels rose in cadherin-null cells upon expression of VE-cadherin suggesting that Rac activation correlated with the junctional localization of Tiam-1 (Lampugnani et al., 2002). Thirdly, Ras-transformed MDCK cells display an epithelial-to-mesenchymal transformation, accompanied by loss of E-cadherin at cell contacts and decreased cell-cell cohesion. Strikingly, expression of exogenous Tiam-1 restored the epithelial phenotype and increased cell-cell adhesiveness, an effect which was partially mimicked by expression of CA Rac (Hordijk et al., 1997). Mutational analysis suggested, moreover, that the capacity to accumulate in cell-cell contacts was necessary for Tiam-1 to restore the epithelial phenotype. Taken together, these findings suggest strongly that Tiam-1 signaling can promote cadherin-based contact formation and cell-cell cohesion. However, whether Tiam-1 activates Rac signalling at cell-cell contacts remains to be definitively established. Nor has it been determined whether Tiam-1 is activated directly in response to cadherin adhesion or by juxtacrine signalling events that may occur in adhesive contacts.

6.2 Regulation of GTP-status: GAPs

Despite increasing evidence that GAPs actively participate in regulating GTPase signalling, much less is know about the GAPs that influence Rho family signalling at cell-cell contacts. A potentially-critical role for GAP activity was suggested by the observation that early-immediate reductions in GTP.RhoA levels accompanied the adhesion of cells to C-cadherin (Noren et al., 2001). Such inhibition of Rho signalling might arise through inhibition of a GEF and/or stimulation of GAP activity. Indeed, Noren et al. (2003) found that adhesion to C-cadherin increased the amount of active p190RhoGAP identifiable in cells. Importantly, expression of a GAP-deficient mutant of p190RhoGAP prevented the early inhibition of Rho activity by C-cadherin adhesion. This suggests that in some circumstances cadherin adhesion may regulate the balance of Rac and Rho signalling by coordinating the activity of both GAPs and GEFs. Interestingly, it has been suggested that differences in the subcellular localization of different GAPs might influence the distinct spatial localization of Rac and Cdc42 signals that are activated by the same GEF (Aoki et al., 2004). If so, then GAPs might influence the spatio-temporal pattern of GTPase activation as well as its signal amplitude

6.3 The role of PI3-Kinase signalling

An important issue that has received recent attention is the potential for phosphoinositide 3-kinase (PI3-kinase) to serve as an activator of Rho family GTPase signalling in cadherin contacts. PI3-kinase functions as an upstream activator of Rac in a number of pathways, including growth factor- and chemokine-induced cell signalling (Cantrell, 2000). PI3-kinase phosphorylates its principal cellular substrate, phosphoinositide 4,5 $(OH)_2$ to yield phosphoinositide 3,4,5 $(OH)_3$ (PIP_3) (Martin, 1998; Vanhaesebroeck et al., 2001). Both PIP_3 and its metabolic product PI-3,4-P_2 can recruit GEFs that contain PH-domains (e.g. SOS1, Tiam 1) to the plasma membrane (Cantrell, 2000; Schmidt and Hall, 2002). GTP-loading and activation of Rac presumably arises through the membrane recruitment of such GEFs, potentially also modulated by their concomitant post-transcriptional modification once localized at the membrane.

The PI3-kinase family consists of 3 classes that often display distinct patterns of subcellular localization (Martin, 1998). Of these, Type 1A PI3-kinase has been immunolocalized to cadherin-based cell-cell contacts (Kovacs et al., 2002a). Indeed, adhesive ligation of E-cadherin by recombinant cadherin ligands sufficed to recruit PI3-K to the plasma membrane. Furthermore, Type 1A PI3-kinase co-immunoprecipitates with cadherins in (Kovacs et al., 2002a; Pece et al., 1999; Tran et al., 2002), a biochemical interaction that may be indirectly mediated via β-catenin since recombinant β-catenin can bind the p85 regulatory subunit of Type 1A PI3-kinase (Woodfield et al., 2001). However, the biochemistry of the E-cadherin-PI3-kinase interaction remains to be rigorously characterized in cells.

Furthermore, the assembly of cadherin-based adhesive contacts can activate PI3-kinase signalling locally at those contacts. This has been identified both by increases in PI3-kinase activity in E-cadherin immunoprecipitates (Pece et al., 1999) as well as by the phosphorylation of Akt (Protein kinase B) (Kovacs et al., 2002a; Tran et al., 2002). The accumulation of PIP3 has also been identified in adherens junctions using GFP-tagged reporter constructs bearing the PIP3-specific PH domain from Akt/protein kinase B (Watton and Downward, 1999). Such signalling in contacts may be directly stimulated by cadherin adhesive ligation (Kovacs et al., 2002a) and/or reflect the local action of growth factor receptors recruited to, and perhaps thereby activated by, cadherins themselves (Pece et al., 1999; Pece and Gutkind, 2000).

PI3-kinase signalling is thus attractively placed to participate in Rac activation in cadherin adhesive contacts. The extent to which this occurs is, however, less clear-cut. Nakagawa et al., (2001) reported that wortmannin

blocked the GTP-loading of Rac that occurred in response to antibody-induced clustering of E-cadherin in MDCK epithelial cells. Similarly, inhibition of PI3-kinase activity shortened, but did not abolish, the apparent duration of Rac activation that occurred cells adhering to cadherin-coated substrata (Kovacs et al., 2002a). Wortmannin also profoundly reduced the ability of cells to form cadherin-based adhesive contacts, an effect that was over-come by expression of constitutively-active Rac mutants (Kovacs et al., 2002a). In contrast, the PI3-kinase, LY294002, did not inhibit cadherin-dependent Rac activation in keratinocytes (Betson et al., 2002) nor was the recruitment of Rac to MDCK cell-cell contacts affected by inhibiting PI3-kinase (Ehrlich et al., 2002).

It is difficult to resolve these discrepancies at this stage. Differences in cell types, assay systems and growth conditions may all contribute to these disparities. The suggestion that PI3-kinase and Rac participate in a positive-feedback loop (Weiner et al., 2002), rather than a simple linear pathway, may further complicate this analysis. Rac signalling in cell-cell contacts seems unlikely to depend absolutely upon upstream PI3-kinase signalling. Instead, the simultaneous activation of PI3-kinase in cell-cell contacts may serve instead to amplify Rac signalling (Weiner et al., 2002) and/or ensure the spatio-temporal fidelity of Rac activation (Iijima et al., 2002).

6.4 Membrane recruitment as an anterior step in GTPase activation

In addition to modulation of their nucleotide status at the membrane, signalling by Rho family GTPases can also be influenced by molecular mechanisms responsible for recruiting them to the plasma membrane. As noted previously, Rho family GTPases are typically retained in the cytoplasm as inactive complexes bound to Rho-GDIs (Collins, 2003; Symons and Settleman, 2000). Formally, activation of GTPase signalling at cell-cell contacts must entail the prior recruitment of the GTPase-GDI complex to the membrane, where the GTPase can be released from its interaction with Rho-GDI (Collins, 2003). There is increasing evidence that these are potentially rate-limiting steps in GTPase activation. Thus release from GDI was identified as critical for the activation of Rac at the leading edge of migrating fibroblasts (Del Pozo et al., 2002). Moreover a number of GDI-dissociation factors (GDFs), responsible for releasing GTPases from GDIs, have now been identified including the yeast integral membrane protein, Yip3, which catalyses the dissociation of endosomal Rab-GDI complexes (Sivars et al., 2003), and the neurotrophin receptor p75NTR, which induces release of Rho from Rho-GDI (Yamashita and Tohyama, 2003).

In the case of cell-cell adhesion, there is evidence to suggest that p120-ctn may participate in such a process of GTPase recruitment. Recombinant p120-ctn interacts functionally with purified Rho (Anastasiadis et al., 2000) and the two proteins could be co-immunoprecipitated from Drosophila embryo lystates (Magie et al., 2002). Strikingly, junctional localization of Rho in Drosophila embryos was lost when p120-ctn was inhibited by RNAi, suggesting that this catenin was necessary for steady-state junctional localization of Rho (Magie et al., 2002). Similarly, the ability of E-cadherin to directly activate Rac signalling was lost in a cadherin mutant that was unable to bind p120-ctn (Goodwin et al., 2003). This loss of Rac activation was accompanied by a failure of the mutant to recruit Rac to sites of homophilic adhesive ligation, consistent with the notion that p120-ctn participated in the recruitment of Rac from the cytoplasm to adhesive contacts. Taken together, these findings suggest a role for p120-ctn in recruiting Rho family GTPases to adhesive contacts. Whether they act at the earliest phase of recruitment, or serve to retain the GTPase after it reaches the membrane, remains to be determined. As α-catenin was also reported to interact with Drosophila Rho (Magie et al., 2002), it is further possible that several components of the cadherin molecular complex contribute to GTPase activation at the initial step of GTPase recruitment to the plasma membrane.

7. SUMMARY AND FUTURE DIRECTIONS

To conclude, over the past several years, the efforts of many laboratories have established key roles for Rho GTPases in regulating cadherin function and cell-cell contacts (figure 4). Of course, many key molecular details remain to be identified, among them the precise mechanisms that allow GTPase signalling to modulate cadherin function and the cellular signalling pathways that, in turn, control the GTPases. One issue is becoming increasingly apparent: these ubiquitous molecular transistors can potentially affect cadherin function at many points in the cell. It will be a necessary challenge to identify which of these potential loci are the physiologically relevant ones. All the experimental maneuvers available to date, including drug inhibitors and gene knock-outs, globally perturb GTPase signaling within cells. A major analytic advance will thus be necessary: to be able to identify and specifically manipulate the several subpools of GTPases that can affect cadherins. This will undoubtedly be an exciting challenge for the immediate future.

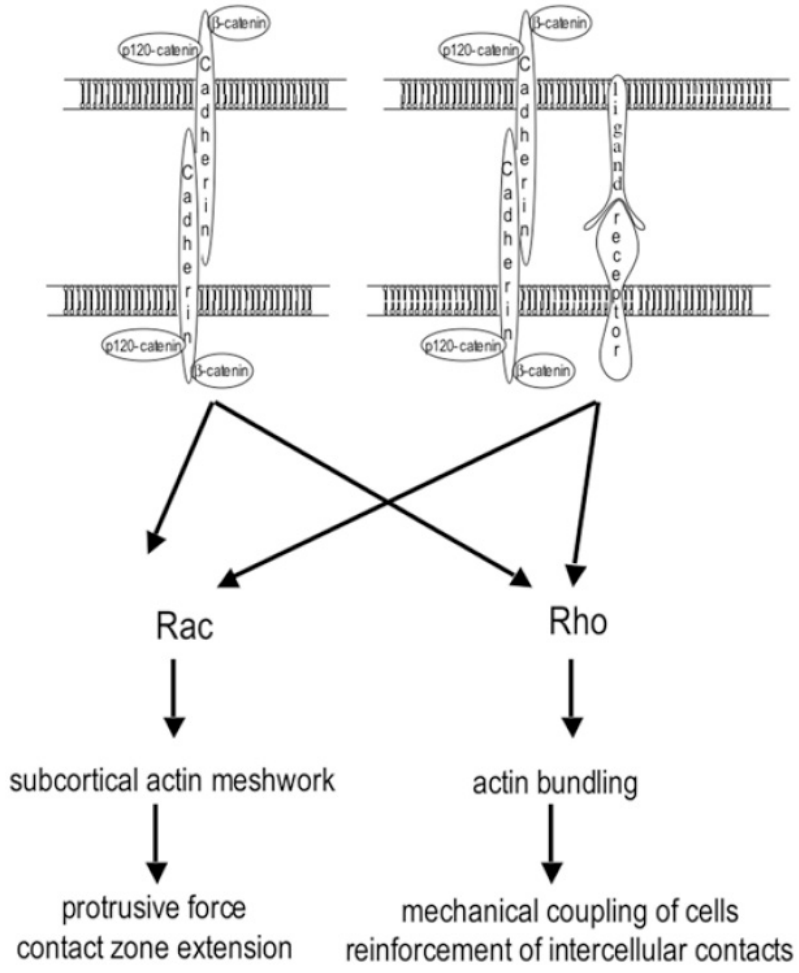

Figure 4. Cadherin-mediated adhesion triggers actin remodelling and contact formation via the Rho family GTPases Rac and Rho. Cadherin-dependent cell contacts signal to Rac and Rho by either direct or juxtacrine pathways. Rac signalling reorganises the subcortical actin meshwork ultimately leading to increased protrusive force and extension of the contact zone. Rho signalling enhances actin bundling, thereby assembling cells into mechanically coupled units with reinforced intercellular contacts.

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Chapter 9

RHO PROTEINS AND VESICLE TRAFFICKING

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Abstract: Membrane trafficking includes a highly dynamic and intricate set of intracellular pathways responsible for the transport of molecules in and out of the cell, and between the different intracellular compartments. A lot of attention has been paid in the past decades to the role played by distinct classes of small GTPases on the regulation of membrane trafficking, with special emphasis on the Rab and Arf families. More recently, Rho GTPases have been implicated in several important aspects of membrane trafficking. The initial indications that Rho proteins might be involved in membrane trafficking came from the observation of the localization of some of these proteins at specific intracellular compartments. These observations are corroborated by the findings of specific effects of these proteins on different membrane transport pathways. The role played by Rho family members in different aspects of membrane trafficking will be considered in this chapter.

1. INTRODUCTION – THE LOCALIZATION OF RHO PROTEINS

For the analysis of the subcellular localization of Rho family GTPases, a few studies have shown the localization of the endogenous proteins at distinct compartments. The use of specific antibodies has allowed the localization of the endogenous Cdc42 at the Golgi complex (Erickson et al., 1996). Moreover, endogenous RhoA has been detected associated with the membrane of secretory chromaffin granules both by cell fractionation and immunofluorescence on chromaffin cells (Gasman et al., 1998). In several cases, the subcellular localization of the endogenous proteins has been hampered by the lack of specific antibodies, or by the low levels of expression of the endogenous proteins. In this cases the use of overexpression systems with tagged proteins has been helpful, although

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caution is required since epitope tagging and overexpression may lead to altered localization (Ellis and Mellor, 2000). These studies have shown that RhoB localizes at the late endosomal compartment (Adamson et al., 1992), while the use of green fluorescent protein (GFP)-tagged Rho GTPases has been used to detect the localization of RhoB to the plasma membrane, Golgi, and motile peri-Golgi vesicles, of TC10 to plasma membrane and endosomes, of Cdc42hs and Rac2 on endomembrane, of Rac1 predominantly at the plasma membrane, and of RhoA in the cytosol (Michaelson et al., 2001). This study also shows that binding to specific compartments is mediated by the carboxy-terminal hypervariable region of Rho proteins, and that targeting of some of these proteins can be regulated by binding to the Rho guanine nucleotide dissociation inhibitor protein (RhoGDI)..

2. ENDOCYTIC PATHWAYS

2.1 Clathrin-Mediated Endocytosis And Membrane Recycling

Clathrin-mediated endocytosis is responsible for the internalization of several transmembrane receptors which can then either be recycled back to the plasma membrane, or be delivered to lysosomes for degradation. It has been shown that activated Rho and Rac inhibit transferrin-receptor-mediated endocytosis when expressed in intact HeLa cells, and the use of a cell-free system has been utilized to establish that Rho and Rac can regulate clathrin-coated vesicle formation (Lamaze et al., 1996). In polarized MDCK cells, the constitutively active V12Rac1 has inhibitory effects on the rate of endocytosis from both the apical and the basolateral plasma membrane compartments, and on the recycling of molecules to the apical compartment. In contrast, basolateral recycling of transferrin and degradation of the epidermal growth factor (EGF) internalized from the basolateral membrane are unaffected (Jou et al.,2000). The accumulation of this Rac1 mutant at an intracellular compartment positive for the Rab11 GTPase, a marker for the recycling endosomal compartment, suggests a specific role of Rac in the formation or transport of vesicles from this compartment. Interestingly, FRET analysis in NIH-3T3 cells transfected with GFP-wtRac1 reveals the presence of the perinuclear accumulation of a pool of GTP-bound Rac1, which may play a role in membrane recycling (Del Pozo et al., 2002).

A well established role of Rac is to regulate the formation of growth factors-induced membrane ruffles and lamellipodia (Ridley et al., 1992). One intriguing hypothesis is that recycling of the endocytosed membrane may contribute to the formation of these protrusions in the cell (Bretscher and Aguado-Velasco, 1998-B; Nabi, 1999). This hypothesis is corroborated by the finding that in KB cells EGF-induced ruffles are enriched in transferrin and low density lipoprotein receptors, which normally are internalized via the clathrin-mediated pathway and recycled back to the cell surface (Bretscher and Aguado-Velasco, 1998-A). In this case, activated Rac might act as a switch, redirecting exocytosis of internalized membrane from random locations on the cell surface to specific sites where ruffles form. According to this model, there must be mechanisms that permit to coordinate membrane traffic and actin polymerization at the sites of protrusion. Protein complexes that may perform this task have been recently identified. These complexes include proteins from the GIT/PKL/p95-APP1 family, which are adaptor proteins with an amino-terminal ArfGAP domain (Turner et al., 2001). These ArfGAPs interact with focal adhesion proteins such as paxillin and FAK, and with the exchanging factor PIX, an activator of Rac and Cdc42 (Manser et al., 1998). It has been shown that ArfGAP mutants of GIT1/p95-APP1 accumulate at Rab11-positive structures in a PIX-dependent manner (Matafora et al., 2001). By regulating the activity of both Arf6 and Rac/Cdc42, these complexes might influence membrane recycling from the Rab11 compartment to the plasma membrane, at sites of protrusion during cell migration (de Curtis, 2001).

In polarized MDCK cells, activated RhoA may modulate endocytosis from both membrane domains and postendocytic traffic at the basolateral pole of the cell. In fact, V14RhoA expression stimulates the rate of apical and basolateral endocytosis, whereas N19RhoA expression decreases the rate from both membrane domains (Leung et al., 1999). Moreover, V14RhoA inhibits both polarized basolateral recycling of transferrin, and degradation of basolaterally internalized EGF, while apical recycling of immunoglobulin A (IgA) is largely unaffected. In contrast, delivery to the apical membrane of basolaterally internalized IgA by trans-cytosis is severely impaired.

The signalling components and the molecular machinery that mediate the described events upstream and downstream of Rho GTPases remain largely to be identified, and represent an important aspect which is attracting the attention of the researchers in the field. The phosphatase synaptojanin 2 has been identified as a novel direct and specific Rac1 effector. Expression of constitutively active Rac1 causes the translocation of synaptojanin 2 from the cytoplasm to the plasma membrane (Malecz et al., 2000). Both activated Rac1 and a membrane-targeted version of synaptojanin 2 inhibited

endocytosis of the EGF and transferrin receptors, a process that is known to be dependent on polyphosphoinositide lipids (Jost et al., 1998). These results suggest that synaptojanin 2 may mediate the inhibitory effect of Rac1 on endocytosis.

A large number of G protein-coupled receptors, including β2 adrenoceptors and various muscarinic acetylcholine receptor subtypes, use the clathrin-mediated sequestration pathway. Overexpression of wild-type RhoA strongly inhibits agonist-induced sequestration of both m1 and m2 acetylcholine receptors (Vogler et al., 1999).

Like Rac1 and RhoA, Cdc42 plays also a critical role in epithelial cell polarity, which is not reflected by major alterations in the actin cytoskeleton, but rather results from the selective inhibition of membrane traffic to the basolateral plasma membrane in both the endocytic and the secretory pathways (Kroschewski et al., 1999; Rojas et al., 2001). In a screening for Cdc42 targets that influence these activities, Yang et al (2001) have identified the protein activated Cdc42-associated kinase-2 (ACK2) as a new binding partner for clathrin. Overexpression of ACK2 inhibits transferrin receptor endocytosis because of a competition between ACK2 and AP-2 for clathrin, suggesting that ACK2 may represent a novel clathrin-assembly protein and participate in the regulation of receptor-mediated endocytosis. Another member of this family of tyrosine kinases, ACK1 has been shown to associate directly with the heavy chain of clathrin. The central region in ACK1 contains a conserved motif that behaves as a clathrin adaptor and competes with β-arrestin for a common binding site on the clathrin N-terminal head domain (Teo et al., 2001). another binding partner for ACK2 has been recently identified, namely the sorting nexin protein SH3PX1 (sorting nexin 9), that has previously been implicated in endocytosis. ACK2 and SH3PX1 work together to promote the degradation of the EGF receptor (Lin et al., 2002).

The modular protein intersectin-S regulates the formation of clathrin-coated vesicles(Roos and kelly, 1998; Yamabhai et al., 1998; Hussain et al., 1999; Okamoto et al., 1999; Sengar et al., 1999). An alternative spliced form, intersectin-1, has a Dbl homology (DH) domain that makes it function as a guanine nucleotide exchange factor (GEF) for Cdc42, a critical activator of N-WASP. The finding that intersectin-1 binds directly to N-WASP, and accelerates Cdc42-mediated actin assembly via N-WASP and the Arp2/3 complex, implicate this adaptor protein as a possible linker between actin and endocytosis (Hussain et al., 2001).

Moving downstream along the endocytic pathway, RhoB has been found to localize at late endosomes (Robertson et al., 1995), where it recruits the serine/threonine kinase PRK1 (Mellor et al., 1998). In this way, RhoB is able to regulate the kinetics of EGF receptor traffic, by retarding the delivery of

the internalized receptor towards the lysosomes (Gampel et al., 1999). This represents an interesting example of the connection between membrane traffic and the regulation of signalling events in the cell. In fact, by slowing down the degradation process, RhoB might prolong the active life of the receptor in the cell and therefore the duration of signalling.

Vesicular transport is a dynamic process that requires coordinated interactions between membrane and cytoskeleton, and the mechanisms integrating these interactions are poorly understood. RhoD may contribute to the coordination between traffic and cytoskeleton, since it is able to induce actin rearrangement at the cell surface, and to regulate early endosome distribution (Murphy et al., 1996). Expression of a constitutively active form of RhoD induces cytoskeletal alterations including the loss of stress fibers and focal adhesions, and causes the retardation of cell migration (Tsubakimoto et al., 1999). Recently, a novel splice variant of human Diaphanous, hDia2C, has been identified, which specifically binds to RhoD and is recruited onto early endosomes. This interaction leads to Src-mediated alignment of early endosomes along actin filaments, which may be responsible for the bi-directional movement of early endosomes between the cell periphery and the interior (Gasman et al., 2003).

2.2 Clathrin-Independent Endocytosis

Clathrin-independent endocytosis includes distinct mechanisms used by the cells to internalize membrane and soluble components. Macropinocytosis consists in the uptake of extracellular fluid in large vesicles forming at Rac-mediated ruffling areas of the cell surface induced by growth factors (Ridley et al., 1992). Macropinosomes form in a Rac-dependent manner via PAK1 (p21-activated kinase 1), a Rac/Cdc42 effector which localizes at areas of membrane ruffling and pinocytic vesicles in fibroblasts and activated human leukocytes (Dharmawardhane et al., 1997 and 1999). Expression of a PAK1 kinase autoinhibitory domain blocks both platelet-derived growth factor- and L61Rac-stimulated uptake of dextran particles, indicating that PAK kinase activity is required for normal growth factor-induced macropinocytosis. On the other hand, active versions of PAK1 enhance both growth factor-stimulated 70-kDa dextran uptake and efflux, suggesting that PAK1 activity modulates pinocytic vesicle cycling (Dharmawardhane et al., 2000). Therefore PAK1 seems to play an important regulatory role in the process of macropinocytosis, perhaps related to the requirement for PAK in directed cell motility (Kiosses et al., 1999; Sells et al., 1999).

While immature dendritic cells actively internalize antigens, mature dendritic cells are poorly endocytic, functioning instead to present antigens to T cells. Immature dendritic cells use constitutive macropinocytosis to

capture exogenous antigens for presentation on MHC molecules (Mellman and Steinman, 2001). In these cells, dominant negative Rac inhibits the formation of macropinosomes but, surprisingly, is not able to eliminate membrane ruffling (West et al., 2000), thus indicating that Rac has an important role in the constitutive formation of macropinosomes in dendritic cells, but may be required downstream of membrane ruffling. Cdc42 is also important for the endocytic activity of the immature dendritic cells, and endocytic down-regulation reflects a decrease in the endogenous levels of activated Cdc42-GTP, detectable only in immature cells. Therefore dendritic cells developmentally regulate endocytosis at least in part by controlling levels of activated Cdc42 (Garrett et al., 2000).

Established Dictyostelium cell lines that conditionally over-express epitope-tagged Dictyostelium discoideum wild-type or mutant DdRac1B have been used to show that constitutively active V12-DdRac1B GTP induces defects in fluid-phase endocytosis and developmental delays (Palmieri et al., 2000). The use of conditionally expressed DdRac1B proteins should facilitate the identification and characterization of the Rac1 signaling pathway in this organism, that is amenable to both biochemical and molecular genetic manipulations.

Rac1 is known to regulate cadherin-dependent cell-cell contacts in epidermal keratinocytes. Injection of keratinocytes with constitutively active Rac1 results in cell spreading and disruption of cell-cell contacts, achieved through the selective recruitment of E-cadherin-catenin complexes to the perimeter of multiple large intracellular vesicles, which are labelled by GFP-tagged L61Rac1 and contain the transferrin receptor, a marker for recycling endosomes (Akhtar and Hotchin, 2001). The reorganization of the actin cytoskeleton is important for vesicle formation, while the inhibition of the uptake of transferrin by GFP-L61Rac1 expression suggests that the endocytosis of E-cadherin is a clathrin-independent mechanism. A distinct constitutive pinocytic pathway, specifically regulated by Cdc42, has been identified for glycosylphosphatidylinositol-anchored proteins, which are endocytosed to the recycling endosomal compartment via a non-clathrin, non-caveolae mediated pathway (Sabharanjak et al., 2002).

Reconstitution experiments using permeabilized cells and cell-free systems have been widely used to identify the molecular mechanisms underlying membrane traffic regulated by Rab and Arf GTPases (Schmid, 1993; Shorter and Warren, 2002). In this direction, permeabilized cells have been utilized to establish the involvement of Rho proteins in clathrin-independent endocytosis at the apical domain of MDCK cells: reconstituted Ricin endocytosis in the presence of intact cytosol, as well as GTP-$\tilde{\gamma}$S-stimulated Ricin uptake were inhibited by Clostridium botulinum C3 transferase, an enzyme that inactivates Rho proteins (Garred et al., 2001).

This approach may lead to the identification of new molecular players linked to Rho GTPases-mediated steps in membrane trafficking.

2.3 Roles for Rho in Phagocytosis

Phagocytosis is an actin-dependent process, by which cells are able to engulf external particles, including bacteria, yeast, and apoptotic cells. This process occurs by extension and fusion of pseudopods. Phagocytosis is generally carried out by specialized cells like neutrophils and macrophages, although other cell types are able to engulf apoptotic cells by phagocytosis (Aderem and Underhill, 1999). Distinct receptors present on the surface of the phagocytic cells may induce various molecular mechanisms of phagocytosis (Aderem and Underhill, 1999). In leukocytes, both Rac1 and Cdc42 are required for phagocytosis induced by Fc receptors for IgG (FcγR) (Cox et al., 1997), while a second type of phagocytosis induced by the complement receptor (CR3) is mediated by Rho (Caron and Hall, 1998).

The use of cells expressing the dominant negative forms of either Cdc42 or Rac1 has shown distinct effects of the two mutant GTPases on the FcγR-mediated phagocytic process, thus indicating that Rac1 and Cdc42 have distinct functions and may act cooperatively in the assembly of the phagocytic cup (Massol et al., 1998): Cdc42 functions during pseudopod extension, while Rac is required for pseudopod fusion and phagosome closure. In response to ligation of the FcγR, the guanine nucleotide exchange factor Vav translocates to nascent phagosomes and catalyzes GTP loading on Rac, but not Cdc42 (Patel et al., 2002). The Vav-induced Rac activation proceeds independently of Cdc42 function, supporting distinct roles for each GTPase during engulfment.

The analysis of the cytoskeletal events initiated upon engagement of phagocytic receptors has shown that the Arp2/3 complex, a multifunctional actin organizer, is involved in actin remodelling during both FcγR- and CR3-mediated phagocytosis, although the upstream signals that recruit the Arp2/3 complex to phagosomes differ for the two receptors (May et al., 2000). Further analysis of the events initiated upon engagement of Fc receptors in macrophages shows the accumulation of Ena/VASP proteins and of the Ena/VASP ligand Fyb/SLAP (Fyn-binding/SLP-76-associated protein) (Krause et al., 2000) at sites of F-actin assembly around nascent phagosomes (Coppolino et al., 2001). It is conceivable that Fyb/SLAP and Ena/VASP participate in the actin assembly process triggered by FcγR. Ena/VASP proteins are ligands for profilin (Reinhard et al., 1995; Gertler et al., 1996; Kang et al., 1997), an actin-monomer-binding protein that under favourable conditions stimulates the polymerization of actin (Pantaloni and Carlier, 1993).

Inhibitors of the Rho effector Rho-kinase and of its downstream target, myosin-II, have been used to demonstrate that inhibition of the Rho → ROK → myosin-II pathway causes a decreased accumulation of the Arp2/3 complex and F-actin around bound particles, leading to a reduction in CR3-mediated phagocytic engulfment (Olazabal et al., 2002). In contrast, the Rac and Cdc42-dependent FcγR-mediated phagocytosis is independent of Rho-kinase activity, and is only dependent on myosin-II for particle internalization, not for actin cup formation.

Apoptotic cells are recognized, engulfed and degraded by phagocytes (Savill and Fadok, 2000). Studies in Caenorhabditis elegans and mammals are now converging to reveal some of the key mechanisms implicated in the ingestion of apoptotic cells both by professional or amateur phagocytes. The process of engulfment involves a ternary complex including the proteins CrkII/CED-2, DOCK180/CED-5, and ELMO/CED-12, which is required for the activation of Rac1/CED-10 (Albert et al., 2000; Reddien and Horvitz, 2000; Gumienny et al., 2001; Leverrier and Ridley, 2001; Wu et al., 2001; Zhou et al., 2001). In mammalian cells, integrin receptors are important for the internalization of apoptotic cells: engagement of the αvβ5 heterodimer results in the recruitment of the p130Cas-CrkII-Dock180 complex, which in turn triggers Rac1 activation and phagosome formation (Albert et al., 2000). One particularly intriguing aspect of this process is the peculiar mechanism of Rac1 activation by the DOCK180-ELMO complex. DOCK180 is an atypical GEF for Rac, which needs to be stimulated by ELMO to activate Rac in the cell (Brugnera et al., 2002; Cote and Vuori, 2002).

One interesting aspect developing from the studies on phagocytosis is the finding that many successful bacterial pathogens are able to alter their recognition by the cell, and therefore to escape macrophage surveillance (Rosenberger and Finlay, 2003). This occurs by a set of bacterial proteins that is delivered into macrophages and interrupts the signalling required for phagocytosis. The injected bacterial proteins may include a RhoGAP to down-regulate the Rho GTPases inside the cell (Fu and Galán, 1999; Goehring et al., 1999; Black and Bliska, 2000). On the other hand, other microorganisms inject into the host activators of Rho GTPases, to ensure efficient actin-mediated entry into non-phagocytic cells (Hardt et al., 1998).

3. EXOCYTIC PATHWAYS

3.1 Regulated Exocytosis

Rho family GTPases have been shown to play a role also in exocytic events. The C3 transferase inhibits the stimulated release of β-hexosaminidase from RBL-2H3 cells (Yonei et al., 1995). More recently, the use of mutant forms of Rho, Rac and Cdc42 in these cells has shown that activation of Cdc42 and Rac enhances antigen-stimulated secretion, and elevates the levels of antigen-stimulated IP3 production, whereas the dominant-negative mutants significantly inhibit secretion. Interestingly, treatment with calcium ionophores and PKC activators rescues the inhibition of secretion in cells expressing the dominant-negative mutants, implying that Cdc42 and Rac act upstream of the antigen-stimulated calcium influx pathway (Hong-Geller and Cerione, 2000).

Constitutively active mutant proteins, V14RhoA and V12Rac1, enhance regulated secretion from permeabilized mast cells by increasing the proportion of cells that are competent to respond to stimulation. Inhibition of endogenous Rac and Rho activity using inhibitors, N17Rac1 and C3 transferase, respectively, or microinjection of RhoGDI into cells induces the inhibition of the stimulated secretory response of mast cells (Price et al., 1995; Mariot et al., 1996).

Localized disassembly of cortical F-actin has long been considered necessary for facilitation of exocytosis. Exposure of permeabilized mast cells to calcium/ATP induces cortical F-actin disassembly and secretion. In mast cells, activation of GTP-binding proteins induces centripetal reorganization of actin filaments. This effect is due to disassembly, relocalization, and polymerization of F-actin and is dependent on two small GTPases of the Rho family. In these cells, the secretory response enhanced by constitutively active mutants of Rac and Rho is unaffected in the presence of cytochalasin, which disrupts the actin cytoskeleton. Therefore, Rac and Rho control actin organization and secretion by divergent, parallel signaling pathways (Norman et al., 1996; Sullivan et al., 1999). Moreover, streptolysin-O-permeabilized mast cells have been used to enable Ca2+-stimulated secretion by addition of GTPγS-preactivated Rac2 and Cdc42 recombinant proteins (Brown et al., 1998).

Rho family proteins have been implicated in other types of regulated secretory pathways. The exposure of normal rat islets or isolated beta cells to different clostridial toxins has suggested for the first time the implication of Rac and Cdc42, but not Rho, in glucose- or potassium-induced insulin secretion (Kowluru et al., 1997). Cdc42 plays an active role in calcium-

regulated exocytosis from adrenal chromaffin cells, by coupling the actin cytoskeleton to the sequential steps underlying membrane trafficking at the site of exocytosis (Gasman et al., 1999)

3.1.1 Insulin-stimulated glucose transport

Insulin stimulates glucose transport into fat and muscle cells by inducing the translocation of intracellular vesicles carrying the facilitative glucose transporter GLUT4 to the plasma membrane. This process requires a continuous cycling through the early endosomes, a Glut 4 specific storage compartment and the plasma membrane. Insulin increases the rate of Glut 4 trafficking from its specific storage compartment to the plasma membrane, by signal transduction from the insulin receptor, vesicle trafficking and actin cytoskeleton modifications (Cormont and Le Marchand-Brustel Y, 2001). The signalling pathways that link the insulin receptor to GLUT4 translocation have recently been clarified. It has been shown that both activation of phosphatidylinositol-3-OH kinase and the parallel activation of the Rho family GTPase TC10 are required to stimulate fully GLUT4 translocation in response to insulin (Figure 1). Activation of TC10 requires the translocation of Cbl, Crk and C3G to the lipid rafts, where C3G specifically activates TC10 (Chiang et al., 2001). Moreover, the insulin stimulation of GLUT4 translocation in adipocytes has been shown to require the spatial separation and distinct compartmentalization of the PI-3 kinase and TC10 signaling pathways (Watson et al., 2001). Events downstream of the activation of TC10 and important for the translocation of GLUT4 to the plasma membrane include the cortical localization of N-WASP, important for F-actin formation in response to insulin (Jiang et al., 2002), and the translocation to the plasma membrane of CIP4/2 (Chang et al., 2002) and TCGAP (Chiang et al., 2003), two putative effectors of TC10 playing a crucial role in the regulation of insulin-stimulated GLUT4 translocation. TC10 interacts also with Exo70 (Inoue et al., 2003), one of the components of the exocyst complex, a conserved eight-subunit complex involved in the docking of exocytic vesicles (Lipschutz and Mostov, 2002). Exo70 translocates to the plasma membrane in response to insulin through the activation of TC10, where it assembles a multiprotein complex that includes Sec6 and Sec8, suggesting the involvement of the exocyst in the targeting of the GLUT4 vesicles to the plasma membrane.

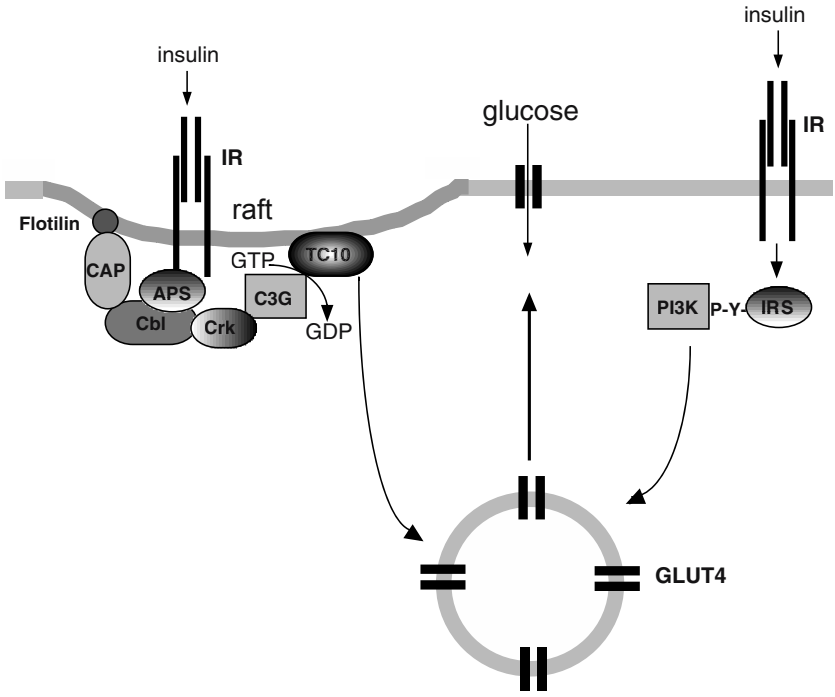

Figure 1. Model for the signalling pathways that link the insulin receptor to GLUT4 translocation. Both activation of phosphatidylinositol-3-OH kinase (PI3K) and TC10 are required to stimulate GLUT4 translocation in response to insulin. A pool of insulin receptors (IR) phosphorylate the adaptor proteins Cbl and APS. Cbl interacts with Cbl-associated protein (CAP), which binds the lipid raft protein flotilin. This leads to the recruitment of the SH2/SH3 adaptor protein Crk-II, by binding of the SH2 domain to phosphorylated Cbl. Crk-II binds constitutively the GDP/GTP exchanging factor C3G, which activates TC10 associated to the lipid rafts. A distinct pool of insulin receptors activates the PI3K via the interaction with the tyrosine phosphorylated insulin receptor substrate (IRS).

3.1.2 Neurotransmitter release and synaptic function

Subcellular fractionation has shown that RhoA, RhoB, Rac1, and Cdc42 are present in rat brain synaptosomes, although only Rac1 was associated with highly purified synaptic vesicles (Doussau et al., 2000). To identify which Rho protein(s) may function in evoked exocytosis of neurotransmitters, bacterial toxins have been utilized to interfere with the function of these GTPases in Aplysia neurons. Intraneuronal application of wild-type or mutated forms of lethal toxin from Clostridium sordellii have established that inactivation of Rac by glucosylation is responsible for the reduction in acetylcholine release. This inhibition is caused by a reduction in the number

of functional release sites, suggesting that after docking of synaptic vesicles, vesicular Rac stimulates a membrane effector (or effectors) essential for the fusion competence of the exocytotic sites (Humeau et al., 2002).

3.2 Polarized Exocytosis

Rho family GTPases play an important role also in different aspects of the polarized delivery of exocytic vesicles. In particular, Cdc42 has been implicated in several aspects of cell polarity. The use of semi-intact MDCK cells expressing Cdc42 mutants has shown their inhibitory effect on the exit of basolateral proteins-containing vesicles from the trans-Golgi network, and the stimulation of the exit of an apically-oriented marker. This regulation may result from the modulation of the actin cytoskeleton, as GTPase-deficient Cdc42 depletes a perinuclear actin pool that rapidly exchanges with exogenous fluorescent actin (Müsch et al., 2001). Effects of Cdc42 mutants were observed also on the secretion of gp80 into the apical medium, and on targeted delivery of newly synthesized polymeric immunoglobulin receptor in MDCK cells (Rojas et al., 2001). Further analysis has shown that GTPase-deficient and dominant negative Cdc42 does not affect the apical targeting of a newly synthesized apical membrane protein, but reverses to apical the distribution of two exogenous basolateral membrane proteins. In contrast, GTPase-deficient Cdc42 does not affect polarized exocytosis of endogenous soluble proteins, either apical or basolateral (Cohen et al., 2001).

A role of Rho proteins in polarized membrane traffic has been identified also in plants. Pollen tube growth relies on the fast and polarized delivery of new membrane and wall material to the apical region, where growth takes place. Injection into extending pollen tubes of antibodies against Rop1, which is a plant homologue of the mammalian Rac (Yang, 2002), inhibits pollen tube elongation (Lin and Yang, 1997). Measurement of endo/exocytosis in living cells using confocal imaging of FM 1-43 has shown higher endo/exocytosis activity in the apical region of the extending pollen tube. Use of antisense oligonucleotides for a GTPase with high homology to Rop induce a decrease of the rate of pollen tubes growth, and of FM 1-43 uptake, implicating Rop GTPases in the trafficking of vesicles required for pollen tube extension (Camacho and Malho, 2003).

3.3 Mechanisms Mediating the Regulation of Exocytosis by Rho Proteins

Several aspects on how Rho proteins are able to affects the different steps of membrane traffic remain unsolved. Recent studies have started to address the mechanisms by which Rho GTPases may be recruited to the different intracellular compartments and may contribute to the transport processes. As mentioned before, Cdc42 has been implicated in the regulation of different steps of membrane traffic. A direct interaction between Cdc42 and the γ-subunit of COPI, a cytoplasmic 'coatomer' protein complex involved in ER-Golgi transport (Lowe and Kreis, 1998), has been identified (Wu et al., 2000). Assembly of this complex onto membranes is controlled by the Arf GTPase to direct the formation of vesicles involved in the transport of proteins from the Golgi back to the endoplasmic reticulum, and in the recycling from the plasma membrane through endosomes (Lowe and Kreis, 1998; Goldberg, 2000). The identification of γCOP as an effector for Cdc42 is consistent with the localization of this GTPase at the Golgi compartment (Erickson et al., 1996). Both constitutively active and inactive forms of Cdc42 block protein transport between endoplasmic reticulum and Golgi, possibly as an indirect, inhibitory effect on movement in the opposite direction, supporting a role for Cdc42 in COPI-regulated vesicle transport. Interestingly, these authors also found that the mutant Cdc42F28L, which can cause growth transformation of NIH 3T3 cells (Lin et al., 1997), interferes with the recruitment of COPI to membranes, and they show that the association of Cdc42 with γCOP is necessary for growth transformation (Wu et al., 2000). An actin-mediated, N-WASP-dependent effect of Cdc42 on the transport of molecules from Golgi to endoplasmic reticulum has also been described (Luna et al., 2002). In this study, analysis by immunoelectron microscopy on transfected HeLa cells has shown that activated GFP-Cdc42-V12 is enriched in the lateral portions of Golgi cisternae and in peri-Golgi transport intermediates, consistent with the effect of this mutant on retrograde transport from the Golgi.

Immunoprecipitation experiments suggest the presence of a Cdk5-p35, Cdc42 and α-PAK complex in Golgi membranes (Paglini et al., 2001). The localization of an active Cdk5-p35 kinase in Golgi membranes, together with the block in the formation of membrane vesicles from the Golgi apparatus upon inactivation or inhibition of Cdk5 or p35 expression, suggest the involvement of Cdk5-p35 in the early steps of the secretory pathway, regulating either vesicle formation or budding in the Golgi apparatus.

RhoGDIs interact preferentially with the GDP-bound form of Rho proteins, thereby maintaining the GTPases in an inactive state (Kikuchi et al., 1992). Recently, the first example of a GDI directly involved in the

delivery of a Rho protein to a specific subcellular compartment has been described. The non-cytosolic fraction of RhoGDI-3 has been found associated via its unique N-terminal segment with the Golgi apparatus, where it is able to recruit RhoG (Brunet et al., 2002). RhoG is a GTPase which shares high sequence identity with Rac1 and Cdc42, and previous studies have suggested that RhoG mediates its effects through activation of Rac1 and Cdc42 (Gauthier-Rouviere et al., 1998). The RhoGDI-3/RhoG complex may be part of the elaborate machinery by which Rho GTPases regulate trafficking from the Golgi compartment.

In this review, evidence supporting the implication of members of the Rho family in particular steps of vesicular traffic has been presented. Several data obtained in recent years have made us appreciate the importance of Rho GTPases in the regulation of both endocytic and exocytic membrane traffic. Vesicular traffic is implicated in several cellular processes controlled by Rho family proteins, including the establishment and maintenance of cell polarity and cell motility. The observed connections between distinct Rho GTPases with particular trafficking compartments has often been corroborated by the localization of the involved GTPases at the corresponding compartments. Future studies will probably expand the implication of these proteins in other trafficking events, but will also increase our knowledge about the molecular mechanisms necessary to dynamically recruit and regulate the function of the different Rho family members at the particular compartments. Another important aspect of the research in this field will be the analysis of the functional and molecular connections between the Rho family and other families of GTPases, such as the Arf and Rab families, which have been implicated in the regulation of vesicular traffic for a long time. The analysis of the signalling events upstream and downstream of Rho proteins will also help us to distinguish between actin-related events and actin-independent events regulated by this eclectic family of molecular switches.

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Chapter 10

THE PROTOTYPE RHO-ASSOCIATED KINASE PAK

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Abstract: p21-activated kinase (PAK) was the first identified serine/threonine protein kinase to bind and be activated by Rho GTPases. Since it was discovered some 10 years ago, PAK has been intensively studied and represents the best understood of the Rho-associated kinases. PAK family kinases are encoded by six mammalian genes with three conventional PAKs (PAK1-3) and three non-conventional PAKs (PAK4-6). Cdc42/Rac activates conventional PAKs through modulation of a kinase inhibitory (KI) domain by the overlapping Cdc42/Rac interaction/binding (CRIB) domain. Nonetheless PAKs can also be activated in GTPase independent manners, including by protease cleavage, translocation to membranes and binding to lipids. As a key signaling component, PAK is evolutionarily conserved from yeast to man. In mammalian cells PAKs play roles in many cellular signaling pathways such as the regulation of focal adhesion and actin dynamics, changes in cell morphology, cell motility, and the regulation of gene expression. Recently a number of studies show PAK is implicated disease states. As a result PAK family kinases are becoming good candidates for drug development.

1. INTRODUCTION

The p21-activated kinase PAK1 was initially purified using an assay for specific Cdc42 binding partners in rat brain cytosol (Manser et al., 1994). I undertook the cloning of this affinity-purified bovine kinase in 1993 by use of degenerate oligonucleotides directed towards peptides isolated from the catalytic domain of the protein. Corresponding cDNAs isolated for PAK (Manser et al., 1994) demonstrated a kinase related to the yeast kinase Ste20p implicated in the pheromone-activated MAP kinase pathway in yeast

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(Leberer et al., 1992). The brain-purified kinase was found to bind GTP-bound forms of Rac or Cdc42, but not RhoA and become activated in vitro by the interaction. It became apparent that three major isoforms of p21-activated kinases termed PAK1 (rat α-PAK), PAK3 (rat β-PAK), and PAK2 (rat γ-PAK) are found in brain, with PAK2 being ubiquitous (Bagrodia et al., 1995; Manser et al., 1995). PAKs serve as important regulators of cytoskeletal dynamics and cell motility, transcription through MAP kinase cascades, death and survival signalling, and more recent evidence suggests roles in cell-cycle progression. Consequently, PAKs are implicated in a number of pathological conditions and in cell transformation (Bokoch, 2003).

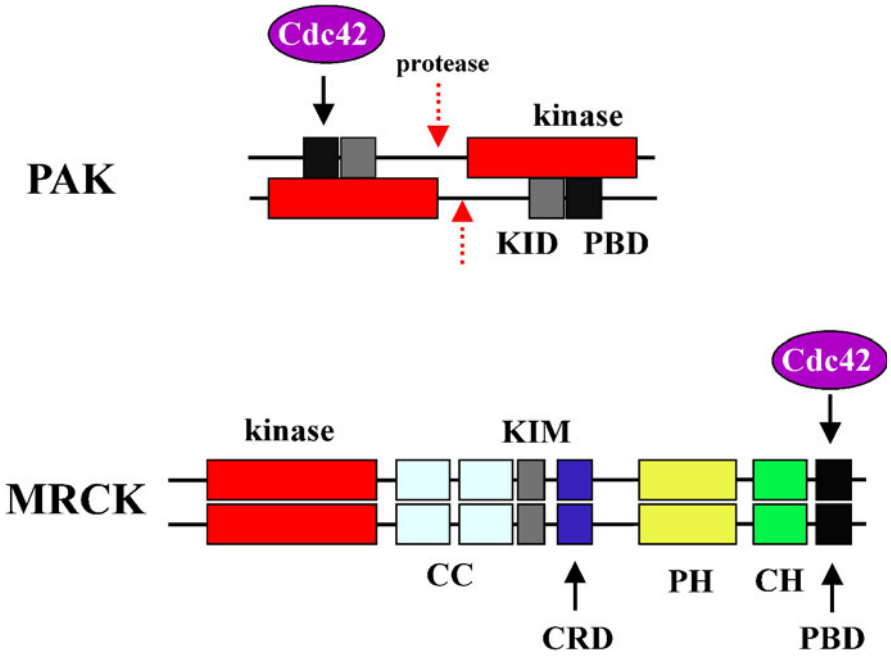


Figure 1. A model for the organization and regulation of two Cdc42-associated kinases PAK and MRCK. The upper panel shows PAK forming a homodimer through interactions between the N-terminal kinase inhibitory (KI) domain of one molecule and the C-terminal kinase domain on the other molecule. In the absence of Cdc42.GTP the dimerized kinases are negatively regulated by the KI domaina in a trans-inhibition fashion. Cdc42.GTP binds to the p21 binding domain (PBD) flanking to the KI domain and eliminates its inhibition effect. PAK can also be activated by protease cleavage. The lower panel illustrates MRCK which forms homodimers through the coiled-coil (CC) interactions, while the kinase is inhibited by a kinase inhibitory motif (KIM). The Cdc42 binding domain (PBD) is located at C-terminal and does not directly participate in kinase activation. KIM is regulated by a diacylglycerol binding cysteine rich domain (CRD), while the plectrin homology domain (PH) and citron homology domain (CH) are thought to interact with as yet uncharacterized components.

The best characterized kinase (of those that interact with Rho GTPases) is PAK1 since a structural template for its regulation is available (Lei et al., 2000). Interestingly all Rho GTPase-associated kinases exists as oligomers. As illustrated in Figure 1 PAK is thought to exist as a homodimer in solution and in cells, probably in a trans-inhibited conformation where the N-terminal kinase inhibitory (KI) region of one PAK1 polypeptide binds the C-terminal catalytic domain of the other (Parrini et al., 2002). By contrast a different kinase effector of Cdc42 known as MRCK also contains a CRIB region but is held in an auto inhibited conformation in a head-to-head fashion (Tan et al., 2001).

Pak4, the first reported member of the non-conventional PAKs (also referred to as group II PAKs) to be identified using a PCR screen with degenerate primers based on the PAK2 kinase domain (Abo et al., 1998). PAK4 is ubiquitously expressed, with levels highest in the prostate, testis, and colon (Abo et al., 1998; Callow et al., 2002). Thus far this is the most extensively studied member of the group II PAKs. A kinase designated PAK6 was identified as an androgen receptor (AR) interacting protein in a yeast two-hybrid screen (Yang et al., 2001) the first description of a PAK to interact with a hormone receptor. In support of this role expression of PAK6 is highest in testis and prostate (Yang et al., 2001). The last PAK member to be described is PAK5 which brain-enriched (Cau et al., 2001; Pandey et al., 2002).

2. THE INTERACTION OF PAKS WITH RHO GTPASES

Group I PAKs are serine/threonine protein kinases with significant sequence homology in their catalytic domains to Ste20p and Cla4p, protein kinases from budding yeast (Cvrckova et al., 1995; Dan et al., 2001b; Zhao et al., 1995). It has subsequently been determined that the genomes of all eukaryotes examined contain representative members of the family of Ste20-related protein kinases (Dan et al., 2001b). Most closely related in their catalytic domains to these mammalian group I PAKs are the more recently discovered PAK4-6 (group II). The kinase of PAKs are >60% identical, but among PAKs 4, 5, and 6 there is >75% identity suggesting that they target similar substrates. The N-terminal Cdc42/Rac interaction and binding (CRIB) domain of PAKs (Fig. 2) are also >60% identical to each other.

PAK1 binds and is activated by Rac1, Rac2, Rac3 (Knaus et al., 1998; Manser et al., 1994; Mira et al., 2000) and Cdc42 (Manser et al., 1994), as well as the less studied GTPases TC10 (Neudauer et al., 1998), CHP (Aronheim et al., 1998) and Wrch-1 (Tao et al., 2001) proteins, but does not

bind Rho A/B/C/E/G or other Ras superfamily members. Conserved residues within the N-terminal PBD/IS domain allow binding and activation by these GTPases (see Fig. 2). Residues conferring GTPase selectivity within the overall p21-binding domain (PBD) domain have been mapped (Reeder et al., 2001). A short lysine-rich tract (PAK1 residues 66-68) just upstream of the CRIB domain is reportedly required for effective Rac GTPase binding (Knaus et al., 1998) but is not present in the Drosophila sequence (Fig. 2). Rac and Cdc42 can minimally bind to the so-called CRIB (for Cdc42 and Rac interactive binding) domain (Burbelo et al., 1995) encompassing PAK1 (75-90) but sequences in the flanking IS "inhibitory switch" that restricts kinase activity of PAK (cf PAK1 67-113), also contribute to overall binding affinity (Knaus et al., 1998; Lei et al., 2000; Sells et al., 1997).

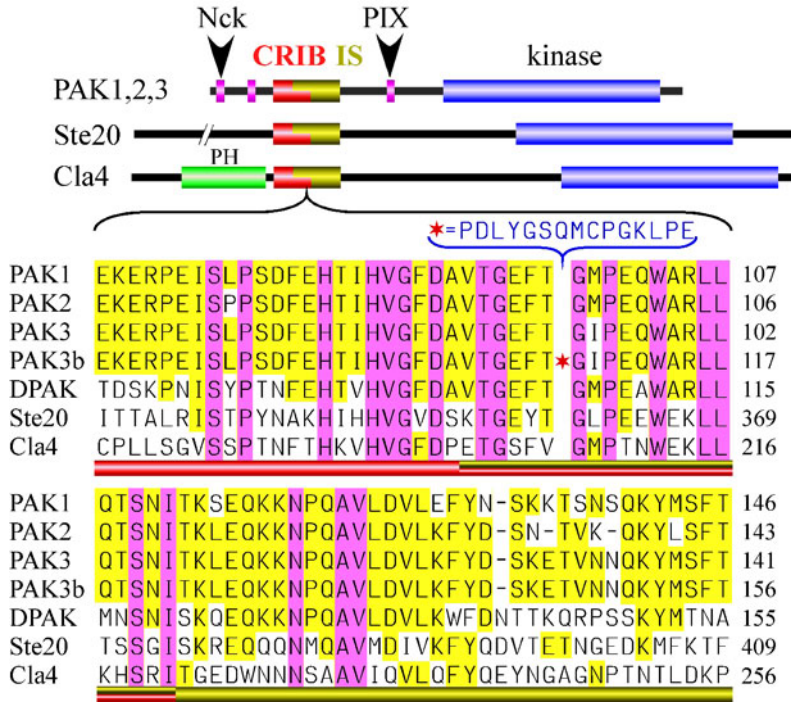
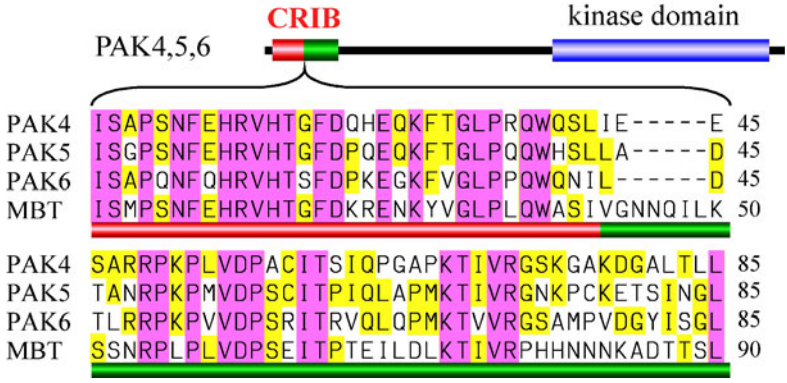


Figure 2. The domain structure and sequence comparison of the group I PAKs.The group I PAKs are represented by human PAK1-3 (accession # Q13153, Q13177, and NP_002569, respectively), Drosophila DPAK (#AAC47094), budding yeast Ste20p (#AAA35038) and Cla4p (#P48562) which is characteristic among fungal PAKs in containing a PH domain (light green). A unique 15 amino acids insertion in the CRIB domain of PAK3b (accession #O75914) was highlighted with a red star and its sequence was shown on top of the multi-alignment. This group of PAKs contains a highly conserved Cdc42/Rac-interactive binding

domain (CRIB, shown in red) and an inhibitory switch (IS) domain overlapping with the CRIB (in yellow). Cdc42/Rac1 binding to the CRIB abolish IS's inhibitory effect to the catalytic kinase domain (in blue). The 3 purple boxes correspond to the conserved proline-rich domains which bind SH3 domain containing proteins NCK, Grb2 and PIX.

In the group II kinases additional conserved features are observed C-terminal to the CRIB motif but these are not involved in kinase inhibition as in PAK1 (Figure 3) Also group II PAKs have no well conserved SH3 binding sites which might point to common protein partners. PAK4 binds to Cdc42.GTP, and only to a lesser extent Rac.GTP (Abo et al., 1998). A significant difference in behaviour of the CRIB region is suggested by the fact that the Cdc42 effector mutant (Y40C) is unable to bind group 1 PAKs but can bind PAK4 (Abo et al., 1998), suggesting that a significantly different structural interface exists between the GTPase and these related



effectors.

Figure 3. The domain structure and sequence comparison of the group II PAKs. The group II PAKs includes human PAK4-6 (accession # O96013, BAA94194 and NP_064553, respectively) and Drosophila PAK which is also known as mushroom bodies tiny (mbt) with accession of CAA09699. Instead of having an IS domain, this group of PAK contains a highly conserved motif (in dark green, see multialignment) C-terminal to the CRIB. The function of this conserved motif is yet to be known. The sequences of the CRIB domain in these PAK kinases were aligned using DNAstar-Megalign software with identical residues being highlighted in red and conserved residues in yellow.

Other distinguishing features of the regulatory domain are illustrated in Figure 2 including two canonical PXXP SH3-binding motifs and a conserved nonclassical SH3 binding site for PIX (Manser et al., 1998). Interaction of the first site conserved among group I PAKs with the SH3-containing adapter protein Nck is well documented (Bokoch et al., 1996) and a second conserved site can bind to the adapter protein Grb2 (Puto et al.,

3. THE CONTROL OF PAK ACTIVITY

Structural data (Gizachew et al., 2000; Lei et al., 2000; Morreale et al., 2000) and biochemical studies (Benner et al., 1995; Buchwald et al., 2001; Chong et al., 2001; Gatti et al., 1999; Tu and Wigler, 1999; Yu et al., 1998; Zenke et al., 1999; Zhao et al., 1998) suggest GTPase binding causes a major change in the conformation of the kinase inhibitory (KI) domain that disrupts its interaction with the catalytic domain, allowing auto-phosphorylation required for full kinase activity (Lei et al., 2000). A single phosphorylated residue in the catalytic domain (equivalent to PAK1 Thr423) comprising the activation loop is important both for maintaining relief from autoinhibition and for full catalytic function toward exogenous substrates (Gatti et al., 1999; Yu et al., 1998; Zenke et al., 1999): modification by the 3-phosphoinositide-dependent kinase PDK1 is also suggested (King et al., 2000). Autophosphorylation of PAK at Ser144 (a conserved residue in the KI) contributes to kinase activation and/or maintenance of kinase activity, but not other conserved autophosphorylation sites (Chong et al., 2001). Interestingly an alternate spliced version termed PAK3b has been identified (also represented by a number of ESTs), which has an insertion in the GBD/KI sequence (Fig. 2) allowing the kinase to be active in the absence of GTPase binding (Rousseau et al., 2003). For the Group I PAKs binding to activated GTPases results in robust kinase activitation (10-100 fold), however Cdc42.GTP binding to PAK4 does not stimulate kinase activity (Abo et al., 1998). Similarly, although PAK 5 and 6 bind to both Cdc42 or Rac, their activities are not enhanced (Pandey et al., 2002). These results indicates that the two subgroups of PAKs are differently regulated, and more specifically that group II kinases may have dispensed with the inhibitory switch mechanism of kinase regulation.

The crystal structure of PAK1 in an autoinhibited conformation has been determined to 2.3-Å resolution (Lei et al., 2000). The KI region packs against and inhibits the catalytic domain with an Ki of ~90 nM (Zhao et al., 1998). PAK1 residues Leu107, Glu116, and Asp126 critically contribute to this inhibitory interface (Lei et al., 2000) and substitution of these residues can generate constitively active forms of the kinase. The kinase inhibitory domain can inhibit PAK activation both in vitro and in vivo, but this polypeptide has no effect once the active (autophosphorylated) form of the kinase is generated (Zhao et al., 1998). The binding constants of Cdc42 to peptides encompassing PAK1 p21 binding domain (PBD) are in the range 10-50 nM (Thompson et al., 1998). Such polypeptides are useful affinity reagent for assaying the nucleotide status of Rac and Cdc42 in cell lysates (Benard et al., 1999) and for FRET experiments (Graham et al., 2001).

Unlike group I PAKs which contain an activation loop phosphothreonine (cf PAK1 T422), the non-conventional PAKs have a serine at the corresponding position. Substitution of this Ser474 to Glu is not sufficient to render PAK4 constitutively active (Qu et al., 2001), although it is a predicted autophosphorylation site required for activity (Abo et al., 1998). Others report some increased activity with the PAK4 S474E substitution (Callow et al., 2002). Thus as with PAK1, such acidic substitution probably performs poorly as a phospho-mimetic (Manser et al., 1997). Since truncated versions of both PAK4 (Abo et al., 1998) and PAK6 (Yang et al., 2001), containing only the catalytic domains, have greater activity than the full-length proteins there is probably some form of intramolecular inhibition at play, but the physiological activators of type II PAKs may yet be undiscovered. Ser/thr kinases, including group I PAKs, have a conserved Asn, which stabilizes the catalytic loop by hydrogen bonding with a conserved aspartic acid. The type II PAKs have a Ser at this site (S445 in Pak4) and substitution to Asn results in constitutive activation of both PAK4 (Qu et al., 2001), PAK5 (Dan et al., 2002) and PAK6 (Schrantz et al., 2004).

While PAKs are considered primarily as Rac and Cdc42 GTPase effectors, GTPase-independent activation mechanisms are known. Indeed PAKs were first studied as kinases whose autophosphorylation and activity could be stimulated by limited protease digestion (Benner et al., 1995; Roig and Traugh, 2001). A physiological correlate came with the discovery of PAK2 as a substrate for caspases during the apoptosis (Rudel and Bokoch, 1997). PAK2 is cleaved by caspase 3 at Asp212, generating a catalytically activated kinase, although binding of Rac or Cdc42 may still be necessary for dissociation of the cleaved N-terminal fragment from the catalytic domain (Buchwald et al., 2001; Zenke et al., 1999). Membrane recruitment of PAK1 via SH3 containing Nck and Grb2 adapter proteins, which is mimicked by the addition of membrane-targeting sequences, results in the stimulation of kinase activity (Daniels et al., 1998; Lu et al., 1997). This might involve the phosphorylation at the critical Thr423 residue by PDK1 (King et al., 2000) or interaction with lipids such as sphingosine which can activate the kinase in a GTPase-independent manner (Bokoch et al., 1998). Activation occurs to levels similar to that induced by GTPases (Zenke et al., 1999). GIT1, which indirectly associates with PAK via PIX, can also potently activate PAKs through a Rho GTPase independent mechanism (Loo et al., 2004). This might explain why the abnormal accumulation of PAK into large focal adhesion-like structures (via GIT1) in some cell lines is associated with increased kinase activity (Stofega et al., 2004).

The importance of serine/threonine phosphorylation in PAK activation indicates dephosphorylation reactions are equally important. Two closely related human protein phosphatases have been identified that efficiently

dephosphorylate PAK1 including Thr423 (Koh et al., 2002). These phosphatases, termed POPX1 and POPX2 (for partner of PIX), bind to various forms of PIX and form multimeric cellular complexes containing PAK. These PP2C-related phosphatases are either ubiquitously expressed (POPX2) or enriched in brain and testis (POPX1) as for PAK2 and PAK1 respectively. Overexpression of either enzyme antagonizes the cellular effects of active PAK1(Koh et al., 2002). The presence of such a negative regulator in complex with the kinase explains the rapid activation/inactivation cycle of PAKs (Zhan et al., 2003).

Other protein kinases have been reported to phosphorylate PAK and regulate its function: Akt phosphorylates PAK1 at Ser21, and this modification both decreases binding of Nck to the PAK1 N-terminus and stimulates PAK1 activity in a GTPase-independent manner in vivo (Tang et al., 2000; Zhao et al., 2000a). The p35-bound form of Cdk5, a neuron-specific protein kinase, associates with and phosphorylates PAK1 at Thr212, a site also targeted by cyclin B-bound Cdc2 in a cell-cycle-dependent manner (Banerjee et al., 2002; Nikolic et al., 1998; Thiel et al., 2002): the role of this modification is not understood.

4. PROTEIN PARTNERS AND SUBSTRATES OF PAKS

4.1 The PAK-interacting exchange factor PIX

PAKs associate with the focal adhesion-associated protein PIX (also referred to as Cool) which is a Rho guanine nucleotide exchange factor (GEF). Multiple PIX proteins, derived from 2 different genes (termed αPIX and βPIX), bind to a nonconventional SH3-binding motif in PAKs illustrated in Figure 2 (Bagrodia et al., 1998; Manser et al., 1998). The interaction of PIX with PAK1 might be expected to lead to kinase activation resulting from local activation by Rac.GTP or Cdc42.GTP generated by PIX (Daniels et al., 1999; Feng et al., 2002) however such activation is not as robust as for other GEFs such as Dbl (Manser et al., 1998). Analysis of PIXα deficient cells have suggested a key role for the PIX/PAK complex in Cdc42 mediated direction sensing of chemotactic leukocytes (Li et al., 2003). PIX is tightly associated with a 90kDa protein GIT1, also termed PKL (for paxillin kinase linker (Turner et al., 1999)) or CAT1 (Bagrodia et al., 1999). The GIT and PIX proteins are phosphorylated by PAK when immunoprecipitated complexes are incubated *in vitro* with [^{32}P]ATP (Manser et al., 1998),

although PIX modification does not affect any known function (Koh et al., 2001). GIT family proteins contain a paxillin binding motif that allows the protein complex to target focal adhesions(Turner et al., 1999): they also binds to and are phosphorylated by FAK (Manser et al., 1998). Over-expression of GIT1 can induce the disassembly of focal adhesions accompanied by loss of paxillin which might be an indirect effect resulting from PAK activation by GIT1 (Loo et al., 2004). To date the PAK/PIX/GIT complex is known to distributes between focal adhesions, the leading edge of motile cells, and cell-cell junctions (Manabe Ri et al., 2002; Zegers et al., 2003; Zhao et al., 2000b) and therefore play a key role in localizing the kinase.

Drosophila dPAK has been genetically linked to the signal transduction pathway from axonal guidance receptors to the actin cytoskeleton in photoreceptor (R cell) growth cones (Hing et al., 1999). The associated dPIX plays a key role in the proper assembly of synaptic junctions in the fly (Parnas et al., 2001): GIT1 in turn is shown to be required for assembly of mammalian synaptic junctions GIT1. PAK's role in neuronal guidance in humans may be reflected in a disease, nonsyndromic X-linked mental retardation, which is caused by point mutations in PAK3, an isoform particularly abundant in the brain (Allen et al., 1998).

4.2 The regulation of actin by PAKs

The phosphorylation of the regulatory myosin light chain (R-MLC) at Ser19 (and Thr18) by myosin light chain kinase (MLCK) has been shown to be a critical regulatory step for physiological modulation of myosin contractility. PAK1 and/or PAK2 are able to directly phosphorylate R-MLC at the critical Ser19 site, with a resulting increase in contractility in both intact myosin and isolated R-MLC (Ramos et al., 1997; Zeng et al., 2000). However PAK1 also modulates R-MLC function in the opposite direction via inhibition of MLCK activity (Sanders et al., 1999). Phosphorylation of MLCK occurs at Ser439 and Ser991: the binding of calmodulin to MLCK is inhibited by Ser991 modification (Goeckeler et al., 2000). Because of the substantial interplay between regulation by PAK and other physiological regulators of MLCK the effect of PAK1-3 is likely to be context dependent.

LIM kinases-1 and -2 are important players in the regulation of actin cytoskeletal dynamics through their ability to specifically phosphorylate members of the cofilin/actin depolymerizing factor (ADF) family. Cofilin/ADF proteins are phosphorylated solely at Ser3 by LIM-kinases and are the only known substrates for this kinase family. When phosphorylated at Ser3, cofilin/ADF can no longer bind effectively to F-actin, and the ability of these proteins to catalyze both F-actin depolymerization and severing is

thus inhibited (as reviewed (Stanyon and Bernard, 1999)). PAK1 has been shown to be able to regulate Ser508 within the LIM-kinase 1 activation loop (Edwards et al., 1999) although other basic-directed kinases such as ROK and MRCK have been similarly identified (see relevant sections). PAK activity has also been linked to tumor suppression via regulatory effects on the action of the neurofibromatosis type 2 (NF2) gene product, Merlin (Kissil et al., 2002; Xiao et al., 2002). Merlin belongs to the ERM (ezrin, radixin, moesin) family of cell membrane-cytoskeletal linker proteins and affected by Rac1 and Cdc42 through phosphorylation at Ser518 mediated by PAK2 (Kissil et al., 2002; Xiao et al., 2002). This site is recently shown to be phosphorylated also by PKA: *in vitro* and *in vivo* interaction studies indicated that phosphorylation of serine 518 promotes heterodimerization between merlin and ezrin, perhaps to convert merlin from a growth-suppressive to a growth-permissive state (Alfthan et al., 2004).

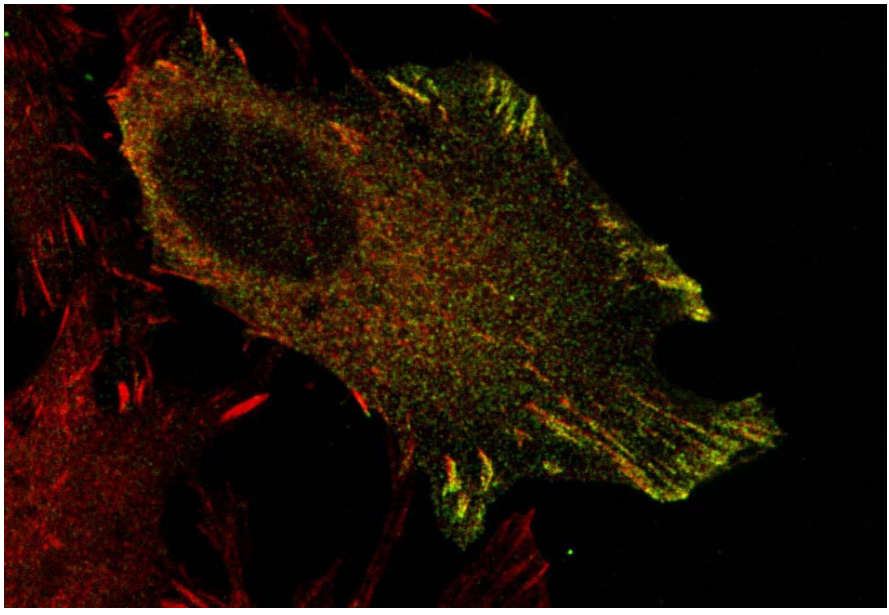


Figure 4. PAK colocalizes with focal adhesion protein paxillin at the leading edge of migrating cells. NIH3T3 cells were transiently transfected with GFP-αPAK and were subjected to a wound healing assay. PAK and paxillin at the leading edge (but not the rear of the cell) were shown by immunofluorescent staining with anti-GFP (in green) and anti-paxillin (in red).

Mammalian PAK1 redistributes from the cytosol into cortical actin structures under a variety of conditions. In monolayer scratch experiments PAK1 localized lamellae at the leading edge of polarized cells but staining

of adhesions complexes at the leading edge of the cell is particularly evident (Figure 4). Such localization to focal adhesions via its partners PIX and GIT1 (Bagrodia et al., 1998; Manser et al., 1998; Zhao et al., 2000b). Inhibition of PAK in PC12 cells leads to stabilization of focal adhesion complexes (Obermeier et al., 1998) which are not usually seen in these cells. Conversely kinase-active versions of PAK1 causes loss of actin stress fibers and increased focal adhesion turnover (Manser et al., 1997; Sells et al., 1997). Cells expressing active PAK1 accumulate large, polarized membrane ruffles (Sells et al., 1997), however the underlying mechanism is not resolved.

Cdc42 is involved in the translocation of PAK4 to the Golgi apparatus rather than in its location to the actin-rich cell periphery (Abo et al., 1998). Nonetheless PAK4 can induce filopodia formation when over-expressed the direct target for this action is not known. Deficiency of the PAK4-like mbt gene product in *Drosophila* results in severe defects in central brain structure (Melzig et al., 1998), which might result from failure of axonal outgrowth. Expression of activated PAK4 results in a decrease in stress fibers and focal adhesions: and this activity may relate to the ability of PAK4 to bind to and phosphorylate LIMK1 (Dan et al., 2001a) as for the group I PAKs. An interaction with PDZ RhoGEF has been reported leading to down regulation of RhoA, which could contribute to these effects (Barac et al., 2004). A number of other PAK1 targets have been investigated with PAK4 and PAK5: for example both can phosphorylate Bad at a critical site, Ser112 thereby blocking its function (Gnesutta et al., 2001). PAK5 is reported to target mitochondria, although the localizing sequences have yet to be defined (Cotteret et al., 2003).

Several PAK homologs have been identified in *Dictyostelium discoideum*, and PAKa colocalizes with myosin II to the cleavage furrow of dividing cells and to the posterior of polarized cells undergoing chemotaxis (Chung and Firtel, 1999). Genetic manipulation of PAKa disrupts both cytokinesis and the ability of the cells to maintain directionality during chemotaxis. It is suggested PAK functions in suppressing lateral pseudopod formation and in proper tail retraction during chemotaxis

4.3 Regulation of MAP kinase cascades

Ste20p was identified as a key kinase in the mating pathway in the haploid budding yeast *Saccharomyces cerevisiae*. This pathway is initiated by the binding of a peptide pheromone to a pheromone receptor, which activates a heterotrimeric G protein and leads to the activation of the yeast MAPK pathway consisting of Ste11p (MAP3K), Ste7p (MAP2K) and Fus3p/Kss1p (MAPKs) (Dan et al., 2001b). The direct phosphorylation of Ste11p by

Ste20p has recently been demonstrated to be a crucial step in the signalling pathway, showing that Ste20p can act as an MAP4K (Drogen et al., 2000). Ste20p phosphorylates three residues on Ste11p which disrupts an intramolecular interaction between the Ste11p N-terminal regulatory domain and its C-terminal catalytic domain, thus activating Ste11p (Drogen et al., 2000). In mammalian systems PAK1 can phosphorylate MEKK1 on Ser67, thereby inhibiting the binding (and activation) of JNK by MEKK1 (Gallagher et al., 2002). In both *S. cerevisiae* and *S. pombe,* roles for the PAKs in actin and septin cytoskeletal assembly, cytokinesis, and polarized growth via budding have been identified (Cvrckova et al., 1995; Goehring et al., 2003; Holly and Blumer, 1999; Sawin et al., 1999; Versele and Thorner, 2004; Weiss et al., 2000) via phosphorylation of a variety of targets including Cdc24p and Bni1p. Hyphal formation during filamentous growth of *S. cerevisiae* and *Candida albicans* requires PAK function that also involves MAP kinase cascades (Leberer et al., 1997).

Some synergy between Rho and Ras in activating ERK has been ascribed to PAK (Frost et al., 1996; Tang et al., 1999). This requirement might relate to PAK1 phosphorylation of MEK1 on Ser298, a site important for the binding of the upstream regulatory kinase, Raf-1 (Frost et al., 1997). PAK2 has been identified as a positive regulator of Raf-1 activity through phosphorylation of Ser338, an essential regulatory site for Ras-dependent Raf-1 activation (King et al., 1998). Phosphorylation at Ser338 site by PAK1-3 (and potential interaction of the kinases) is suggested to be critical for Raf-1 activation and ERK stimulation by a variety of growth factors and integrins (Chaudhary et al., 2000; Zang et al., 2002), although its significance remains controversial (Chiloeches et al., 2001). Thus the blockade of PAK activity has been suggested as a potential means to treat Ras-induced cancers (Nheu et al., 2002). Unlike group I PAKs it appears that over-expression of PAK4 can drive anchorage-independent growth and transformation (Qu et al., 2001). Although it is likely that PAK4 has a physiologically relevant role in oncogenic transformation, for example via oncogenic Ras (Callow et al., 2002), the mechanism underlying this needs be addressed. Interestingly one study reports expression of PAK4 was elevated in a panel of tumour cell lines (Callow et al., 2002)..

4.4 Other roles for PAKs

Introduction of Xenopus PAK into oocytes blocks progesterone-induced maturation, perhaps linked to mitotic spindle pole migration (Faure et al., 1997; Faure et al., 1999). Similarly the *S. pombe* PAK Shk1, is implicated in orientation of polarity during cell fission (Sawin et al., 1999). Shk1 is localized to interphase microtubules and to mitotic spindles. PAK is

suggested to alter dynamics of microtubules by phosphorylation of stathmin/Op18 at Ser16 (Daub et al., 2001) which suggests a role in mitotic spindle assembly. PAK acts on stathmin in a Rac/Cdc42-dependent manner and directly inhibits the microtubule destabilizing function of Op18, both *in vitro* and *in vivo* (Daub et al., 2001). PAK is associated with stabilization of microtubules at the leading edge of cells which can promote actin polymerization and lamellipod formation (Wittmann et al., 2003). Conversely, the activation of Rho was recently shown to be mediated, at least in part, via release of the microtubule-inhibited Rho-specific guanine nucleotide exchange factor, GEF-H1 which is a target of PAK1 (Krendel et al., 2002).

PAK2 cleavage and activation by caspases is a relatively late event in apoptosis (Walter et al., 1998) it takes place well after cells have committed to the apoptotic program in response to proapoptotic signals. PAK1 by contrast can promote cell survival by phosphorylation of the death-promoting Bcl-2 family member, Bad, on Ser112 and 136 (Jakobi et al., 2001; Schurmann et al., 2000). This forces Bad to associate with the cytosolic adapter protein, 14-3-3, freeing it from Bcl-2 and Bcl-xL. In the absence of survival factors, Bad is not phosphorylated and interacts with the Bcl-2 family members (Bcl-2 or Bcl-xL) to inhibit their anti-apoptotic activities. In a different context PAK1-mediated activation by the HIV Nef protein appears to involve the activity of PI-3-kinase (Linnemann et al., 2002) which in combination may protect infected cells from apoptosis

5. CONCLUSIONS

In the last 10 years we have accumulated a tremendous amount of information about PAK kinases in a variety of organisms. Nonetheless the molecular details underlying PAK activation by Rho GTPases could be still improved if the structure for a complete kinase molecule were available. Mechanisms leading to PAK activation by GTP-independent routes need to be unravelled and their physiological significance assessed. PAKs might well play a nuclear role since phospho-PAK can be seen to accumulate in the nucleus of cells following centrosome duplication (unpublished data). The binding of PAK6 to AR demonstrates an interaction between a PAK family member and a hormone receptor which directly modulate transcription (Yang et al., 2001). In the presence of both AR and ligand, expressed Pak6 is recruited from the cytoplasm to the nucleus and represses AR-mediated transcription. Finally the genetics of PAK in lower organisms has recently been complemented by similar knock out studies in mice [for review see

(Hofmann et al., 2004)]. In combination with 'knock-in' techniques the specific interactions described in this chapter can be assessed more critically.

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Chapter 11

GENETIC ANALYSIS OF RHO PROTEIN FUNCTION IN MICE

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Abstract: The Rho small GTPases regulate a variety of cellular functions, including proliferation, differentiation, vesicle trafficking, gene expression, adhesion, and motility. Among their well established essential roles is that of organizing the actin cytoskeleton, and this aspect of Rho GTPase function has led to the identification of important roles for various Rho family GTPases in a variety of actin-dependent cellular processes, including cell shape change, adhesion, and migration. As critical regulators of these actin-mediated processes, the Rho GTPases also function as essential regulators of many developmental processes. The morphogenesis of tissues in all developing multi-cellular organisms requires precise changes in cell shape and cell movements that depend on the various Rho GTPases. This has been observed in studies using sevefigureral different developmental model systems, including flies, worms, frogs, and mice. Here, we review the literature reporting functions for Rho GTPases and their associated signaling components in the context of embryonic development, adult physiology, and pathogenesis. In particular, the focus of the studies described here is on the mouse system, where transgenic and "knockout" strategies have played an especially important role in elucidating the in vivo organization and function of the various Rho signaling pathways.

1. INTRODUCTION

The Rho family of small GTPases appears to mediate nearly all of the major cellular functions. These include proliferation, differentiation, vesicle trafficking, gene expression, adhesion, and motility (Etienne-Manneville and Hall, 2002). Among their well established essential roles is that of organizing the actin cytoskeleton, and this aspect of Rho GTPase function has led to the identification of important roles for various Rho family

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GTPases in a variety of actin-dependent cellular processes, including cell shape change, adhesion, and migration (Nobes and Hall, 1999). As critical regulators of these actin-mediated processes, it was not surprising to find that the Rho GTPases are essential regulators of many developmental processes as well. The morphogenesis of tissues in all developing multicellular organisms requires very precise changes in cell shape and cell movements that are undoubtedly dependent on the various Rho GTPases. This has indeed been confirmed in studies using several different developmental model systems, including flies, worms, frogs, and mice (Settleman, 1999). In other chapters in this volume, the signaling role of Rho GTPases is addressed largely in the context of cell culture systems, where much has been learned about the biochemical organization of Rho-mediated signaling pathways. In this chapter, we review the literature reporting functions for Rho GTPases and their associated signaling components in the context of embryonic development, adult physiology, and pathogenesis. In particular, the focus of the studies described here is on the mouse system, where transgenic and "knockout" strategies have played an especially important role in elucidating the in vivo organization and function of the various Rho signaling pathways.

2. REGULATORS AND TARGETS OF THE RHO GTPASES

As detailed in Chapter 3, the cycling of Rho GTPases, like that of other small GTPases, is largely controlled by three classes of regulatory proteins. These are the GAPs, the GEFs, and the GDIs. We will summarize studies of each of these various GTPase regulators in the context of mice. In addition to the regulators of the GTPases, the downstream targets of the GTPases, or effector proteins, play an essential role in mediating the actions of activated Rho proteins that ultimately elicit a biological response. The established signaling roles for these proteins, which typically interact with the GTP-bound, activated form of the GTPases, are also described in other chapters of this volume, and in this chapter, we will summarize findings from mouse studies that shed light on the role of these various Rho effectors in the context of the whole organism. Although a variety of other proteins appear to contribute less directly to the regulation and function of the Rho GTPases, in the interest of space, these will not be considered here.

2.1 Model organism systems for studying Rho GTPase function

Cell culture and biochemical studies have revealed much of what we now understand regarding the regulation and function of the various Rho-mediated signaling pathways. For example, the early micro-injection studies by Ridley and Hall provided important information regarding the role of the Rho family proteins as regulators of the actin cytoskeleton in cultured fibroblasts (Ridley and Hall, 1992a; Ridley and Hall, 1992b; Ridley et al., 1992). Additional biochemistry-oriented studies led to the identification of several direct binding partners for the activated Rho family GTPases, including p21-activated kinases (PAKs), Rho-kinases (ROCK), and the PKN family proteins (Burridge and Wennerberg, 2004). Thus, the power of these approaches has been clearly demonstrated. However, it can be argued that it is a considerably more difficult challenge to establish the regulatory mechanisms and functional requirements for the various Rho pathways in the context of whole organisms. These days, several experimental model systems, including Drosophila melanogaster, Xenopus laevis, C. elegans, and mice, are routinely used for such analysis. Each model provides a distinct set of advantages, and all share the property that their genomes encode several highly conserved Rho family GTPases as well as their regulators and targets. Thus, it should be possible to readily extrapolate findings from these relatively simple organisms to humans. Moreover, as complete annotated genome sequences are becoming available for each of these organisms, the power of a genetic approach to address the function of individual proteins in whole organisms has become even greater. We refer to previous review articles that have summarized the analysis of Rho GTPase function in several of these systems (Settleman, 2001; Settleman and Barrett, 2001), and here, we will focus exclusively on the use of the mouse system

2.1.1 Mouse models of Rho GTPase regulation and function

As a model system for studying the developmental function of particular genes, the mouse is particularly attractive because of its high degree of evolutionary relatedness to humans. The major aspects of embryogenesis, including fertilization, blastocyst development, embryo implantation, gastrulation, and organogenesis are remarkably similar in mice and humans. Moreover, all of the Rho family GTPases and their protein regulators and targets that have been identified in humans appear to have highly related orthologs in mice. Thus, while the use of classical genetic analysis is substantially more laborious in the mouse when compared to fruit flies and worms, the mouse provides a powerful model system in which the

developmental function of Rho-mediated signaling pathways can be examined.

Two methodologies account for the vast majority of the in vivo analysis of Rho signaling in the mouse. These are the introduction of transgenes under the control of tissue-restricted or inducible promoters, and the use of gene targeting in embryonic stem cells to disrupt particular genes of interest. As described below, the application of these methodologies to the analysis of Rho regulation and function in development has yielded a wealth of information regarding the organization of Rho pathways in vivo and their role in tissue morphogenesis during development. Moreover, mouse models are beginning to provide additional insights into the post-developmental role of Rho GTPase signaling in the physiology of the adult animal and, in some cases, in pathogenesis.

2.2 Expression studies of Rho GTPase pathway components in mice

When considering the roles for Rho GTPase signaling components in the context of a developing organism, it can be useful to determine the expression profile of a gene of interest in embryos. This is typically done using in situ hybridization of mRNA in intact tissues (whole-mounts) or tissue sections prepared on slides. It is reasonable to assume that detectable expression of a gene in a particular tissue correlates, to a first approximation, with a likely function for the gene product in that tissue. Of course, mRNA levels do not necessarily accurately predict protein expression levels. Moreover, levels of expression of a particular gene that do not exceed the threshold for detection may still be significant. Finally, a gene product does not necessarily play a significant role in a tissue where it is expressed, and redundant function provided by closely related family members is a considerable issue as well. Thus, expression analysis can loosely direct the focus of attention to a particular tissue type or a particular developmental stage, but this will rarely yield any definitive conclusions. For some pathway components, antibodies may be available that can be used for immunostaining of tissues directly. In mice, the analysis of embryonic expression of Rho pathway components has been rather limited, and overall, and has only provided some hints about developmental functions for Rho family GTPases and their numerous associated regulators and targets.

There are at least 20 GTPases that are classified as Rho family GTPases on the basis of primary structure relatedness (see Chapter 2). Of these, he expression of only a few has been examined during mouse evelopment. The expression of the RhoA GTPase in developing embryos has only been reported in the context of kidney development, where

immunohistochemistry revealed that RhoA was largely restricted to the mesonephric ducts and vesicles, and the periglomerular tubules, which exhibit intense staining (Bianchi et al., 2003). This finding suggest a potential role for RhoA in the epithelial-mesenchymal induction process required for kidney morphogenesis, and is consistent with additional studies that support a role for Rho GTPases in the epithelial-mesenchymal transition (Zondag et al., 2000). RhoB gene expression in mouse embryos has been examined in great detail, and it appears to be expressed widely throughout development (Henderson et al., 2000). Notably, as described below, RhoB knockout mice develop normally, highlighting the limited utility of expression analysis in predicting the developmental requirement for a gene of interest. Expression analysis of Rac1 revealed prominent widespread expression in very early embryos, a finding which, as described below, is consistent with its apparent role in early gastrulation (Moll et al., 1991). Cdc42 expression during development has not been characterized in detail, although the mRNA has been detected at very early stages of embryogenesis (Salas-Vidal and Lomeli, 2004), and as described later, this is consistent with a very early developmental requirement for Cdc42. The Rac-like RhoG gene has been examined in the developing brain. RhoG, which had been implicated in neurite outgrowth in cell culture studies (Katoh et al., 2000), is expressed throughout the ventricular zone in late neurogenesis, suggesting a role in newly generated neurons (Ishikawa et al., 2002). Postnatally, RhoG mRNA expression is enriched in white matter tracts of the corpus callosum, anterior commissure, and cerebellum, and this was determined to represent expression in oligodendrocytes (Ishikawa et al., 2002). Thus, RhoG may play a role in both glial and neuronal cells of the central nervous system.

The expression of relatively few of the numerous GTPase regulators has been examined in developing mice. One of these is FGD1, a GEF that specifically activates the Cdc42 protein, and which has been identified as the primary gene defect associated with faciogenital dysplasis, or Aarskog Syndrome (Gorski et al., 2000; Olson et al., 1996; Pasteris and Gorski, 1999). Notably, this congenital syndrome in humans is associated with defects in skeletal development, suggesting a likely function in developing bone. The mouse FGD1 ortholog was found to be first expressed during the onset of ossification, and is expressed in areas of active long bone formation (Gorski et al., 2000). Expression is also seen in osteoblasts at the onset of matrix mineralization, suggesting a role in differentiation of bone. Postnatally, FGD1 exhibits a broader expression pattern in skeletal tissues, suggesting that it regulates distinct Cdc42-mediated processes in bone development and in the mature skeleton. A more comprehensive analysis of the expression profiles of several GEFs for the Rho GTPases during mouse brain development has been reported, which indicates a complex expression

profile for this class of GTPase activators within the developing brain, and indicates the likelihood that particular GEFs may play unique functions in a subset of neural tissues (Yoshizawa et al., 2003).

Expression of another RhoGEF, LARG (leukemia-associated Rho GEF), has been examined in some detail in the context of developing mice (Kuner et al., 2002). LARG is first detected at embryonic day 14, and expression is seen in skin, intestinal epithelium, and smooth muscle layers of the bronchi, vasculature, and intestine. This specific pattern of LARG expression is maintained through development and persists in the adult animal, indicating a potential role for LARG both in the development and physiological function of these tissues. Interestingly, LARG was first identified as a translocation partner in a human myeloid leukemia, suggesting that the leukemic cells have usurped LARG to activate Rho inappropriately. LARG has previously been found to interact with the IGF-1 receptor, indicating a likely role in mediating the activation of Rho by IGF-1. In the expression study, LARG was found to co-localize with the IGF-1 receptor, indicating that LARG may specifically mediate the ability of IGF-1 to activate Rho in a subset of tissues that require IGF-1 for their proper development.

The RhoGAP, p190-A, is expressed predominantly in the developing central nervous system, and as detailed in a later section, mice lacking p190-A RhoGAP exhibit substantial defects in several aspects of neural development (Brouns et al., 2001).

Among the various Rho effector targets, relatively little detailed developmental expression analysis has been reported. The ROCKs exhibit prominent expression in developing cardiac tissues, which, as described below, is consistent with an apparent role for ROCKs in cardiac development (Nakagawa et al., 1996). Others have been detected in developing tissues at various embryonic stages using RT-PCR methodology, and we refer to an online database that provides substantial information regarding the developmental expression profile for several additional genes that encode Rho pathway components (web site adress: tbase.jax.org). Many more gene expression studies that have relied largely on northern blotting and western blotting of adult tissues have revealed tissue-restricted expression profiles for various regulators and targets of Rho GTPases. Collectively, these studies have not shed much light on likely developmental roles for individual pathway components, but rather, have reinforced the notion that while the GTPases themselves seem to be relatively broadly expressed, their regulators exhibit somewhat more restricted tissue expression patterns. This potentially provides a mechanism by which extracellular signals can be linked to the Rho GTPases via distinct sets of regulatory proteins depending on the tissue context. One notable exception to this is the Rac2 protein, which is largely restricted in its expression to

hematopoietic cells (Yu et al., 2001), and as described below, is specifically required in blood cells.

2.3 Rho GTPases in fertilization, preimplantaion, and early morphogenesis

Mammalian development is initiated with the fertilization of the egg by sperm, a process that has previously been shown to depend on actin polymerization and cytoskeletal rearrangement (Webster and McGaughey, 1990). Although a genetic analysis of this process in mice is technically challenging, the role of Rho GTPases in sperm-egg interaction has been examined using one of the Rho-inactivating toxins, Clostridium difficile toxin B (Kumakiri et al., 2003). Toxin B inhibits several of the Rho family GTPases, and treatment of eggs with toxin B results in a substantial inhibition of sperm fusion or sperm nucleus decondensation in the ooplasm. Rac1 and RhoB, but not Cdc42, are detected in ooplasm, suggesting that these GTPases may mediate the actin rearrangements required for fertilization.

Following fertilization, the mouse egg undergoes several cell divisions during its 4-5 day migration to the uterus. There, the multi-cellular blastocyst breaks free of its protective membrane, the zona pellucida, and implants itself within the uterine wall. The Rho GTPases have been implicated in the preimplantation mouse embryo. The ability to maintain preimplantation embryos in culture for several days facilitates the analysis of this early stage of development. Expression analysis revealed that Rac and Cdc42 genes are expressed in the preimplantation embryo, and immunostaining revealed that Rac1 protein translocates from plasma membrane to cytoplasm as this early embryo undergoes cleavage at the blastocyst stage (Natale and Watson, 2002). Possibly, these GTPases are required to mediate the turnover of adherens junctions, which are known to play an important role in the cell-cell adhesions that mediate blastocyst formation (Ohsugi et al., 1997).

Use of the Rho-inactivating Clostridium botulinum C3 toxin has also pointed to a role for Rho in preimplantation development (Clayton et al., 1999). Injection of the 4-cell blastomere with C3 disrupts cortical actin microfilament organization and prevents the requisite polarization of the 8-cell blastomere. This polarization process, referred to as compaction, requires both actin and microtubule reorganization, both of which are Rho-mediated. Thus, the ability of Rho to regulate the cytoskeleton, appears to be required for blastomere compaction. Notably, early cell divisions proceed normally in the presence of C3.

Mice containing a targeted disruption of the Cdc42 gene have been reported (Chen et al., 2000). Although Cdc42-deficient embryonic stem cells proliferate normally, homozygous mutant embryos fail some time before embryonic day E7.5, and exhibit a disorganized primary ectoderm and signs of degeneration by E5.5. In vitro analysis indicated that the Cdc42-deficient cells fail to support phosphatidylinositol 4,5-bisphosphate-induced actin assembly, suggesting a role for Cdc42 in regulating the actin cytoskeleton during preimplantation development. It is important to note that in such genetic studies of early embryo development, it is possible that some early phenotypes may be masked in homozygous mutant animals due to the contribution of maternal gene products. Thus, a disrupted gene may encode a protein that is required prior to the first signs of a defect.

Disruption of the Rac1 gene in mice also results in relatively early lethality (Sugihara et al., 1998). Mutant embryos collected between E6.5 and E8.5 exhibit clear defects in the process of gastulation. Gastrulation is the first major morphogenetic process in the implanted developing embryo, and ultimately gives rise to the three germ layers. This process is known to require precise cell shape changes, and regulated cell adhesion and cell migration. Thus, it was not surprising to find a role for Rho GTPases in gastrulation. In the absence of Rac1, gastrulating tissue appears misfolded, and numerous apoptotic cells are detected in early embryonic tissues. Rac1 gene expression is widely detected in E7.5 embryos, but the precise role of Rac1 in gastrulating tissues remains to be determined. Moreover, the identity of Rho GTPase regulators or effector targets that are required for gastrulation in early eambryos is unknown. Significantly, in the Drosophila system, a specific RhoGEF has been identified that regulates Rho-mediated cell shapes required for gastrulation (Barrett et al., 1997), and this GEF is regulated by heterotrimeric G-proteins (Hacker and Perrimon, 1998). In mammals, three related RhoGEFS, PDZ-RhoGEF, LARG, and p115 RhoGEF appear to function similarly in their ability to couple G-protein coupled receptor activation to Rho activation (Chikumi et al., 2004). Thus, it is possible that these RhoGEFs perform an evolutionarily conserved function in directing Rho-mediated cell shape changes in mammalian gastrulation.

2.4 Germ cell development

Expression studies have pointed to a likely role for Rho signaling in the development of male germ cells. For example, RhoB immunostaining indicated its expression in elongating spermatids, spermatocytes, and Sertoli cells (Lui et al., 2003). In addition, the RacGAP, MgcRacGAP, is known to be highly enriched in male germ cells, pointing to a likely specialized function for Rac in germ cell development (Naud et al., 2003). Several

mouse knockout studies have confirmed a role for Rho signaling in spermatogenesis. Specific disruption of the Rho-binding kinase, Citron, results in profound testicular impairment and a complete absence of mature spermatocytes. Cells exhibit a severe cytokinesis defect, and excessive apoptosis is detected. This defect appears to reflect the previously identified role for Citron in Rho-directed cytokinesis (Cunto et al., 2002). Notably, germ cell development in Citron-deficient females proceeds normally. Mice lacking Lim-kinase-2, one of two closely related Lim-kinases that have been implicated in linking several of the Rho family GTPases to the actin cytoseleton, are viable but exhibit a testes defect associated with partial degeneration of spermatogenic cells in the seminiferous tubules and increased apoptosis (Takahashi et al., 2002).

3. RHO GTPASES IN ORGANOGENESIS

3.1 The nervous system

A combination of cell culture and gene expression studies have strongly implicated the Rho family GTPases and their regulators and targets in many aspects of neural development and function. Again, this should not be surprising when considering that the processes of neuronal migration, neurite outgrowth and guidance, and synapse formation all require actin reorganization. Moreover, glial cells are also migratory and are likely to require Rho signaling for their proper development and function. Several studies have been conducted in which transgenic mice have been established that express gain- and loss-of-function forms of the various Rho GTPases specifically in neural cell types. In one study that utilized a promoter system that gives rise to specific gene expression in the glial cells of the peripheral nervous system, it was observed that constitutively active Rho prevents peripheral glial migration, associated with stalling, defective process extension, and defective axonal ensheathment (Sepp and Auld, 2003). The transgenic animals die embryonically, suggesting that RhoA is an important mediator of normal glial development. Expression of either dominant-negative or activated Rac1 also produced defects in glial migration and axon ensheathment (Sepp and Auld, 2003). In addition, these transgenes produced fasciculation defects in sensory axons, suggesting that RhoA and Rac1 play distinct roles in peripheral glia. Axon pathfinding was not obviously affected by any of these transgenes, indicating that axon guidance in the periphery is largely resistant to disruption of normal glial function. Significantly,

analogous studies of Cdc42 mutants revealed no apparent role for Cdc42 in glial development (Sepp and Auld, 2003).

In another transgene study, an activated form of Rac1 was expressed specifically in developing Purkinje cells of the developing cerebellum (Luo et al., 1996).The resulting mice were ataxic and their Purkinje cells exhibited a substantial reduction in axon terminals, and a reduction in size, but an increase in number, of dendritic spines. This finding suggests that Rac1 plays distinct roles in regulating neurite outgrowth in the context of axons and dendrites. In related studies using rat hippocampal slices, it was determined that expression of dominant-negative Rac1 causes elimination of dendritic spines, whereas an activated form of RhoA reduces the branching complexity through a ROCK-mediated process (Nakayama et al., 2000).Together, these results point to important and distinct roles for Rho and Rac GTPases in dendrite growth, number, and branching.

Several gene knockout studies have similarly supported a role for the Rho GTPases in various other aspects of neural development and function. Although no studies have reported neural phenotypes in mice containing specific disruptions of individual GTPases, this probably largely reflects the fact only a few such knockouts have been described and those animals tend to fail early in development, prior to neurogenesis. However, knockouts of some of the Rho regulators and targets have been described, and several of these exhibit neural defects. For example, Lim-kinase-1, which like Lim-kinase-2, mediates signals downstream of several Rho GTPases, is not essential for development, but is required for normal dendritic spine morphogenesis and synaptic function (Meng et al., 2003; Meng et al., 2002). Consequently, the adult animals exhibit learning defects and an altered fear response. A few additional knockout mouse studies of Rho pathway components have resulted in viable animals that exhibit defects in neural function. For example, disruption of WAVE1, a member of the WASP family of Cdc42 targets, results in mice with severe limb weakness, a resting tremor, and neuroanatomical malformations (Dahl et al., 2003). During late stages of development, WAVE-1 is largely restricted to the CNS, but does not seem to play a role in neurite growth. The precise role of WAVE1 in neurons is unknown. WAVE2-deficient mice fail at E12.5 and exhibit growth retardation and malformed ventricles (Yan et al., 2003).

Some of the Rho effector targets have been implicated in neural development and function in mouse studies. Mice lacking the Rho target, Citron kinase, are severely ataxic and eventually die of seizures (Di Cunto et al., 2003). Further analysis has revealed that Citron is required for cytokinesis of neuronal precursors, suggesting that the observed phenotype in mutant animals reflects a developmental defect in the CNS. Thus, defects in neural development do not necessarily lead to embryonic lethality, and it

can be difficult to distinguish between subtle defects in neural development and defects in neural physiology that may not be associated with any significant developmental defect. Considering that a variety of studies have suggested a role for Rho signaling in synapses, it is possible that Rho GTPases play an important role in synaptic transmission and synaptic remodeling in the mature brain. The Rac/Cdc42 target, Pak1, appears to play a role in dendrite formation in cortical neurons. Expression of a constitutively active form of Pak1 in developing mouse neurons caused an increase in dendrite number whereas a dominant-negative form of Pak1 decreased dendrite number (Hayashi et al., 2002). It has been difficult to use knockouts to confirm this function of Pak1 in knockout animals because there is evidence of substantial redundancy between some of the highly related Pak kinases. However, disruption of Pak4 results in embryonic lethality associated with differentiation and migration defects in spinal cord motor neurons and interneurons (Qu et al., 2003). These mice also exhibit a defect in the proper folding of the caudal region of the neural tube, suggesting that Pak4 plays a unique role among the Paks in both neuronal and neuroepithelial cells.

Some of the Rho regulators have also been implicated in neural development. The two RhoGAPs that constitute the p190 family of RhoGAPs (p190-A and p190-B) have each been disrupted in mice, and both mutants are associated with neonatal lethality that is associated with neural defects (Brouns et al., 2000; Brouns et al., 2001; Sordella et al., 2002). The p190-A knockout mice exhibit multiple neural defects that involve both neuropeothelial tissues as well as neurons themselves (Brouns et al., 2000; Brouns et al., 2001). Several defects in neuroepithelial fusion were observed, including a defect in closure of the anterior neural tube, defective fusion at the neural midline, resulting in a failure of midline commissure formation, and a defect in closure of the optic fissure, resulting in a small, malformed eye. Neuron-associated phenotypes included defective fasciculation (axon bundling) in the cranial nerves and in some of the major axon tracts in the forebrain, defective guidance of some axon tracts, and defective migration of cortical neurons, resulting in abnormal cortical layering. Despite the very similar overall structure of the p190-A and p190-B proteins, mice lacking p190-B exhibit a distinct neural phenotype (Sordella et al., 2002). Those mice exhibit a substantial reduction in some of the major midline crossing tracts, associated with a reduction in neuronal differentiation, a reduced striatum, and enlarges lateral ventricles (figure 1). The p190-A protein was identified as the major substrate of Src phosphorylation in the developing and adult nervous system, and in cellculture studies, both p190 proteins appear to mediate adhesion signals to the actin cytoskeleton (Brouns et al., 2001). Thus, these various defects in p190-deficient mice probably reflect

defects in the transduction of signals from various cell surface adhesion molecules to the actin cytoskeleton via the Rho GTPases.

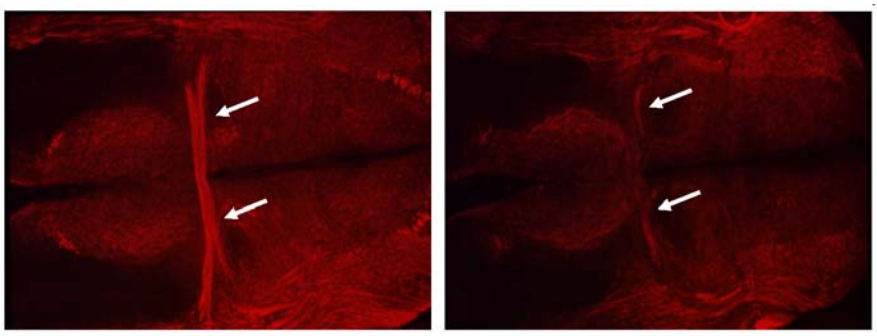

Figure. 1. In mouse embryos (E17.5) lacking the p190-B RhoGAP (-/-), the midline crossing axons of the anterior commissure (arrows) are severely reduced relative to wild-type embryos (+/+)

Several of the RhoGEFs have been disrupted in mice, and many of these are viable, suggesting the likelihood of functional redundancy among this relatively large class of GTPase regulators. In most cases, such animals have not been examined for even subtle defects in neural development. However, in mice lacking the Dbl RhoGEF, it was observed that distinct populations of cortical neurons exhibit a reduction in dendrite length, suggesting a highly specialized role for Dbl in some neurons (Hirsch et al., 2002). Such a finding highlights the fact that the developing brain consists of a relatively large number of distinct cell types, and that phenotypes that are limited to only one or a few of these cell types can easily go undetected in the absence of a detailed analysis.

In adult mice, Rho has been implicated in nerve regeneration following injury. It had been observed that microcrush lesions to the spinal cord of mice or rats results in a 10-fold activation of the RhoA GTPase (Dubreuil et al., 2003). That finding, together with numerous cell culture studies that indicated an important inhibitory role for Rho in neurite outgrowth, prompted studies to address the potential therapeutic utility of inactivating Rho following nerve injury. Several studies have made use of C3 toxin in such models, and it has been observed that C3 treatment of injured spinal nerves results in regeneration of axons and substantial functional recovery (Ellezam et al., 2002; Lehmann et al., 1999; Winton et al., 2002). A similar study using the ROCK inhibitor, Y-27632, indicated that inhibition of ROCK function could also promote recovery from nerve injury (Lehmann et al., 1999). Such findings suggest that activation of Rho and ROCKs

following nerve injury may prevent regeneration, and furthermore, that interfering with this pathway pharmacologically could be therapeutically beneficial

3.2 Eye and ear development

Inner ear development involves the morphogenesis of very complex and elaborate structures (the vestibule and cochlea) from a simple epithelium. Abnormal inner ear development in humans is associated with a variety of defects in balance and orientation as well as hearing. Rho GTPase signaling has been implicated both in developmental and physiological aspects of the mouse inner ear. Abr and Bcr are two closely related GAPs that regulate Rac and Cdc42 GTPases. Mice containing a targeted disruption of both of these genes (double knockouts) are viable, but exhibit behavioral defects including hyperactivity, circling behavior, and an inability to swim (Kaartinen et al., 2002). Such phenotypes are typically associated with vestibular defects, and these mice were found to display abnormal morphology of the saccule and ventricle of the inner ear, two structures that, together with the semicircular canals, constitute the vestibular system. The mice are not deaf, suggesting that these Rac/Cdc42 GAPs play a specific role in the morphogenesis of inner ear structures required for maintaining balance and orientation.

Within the cochlea, the hair cells are the essential structures for mechanosensation, and rely on a specialized cytoskeleton to transduce mechanical forces into signals for sensory neuron transmission. Interestingly, human mutations in the Rho-binding target, diaphanous, a regulator of actin polymerization, are associated with nonsyndromic deafness, confirming a likely role for Rho-directed actin assembly in cochlear hair cell function (Lynch et al., 1997). Gene knockouts of the mouse diaphanous genes have not yet been reported, and these should eventually be useful in determining whether diaphanous is required for developmental or physiological aspects of the cochlear hair cells.

Post-developmental hearing loss is often associated with damage to the cochlear hair cells by toxins or trauma, and the JNK signaling pathway has been implicated in the stress response in hair cells, raising the possibility that Rho GTPases (which can activate JNK) may be involved. Indeed, when explants of the organ of Corti, the cochlear structure that contains the hair cells, are treated with the bacterial C. difficile toxin B, which inactivates Rho, Rac, and Cdc42 proteins, a significant reduction in JNK-mediated hair cell death was observed upon exposure to toxic agents (Bodmer et al., 2002). This finding suggests that Rho GTPases may play a protective role in mature hair cells by mediating the activation of JNK in response to stress-inducing stimuli.

The potential role of Rho GTPases in the developing mouse eye has not received much attention thus far. However, a few reports have begun to implicate Rho signaling in eye morphogenesis. Cell culture studied have indicated that Rho signaling probably plays a role in lens epithelia both by regulating gene expression (alphaB-crystallin) and the actin cytoskeleton, and transgenic mice that express the Rho-inactivating C3 toxin in the developing lens exhibit lens cataracts and other ocular defects including defects in the focal attachments of the lens to the iris (Rao et al., 2002). Histological analysis also revealed that the lens defects included defective fiber cell differentiation and elongation, indicating a role for Rho in lens growth and maintenance of lens transparency.

The formation of the optic cup, which occurs between E11.5 and E13.5, gives rise to the retina, and requires dramatic morphogenesis of neuroepithelial tissue. The completion of the eye cup requires closure of an "optic fissure", in order to create a smooth seamless retina. In mice containing a targeted disruption of the p190-A RhoGAP gene, the optic fissure fails to close, leading to an abnormally formed eye structure associated with misfolded retinal tissue (Brouns et al., 2000). Thus, Rho is likely to play a role in the neuroepithelial fusion process required to close the optic fissure. This optic fissure defect appears to be related to other midline neuroepithelial fusion defects in p190-A RhoGAP knockout mice, including a failure to close the anterior neural tube and defective fusion of the forebrain hemispheres, suggesting that a similar Rho-mediated process regulates several neuroepithelial fusion events required for normal development.

3.3 Hematopoiesis and the immune system

Mouse models for studying the development of blood and lymphoid cells are particularly attractive for several reasons. A variety of cell surface markers that are routinely used to assess the various stages of hematopoietic differentiation are readily available. In addition, since many aspects of blood cell development are maintained throughout adult life, the development and physiology of blood cells can be easily examined in cases where gene disruption does not result in embryonic lethality. Finally, in cases where embryonic lethality is observed, fetal liver-derived stem cells can be used to reconstitute a complete hematopoietic system in irradiated host recipients. Surprisingly, of all of the developmental processes in mice, blood cell development has received, by far, the most attention in the context of Rho signaling. Hematopoiesis is generally not associated with the same kinds of tissue morphogenesis that play a major role in most other aspects of mammalian development. While blood cells do exhibit adhesive and

migratory behaviors, for the most part, they function as individual units and often exhibit a simple round morphology that does not appear to depend substantially on a dynamic actin cytoskeleton. In fact, as described blow, the vast majority of studies of Rho GTPases in hematopoiesis and immune function have revealed their role in the differentiation process and in signaling during the immune response. Thus, their role as cytoskeletal regulators may be less significant in the context of blood cells than it is in other tissues.

The rationale for the analysis of Rho GTPases in blood cell development and function can probably be traced, historically, to an early report that the Rac2 GTPase is largely restricted in its expression to myeloid cells (Didsbury et al., 1989), and subsequent findings that it is an essential component of the NADPH-mediated respiratory oxidative burst process that is used for microbial killing by neutrophils and macrophages (Bromberg et al., 1994). Indeed, several more recent reports that make use of mouse transgenics and knockouts have validated an important role for Rac2 in blood cells. Rac2-deficient animals are viable, and their neutrophils display defects in chemoattractant-stimulated superoxide production as well as in chemotaxis (Li et al., 2002; Roberts et al., 1999). The chemotaxis defect is associated with a reduction in F-actin formation, suggesting that Rac2 mediates distinct actin-dependent and -independent biological processes in neurtrophils. Interestingly, Rac1, which is 92% identical in sequence to Rac2, and is also expressed in neutrophils, cannot completely substitutre for Rac2. To address the role of Rac1 directly in neutrophils, a conditional Rac1 knockout mouse was generated in which Rac1 was specifically disrupted in the granulocyte/monocyte lineage (Glogauer et al., 2003). In those animals, neutrophils were observed to be profoundly defective in chemotactic migration and inflammatory recruitment, but interestingly, they exhibited normal levels of superoxide production. Thus, the closely related Rac1 and Rac2 GTPases both function in neutrophil chemotaxis, but Rac2 appears to function uniquely in regulating the respiratory burst.

The Rac GTPases are also detected in lymphocytes, and several mouse studies have addressed their role in lymphocyte development and function. An early transgenic study in which activated Rac2 was expressed specifically in thymocytes revealed an increase in apoptosis in thymus, suggesting a potential role in T cell selection (Lores et al., 1997).In another study, it was determined that Rac2 is selectively expressed in T helper cells of the TH1 class, which mediate cellular immunity (Li et al., 2000a). Using an inducible transgene system, it was demonstrated that expression of an activated Rac2 in mature T cells led to enhanced interferon-gamma production, while dominant-negative Rac2 inhibited interferon-gamma production, indicating a likely role for Rac2 in a key signaling pathway in

TH1 cells that involves NF-•B and MAP kinases (Lores et al., 1997). However, in a related study, no defects were observed in the ability of Rac2-deficient TH1 cells to respond to bacterial or viral infection in vivo (Croker et al., 2002b). In Rac2-deficient T cells, cytokine-stimulated proliferation is reportedly reduced, and a defect in MAP kinase activation has been noted, confirming a likely signaling role for Rac2 in some aspect of T cell activation. Rac1 also appears to play a role in developing thymocytes (Yu et al., 2001). Expression of an activated mutant form of Rac1 in pre-T cells can drive the differentiation process and promote proliferation of thymocyte precursors at the time of T cell antigen receptor beta selection (Gomez et al., 2000). An extension of this study revealed that Rac1 activation can switch thymocytes from a fate of positive to negative selection through an unknown mechanism (Gomez et al., 2001). The caveat with such studies that rely exclusively in the expression of a mutated form of the GTPase is that resulting defects may not correspond to the normal function of that GTPase.

Loss-of-function studies have pointed to roles for the Rac GTPases in B cell development and function as well. Rac2-deficient mice exhibit a significant reduction in peritoneal B lymphocytes, marginal zone B lymphocytes, and IgM-secreting plasma cells (Croker et al., 2002a). The isolated Rac2-deficient B cells also exhibited reduced chemotaxis in a chemokine gradient, indicating a likely role for Rac2 in both B lymphocyte differentiation and immune function. Using a conditional gene targeting approach, it was demonstrated that disruption of both Rac1 and Rac2 in the B cell lineage results in a nearly complete block in B cell development, indicating some degree of functional redundancy between Rac1 and Rac2 in this lineage (Walmsley et al., 2003). B lymphocyte precursors lacking both GTPases exhibited defects in the ability to transduce B cell receptor signals for proliferation and survival. Analysis of double-deficient hematopoietic stem/progenitor cells (HSC/Ps) also indicates a role for Rac1 and Rac2 in the movement of HSC/Ps from the marrow into the blood. In the absence of both GTPases, HSC/Ps exhibit a massive egress from the bone marrow, and are severely defective in the proliferative response to cytokines and adhesion to fibronectin (Gu et al., 2003).They also exhibit excessive apoptosis. Thus, these GTPases may perform redundant functions in the motility and engraftment of hematopoietic stem cells by mediating the response to both soluble and adhesive signals that direct motility, proliferation, and survival.

The Rho GTPase has also been implicated in lymphocyte development. In particular, several studies have revealed a role for Rho in the thymocyte lineage. By targeting the C3 toxin to the developing thymus in transgenic mice, it was determined that disruption of Rho function results in a severe reduction in thymic cellularity and is associated with defects in the maturation, proliferation, and survival of thymocytes, resulting in many

fewer mature peripheral T cells (Henning et al., 1997). A further study of these mice revealed that Rho plays distinct roles in both the survival and proliferation of thymic progenitors (Cleverley et al., 2000). Specifically, in pre-T cells, Rho is required for survival, but not for proliferation, whereas in late pre-T cells, Rho is required for proliferation but not survival. If C3 is expressed from a promoter that drives expression specifically in relatively late stages of T cell development, a block in T cell differentiation after the T cell receptor rearrangement stage was observed, suggesting a critical role for Rho at this important selection point in T cell development (Cleverley et al., 1999). Similarly, in transgenic mice in which an activated RhoA GTPase was expressed in thymocytes, an increase was observed in the positive selection of mature T cells bearing a rearranged T cell recept (Corre et al., 2001). Thus, Rho appears to play a significant role in the differentiation of both pre-T cells and mature T cells.

In addition to the studies of Rho and Rac GTPases in hematopoietic lineages, a single study has begun to address the role of Cdc42 in such cells. Specifically, in transgenic mice expressing an activated form of Cdc42 in late T cells, massive apoptosis was observed, resulting in a substantial decrease in the number of mature thymocytes and peripheral T cells (Na et al., 1999). Interestingly, one of the known Cdc42 targets, WASP, is specifically disrupted in patients with Wiscott-Aldrich Syndrome, in which patients exhibit immunodeficiency associated with defective T cells that appear to have a disorganized cytoskeleton (Schwartz et al., 1996). Thus, Cdc42 may play a role both in T cell development and in actin-mediated aspects of mature T cell function.

The nature of upstream regulation of the Rho family GTPases in hematopoiesis is not well established, but one regulator in particular, has received considerable attention. The vav1 protein, a member of the Rac/Cdc42 GEF family, is expressed almost exclusively in hematopoietic lineages, and was suspected of regulating GTPase function in such cells. In the first reported vav1 knockout, mice were observed to exhibit normal erythroid and myeloid development (Zhang et al., 1994). Subsequent studies, in which vav1-deficient embryonic stem cells were used to produce RAG2-deficient chimeras, resulted in thymic atrophy and B and T cells with reduced responsiveness to antigen receptors or stimulation with phorbol esters and calcium agonists (Gulbranson-Judge et al., 1999). B cells were found to respond normally to bacterial and other mitogens, indicating a selective role for vav1 in the development and function of lymphocytes. Additional analysis of vav1-deficient mice revealed that they exhibit a substantial defect in the positive selection of T cells, but do not seem to exhibit a defect in negative selection (Zhang et al., 1995). In light of the apparent role for the Rac GTPases in thymocyte selection, it seems likely

that activation of Rac by vav1 is required for some aspect of the positive selection of T lymphocytes. In vav1-deficient mice, conventional B cells appear to develop normally, however, it was reported that a subclass of mature B cells, the B1 B cells, are reduced in number, possibly due to an increased threshold for proliferative response to B cell receptor engagement (Zhang et al., 1995). Vav1 has also been implicated in the development of natural killer (NK) T cells. Vav1-deficient mice have a normal number of splenic NK cells, but the number of NK T cells is drastically reduced (Colucci et al., 2001). In addition, NK T cells from vav1-deficient mice do not produce IL-4 in response to in vivo activation, indicating a specific role for vav1 in NK T cell development and function (Chan et al., 2001).

Vav1 is closely related to two additional vav family members, vav2 and vav3, indicating the possibility of functional redundancy. Indeed, in two separate studies, it was determined that mice lacking both vav1 and vav2 exhibit a reduction in B lymphocyte number associated with an absence of a proliferative response to B cell antigen receptor engagement (Doody et al., 2001; Tedford et al., 2001). The defective response was associated with a failure to mobilize intracellular calcium, suggesting a specific and redundant function for vav1 and vav2 in B cell activation. A recent study has analyzed mice lacking all three vav proteins (Fujikawa et al., 2003). Those animals produce no functional B or T cells and fail to mount either T cell-dependent or T cell-independent humoral immune responses. Cells exhibit a defect in calcium mobilization in response to B cell receptor or T cell receptor engagement, but exhibit a normal MAP kinase activation, again consistent with a specialized and redundant role for the vav proteins in linking the activation of B and T cells to calcium-dependent responses.

Another RhoGEF that is highly enriched in hematopoietic cells is Lsc (Girkontaite et al., 2001). Lsc knockout mice have been produced, and mature lymphocytes were found to exhibit a reduction in motility in the absence of stimulation, whereas the marginal zone B cells exhibited enhanced migration in response to serum. Thus, Lsc appears to be another important activator of Rho GTPases within the immune system.

3.4 Fat, bone, skin, and muscle

A common mesenchymal stem cell gives rise to cells that form bone, fat, and muscle, and cell culture studies have implicated the Rho GTPases in each of these tissue types. The formation of bone, or osteogenesis, involves the differentiation of mesenchymal cells into osteoblasts, and is associated with a substantial change in cell shape. Although several cell culture studies have indicated a likely role Rho GTPase function in osteoblasts, relatively few reports have examined Rho signaling in animal models of bone

development. As described earlier, the FGD1 protein, a Cdc42 GEF, is expressed in developing bone, and loss of the human ortholog results in Aarskog Syndrome in humans, which is associated with skeletal defects (Pasteris and Gorski, 1999). The role of Src kinases in bone development and physiology is well established (Lowell and Soriano, 1996), and the ability of Src to modulate Rho-dependent actin reorganization is likely to play a role in bone development. However, this connection has yet to be established in the context of mouse models.

The role of Rho GTPases in skin development has not also not yet been addressed in mouse models, although Rho signaling has been shown to play a significant role in actin organization during keratinocyte differentiation (McMullan et al., 2003), which is associated with a substantial change in morphology and adhesion properties. In addition to development, two aspects of skin biology in adult mammals, wound healing, and oncogenesis are also likely to involve Rho signaling. Indeed, mice lacking the Rac activator, Tiam1 (a Rac-specific GEF), exhibit resistance to phorbol ester-promoted, Ras-induced skin cancers (Malliri et al., 2002; Sussman et al., 2000). This finding suggests that activation of the Rac GTPase by Ras is an important requirement for skin tumor formation. In a related study, it was observed that mice lacking RhoB, which develop normally, exhibit an increase in carcinogen-induced skin tumors, suggesting that RhoB is a negative regulator of oncogenesis in skin (Liu et al., 2001).

Adipogenesis, or the differentiation of fat cell precursors into mature adipose tissue, has been largely studied in the context of cell culture, where insulin/IGF-1 signaling appears to play a major role in this process. However, a recent report of the targeted disruption of the p190-B RhoGAP gene in mice revealed that this protein is required for adipogenic differentiation in vivo (Sordella et al., 2003). Further analysis, making use of embryo-derived cell cultures, indicated that Rho activity must be reduced in order for fat cell precursors to become mature adipocytes, thereby establishing a role for Rho signaling in adipogenesis. A specific pathway was identified in which excessive Rho activity leads to an increase in Rho-kinase-mediated phosphorylation of the insulin receptor substrate (IRS). This is an inhibitory phosphorylation that prevents the interaction of IRS with the insulin/IGF receptors, thereby down-modulating insulin/IGF signaling. It was also determined that insulin/IGF receptors can directly phosphorylate p190-B RhoGAP and thereby promote its activity by facilitating its translocation to membrane lipid rafts, where active Rho protein is enriched.

In that same analysis of p190-B RhoGAP, it was observed that while excessive Rho activity inhibits adipogenesis, it promotes myogenesis, or muscle cell differentiation. Myogenesis is also sensitive to IGF-1 signaling,

and the observation that Rho modulates the response to IGF-1 suggests that Rho probably plays a role in determining mesenchymal stem cell fate by determining how the cell will respond to IGF-1. Interestingly, IGF-1 signaling is also a major determinant of cell size and animal size, by promoting signals for cell growth, and mice lacking p190-B RhoGAP are approximately 30% reduced in size, and their tissues consist of cells that are reduced in size (Sordella et al., 2002). Thus, Rho appears to play an important role in several developmental processes that depend on IGF-1 signaling, and furthermore, this function of Rho appears to be independent of its ability to regulate the actin cytoskeleton. An important aspect of this analysis is that the developmental phenotypes observed in the knockout mice provided clues that led to the elucidation of a novel signaling role for the Rho GTPase. This provides a good example of the value of targeted gene knockouts in mice in establishing the molecular organization and function of GTPase-mediated signaling pathways in vivo.

Another Rho regulator, the RhoGEF, Trio, has also been implicated in muscle development (O'Brien et al., 2000). Trio knockout mice die in late embryogenesis and unusual spherical myofibers of skeletal muscle were detected at E18.5. The timing of the defect coincides with the so-called "second wave" of myogenesis, and was associated with a reduction in the number of the smaller secondary muscle fibers that are located adjacent to primary myofibers. Possibly, Trio regulates the alignment or fusion of secondary myoblasts. Interestingly Rac signaling has been directly implicated in the process of myoblast fusion in the Drosophila system (Erickson et al., 1997). However, the precise role of Rac signaling in that process is not clear.

3.5 Cardiac development and function

The formation of a mature heart, with its elaborate chamber and valve structure, and a purposeful asymmetry, is among the most remarkable of all tissue morphogenesis processes in mammals. Cell culture studies had implicated RhoA in the hypertrophic growth and cytoskeletal organization of cardiac muscle cells (Aikawa et al., 1999), and subsequent mouse studies have confirmed a likely role for Rho signaling in both cardiac development and physiology. Transgenic mice expressing wild-type RhoA or a constitutively activated RhoA mutant under the control of a cardiac-specific promoter exhibited atrial enlargement, and eventually, dilation of the left ventricular chamber and a consequent decrease in left ventricle contractility, ultimately leading to atrial fibrillation and death (Sah et al., 1999). Thus, excessive Rho activity can lead to ventricular failure. In a related study, an activated form of Rac1 was expressed specifically in the myocardium in

transgenic mice (Sussman et al., 2000). Those animals exhibited a range of postnatal cardiac phenotypes, whose severity was dependent on the level of transgene expression. In mice that died within two weeks of birth, a severe dilation phenotype was observed, which was associated with substantial enlargement of both ventricles and atria. Other mice exhibited a milder phenotype in which, at 3 weeks of age, no evidence of heart enlargement was seen, but did eventually appear by 2 months. These animals exhibited a progressive deterioration of ventricles, and their hearts were found to be hypercontractile in physiology studies. It was observed that the Rac target, PAK1, was translocated from a cytosolic to cytoskeletal localization in fractionated cardiomyocytes from transgenic animals. Moreover, immunostaining of paxillin indicated that focal adhesion formation may be affected in the Rac-expressing cardiomyocytes. Thus, Rac signaling appears to influence both cardiac dilation and hypertrophy, and Rac-regulated focal adhesions may be required for normal cardiac physiology. Notably, transgenic mice that overexpress the SRF transcription factor specifically in developing heart tissue exhibit cardiac hypertrophy (Zhang et al., 2001). Since SRF can be activated by Rho GTPases (Hill et al., 1995), this fnding suggests that the role of Rho GTPases in hypertrophy may also involve effects on SRF-dependent gene transcription.

Transgenic mouse embryos expressing RhoGDIα specifically in cardiomyocytes (starting at E8.5) fail at E10.5, and exhibit a disruption of cardiac morphogenesis associated with incomplete looping, lack of chamber demarcation, and ventricular hypoplasia (Wei et al., 2002). RhoGDIα can inhibit the activation of various Rho family GTPases, and so while this study suggests a likely role for Rho GTPases in early cardiac development, it is not clear from this analysis which of the Rho family members are involved.

Rho effector targets, including the ROCKs, have also been implicated in cardiac development. There are two ROCK genes in mammals, ROCK-I and ROCK-II, both of which are expressed in early embryonic heart. ROCK-II knockout mice have recently been reported, and the mutant animals die in late embryogenesis with apparent defects in blood coagulation and blood flow (Thumkeo et al., 2003). However, it is quite possible that there is significant functional redundancy between the two closely related ROCKs. Several other studies have made use of the pharmacological ROCK inhibitor, Y-27632, which effectively inhibits both forms of ROCK, and therefore, can potentially overcome such redundancy. To address a developmental requirement for ROCKs, early mouse embryos (E8.5-E9.5) were isolated and maintained in culture, and then treated with Y-27632 (Wei et al., 2001). The drug-treated embryos exhibited a block in the differentiation of both ventricles and atria. The treated embryos had smaller hearts and a dilated pericardium. A similar study by another group revealed

that Y-27632 treatment of early embryos blocked fusion of the bilateral heart primordial (Zhao and Rivkees, 2003). These finding suggest that the ROCKs play an essential role in several aspects of early cardiac development, and future studies using double ROCK-I/ROCK-II knockout mice will undoubtedly be performed to extend such findings.

The ability of ROCK inhibitors to prevent ROCK-mediated actomyosin contractility, and consequently affect vascular function has led to several studies in which such inhibitors appear to provide relief from vascular hypertension (Hu and Lee, 2003). Additional studies have now begun to address the consequences of ROCK inhibition in other aspects of cardiovascular disease. When adult mice fed on an atherogenic diet were injected daily with Y-27632, a substantial decrease in atherosclerotic lesion size in the aortic sinus and thoracic aorta was observed, suggesting that ROCKs may play a role in atherosclerotic plaque development, and furthermore, that ROCK inhibitors could provide effective treatments for atherosclerosis (Mallat et al., 2003). The potential therapeutic utility of ROCK inhibitors in cardiovascular disease was additionally revealed in a study in which it was shown that a high salt diet-induced left ventricular hypertrophy in rats could be prevented by chronic administration of Y-27632 (Satoh et al., 2003). This finding suggests that ROCKs may play a role in hypertension-induced cardiac hypertrophy, and indicates another potential therapeutic application of Rho pathway inhibitors in human disease.

The Rac/Cdc42 target, PAK4, is required for normal heart development. PAK4-null embryos fail prior to E10.5 with heart defects. Specifically, mutant embryos exhibited a thinning of the myocardial walls of the bulbus cordis and the ventricles. Most likely, this resulted in poor ventricular function, and embryonic lethality (Qu et al., 2003).

3.6 Vasculature and lungs

Angiogenesis is essential for vasculature formation during development, and plays an important role in wound healing and tumor growth in the mature organism. A variety of cell culture studies have revealed roles for Rho GTPases and their regulators and targets in several aspects of endothelial cell biology, including migration, cell-cell interactions, survival, and the response to adhesion molecules and growth factors, suggesting a likely role for the Rho GTPases in angiogenesis in vivo (Cascone et al., 2003). Thus far, however, the analysis of Rho function in this context in mouse models has been rather limited. Among the numerous Rho family GTPases, only RhoB has been directly implicated in angiogenesis in mouse studies (Adini et al., 2003). RhoB-deficient mice are viable, but exhibit a specific defect in retinal vascular development that is associated with abnormal sprout

morphology. This is consistent with the finding that RhoB plays a role in survival of sprouting endothelial cells, and suggests that RhoB might be a good therapeutic target for diseases associated with excessive sprouting angiogenesis, such as diabetic retinopathy and macular degeneration. In another study, the ROCK inhibitor, Y-27632, was used to specifically examine the role of Rho signaling in angiogenesis (Uchida et al., 2000). Mice were orally fed Y-27632, and angiogenesis in dorsal skin was quantified following local administration of VEGF delivered via transplanted VEGF-secreting cells. A significant inhibition of new vessel growth, without an effect on pre-existing vessels, was observed following Y-27632 treatment, suggesting that at least one Rho effector target plays a role in angiogenesis. A similar study using a different ROCK inhibitor, Wf-536, reported inhibition of tumor growth and angiogenesis in mice bearing xenotransplants of human prostate tumor cells (Somlyo et al., 2003). Thus, pharmacological inhibition of ROCK could potentially be an effective anti-cancer treatment.

As mentioned above, ROCK inhibitors have received a great deal of attention in the context of therapy for hypertensive disorders. Most of these studies have relied on rat models of hypertension, since, historically, this has been the model of choice for studying cardiovascular disease. However, in light of emerging conditional knockout technologies in mice, it is likely that future studies of specific gene function in cardiovascular disease will make more frequent use of mouse knockout models

3.7 The gastrointestinal system

Intestinal development begins relatively late in embryogenesis, with the conversion of a pseudo-stratified epithelium to a simple epithelial monolayer that undergoes differentiation and morphogenesis. This results in the formation of multiple discrete epithelial units known as crypts and villi that give rise to the mature intestine. The formation of these structures requires the differentiation of crypt stem cells, the migration of differentiating epithelial cells, and the morphogenesis of precisely folded epithelial tissue. The mature intestinal epithelium, once formed, undergoes periodic renewal throughout life, during which time, many aspects of the development process are recapitulated. The analysis of Rho GTPase signaling in intestinal morphogenesis has not yet been thoroughly addressed, although a few studies point to a likely role for Rho-mediated signaling. By expressing activated and dominant-negative forms of the Rac GTPase in regions of the developing small intestine, it was observed that activated Rac causes a cell-autonomous precocious differentiation of gut epithelia while dominant-negative Rac inhibits differentiation and migration of cells along crypt-villus

units (Stappenbeck and Gordon, 2000; Stappenbeck and Gordon, 2001). A follow-up mechanistic analysis of this defect revealed that expression of activated Rac in intestinal epithelia results in an accumulation of cytoplasmic phospho-JNK (Jun n-terminal kinase). Notably, the JNK substrate, c-jun, did not exhibit excessive phosphorylation, suggesting that the accumulation of cytoplasmic phospho-JNK may reflect a sequestering mechanism that suppresses JNK-mediated apoptosis in order for Rac signaling to promote cell proliferation without accompanying cell death.

IQGAP1, a Rac/Cdc42 effector targets that has been implicated in cadherin-based cell adhesion, also appears to play a role in the gastrointestinal system. Mice specifically lacking IQGAP1 appear to develop normally, however, homozygous mutant adults exhibit a late-onset gastric dysplasia (Li et al., 2000b). This suggests a function for Rac and/or Cdc42 signaling in maintaining the integrity of the gastric mucosa. IQGAP1 is highly related to IQGAP2, and it is possible that redundant functions between these proteins mask developmental phenotypes that would otherwise be associated with specific disruption of IQGAP1. However, a specific function for the Rho GTPases in maintaining the physiology of the epithelial lining of the mature gut is suggested by accumulating reports indicating that many of the pathogenic bacterial strains that infect the intestine produce toxins that specifically interefere with Rho GTPases and their normal regulation (Rudolph et al., 1999)

3.8 Mammary development and breast cancer

In the developing mammary gland, a variety of interactions between epithelial cells and surrounding matrix proteins play an important role in ductal morphogenesis, suggesting a likely role for Rho-mediated signaling. Similarly, changes in mammary epithelium that occur during pregnancy, lactation, and weaning may involve similar morphogenetic processes. The role of Rho GTPases has begun to be addressed in mouse models of mammary development and physiology. It had been observed that the Rac3 GTPase is frequently expressed at high levels in breast cancer cell cultures, and transgenic mice expressing an activated form of Rac3 were produced and found to exhibit defective postlactional involution and benign mammary lesions (Leung et al., 2003). These lesions consisted of epithelial islands that persisted during late stages of involution, at a time when elevated levels of apoptosis normally bring about the reduction in cell number. Thus, Rac3 may contribute to the involution process during weaning, and its apparent role in proliferation or survival of mammary epithelial cells, together with previously described expression studies, indicate a potential causative role in human breast cancer.

In a differential expression screen to identify genes whose expression is relatively high in the proliferating terminal end buds of developing mammary glands, the p190-B RhoGAP gene was detected (Chakravarty et al., 2003; Chakravarty et al., 2000). To examine a potential role for p190-B RhoGAP in mammary development, the mammary anlagen from p190-B knockout mice was rescued by transplantation into the cleared fat pad of recipient Rag1-deficient mice (Chakravarty et al., 2003).None of the p190-B-deficient epithelial transplants displayed any outgrowths in host recipients, indicating that p190-B RhoGAP is required for ductal morphogenesis. Interestingly, it has been established previously that IGF-1 signaling plays an important role in terminal end bud proliferation. Thus, the findings described above, that p190-B RhoGAP plays a critical role in regulating IGF-1 signaling during development, may be relevant to its role in mammary morphogenesis.

RhoC may also contribute to breast cancer. RhoC expression appears to be a reliable marker of human breast cancer invasiveness and metastasis potential (Kleer et al., 2002).In mice, a role for increased RhoC expression in the conversion of a primary tumor to a metastatic phenotype was reported (Clark et al., 2000), and several subsequent expression studies have confirmed a potential role for increased RhoC activity in tumor progression. The ability of Rho GTPase signaling to influence cell proliferation, adhesion and motility, and angiogenesis, indicates that these proteins could potentially play a role in tumorigenesis at multiple levels, and efforts are underway to explore the feasibility of using small molecule inhibitors of various Rho pathway components as therapeutic agents in the treatment of human cancers

4. CONCLUDING REMARKS

The mouse studies described above have revealed widespread and essential roles for several of the Rho family GTPases and their regulators and targets in embryonic development, as well as in the normal physiological function and pathology of adult animals. In some sense, this type of analysis is in its relative infancy, and in many cases, the phenotypes observed in transgenic and knockout mice have provided largely descriptive information regarding the requirement for the Rho GTPases in complex in vivo processes. However, the power of this technology is clear, and future studies, particularly those that make greater use of conditional gene targeting methods, will undoubtedly yield substantial new insights into the organization and function of Rho-mediated signaling pathways both in normal biology and in human disease

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Chapter 12

RHO PROTEINS AND MICROTUBULES

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Abstract: Rho GTPases have increasingly become recognized as prominent regulators of the microtubule (MT) cytoskeleton. Whereas Rho GTPases regulate the de novo formation of distinct actin arrays (stress fibers, lamellipodia, and filopodia), with MTs, which are present as extensive and dynamic arrays in the absence of Rho GTPase signaling, Rho GTPases principally modify the behavior and dynamics of individual MTs within an existing array. Despite this seemingly modulatory role, Rho GTPases have to profound effects on the MT cytoskeleton. The action of Rho GTPases is primarily exerted at the ends of MTs and causes changes in: (1) dynamics of MT plus ends either through MAPs or sequestering proteins, (2) interactions of MT plus ends with targets in the cortex, in kinetochores or at other sites, a process termed MT capture or (3) the activity of MT minus ends at the centrosome. In many cases, specific GTPases and effectors are known to regulate each of these processes and constitute signaling pathways to regulate MTs. Additionally, MTs can in turn influence the activity of Rho GTPases by interacting with the guanine exchange factors (GEFs) and GTPase activating proteins (GAPs) regulating their function. Together, MTs and Rho GTPases have a dynamic relationship that allows a cell to quickly respond to and integrate a variety of stimuli.

1. INTRODUCTION

In this chapter, we consider the effect of Rho GTPase signaling on MT function and dynamics. We review MT dynamics and the major sites of Rho GTPase action on MTs and then consider specific Rho GTPase effects on MTs. Each major member of the Rho family of small GTPases, Rac, Rho, and Cdc42 regulates MT capture, and this is a major mechanism by which Rho GTPases regulate MTs. Because of the diversity of molecules involved in capture and the Rho GTPases regulating them, there is quite a range of

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consequences for the captured MT. Rho, Rac and perhaps Cdc42 are known to regulate MT plus end dynamics through effects on the availability of tubulin monomers. Rho may also regulate MT dynamics through affects on MT associated proteins (MAPs). The activity of Rho GTPases on MT minus ends and organizing centers such as the centrosome is less well-studied, however, certain effectors of Rho GTPases are localized there and appear to be involved in regulating events at this site.

We explore the evidence that Rho GTPases exert their effects on MTs through specific effectors and signaling pathways. Rho GTPase effectors are proteins that bind specifically to the active GTP bound form and mediate the activity of Rho GTPases on cellular processes. The number of Rho GTPases effectors that regulate MTs has grown dramatically in recent years and includes kinases, adaptor and scaffolding proteins (Table 1).

GTPase	Effector	Effects on MTs	Ref
Rho	mDia1, mDia2	MT capture and long-lived stabilization	(Palazzo, 2001a)
	Bni1	MT capture and transient stabilization	(Lee et al., 1999)
	Rho kinase	MT assembly inhibition (CRMP-2)	(Fukata et al., 2002)
	Rho kinase, PKN	MT assembly inhibition (MAPs)	(Kawamata et al., 1998; Taniguchi et al., 2001; Amano et al., 2003)
	Rho kinase	Centriole splitting and migration	(Chevrier et al., 2002)
Cdc42	Par6/aPKC	MT capture (?) and centrosome and spindle positioning	(Etienne-Manneville, 2001; Palazzo, 2001b) (Joberty et al., 2000; Lin et al., 2000; Qiu et al., 2000)
	mDia3	MT capture (?) at the kinetochore	(Yasuda et al., 2004)
Cdc42/ Rac1	IQGAP	MT capture and transient stabilization	(Fukata, 2002)
	Pak1	MT dynamics (stathmin/Op18)	(Daub et al., 2001; Wittmann et al., 2003; Wittmann et al., 2004)
	Pak1(pT212)	MT-centrosome interaction	(Banerjee et al., 2002)
	X-Pak5	? (binds MTs)	(Cau et al., 2001)
RhoG	Kinectin	Kinesin-based motility	(Vignal et al., 2001)
Rnd2	Rapostlin	? (binds MTs)	(Fujita et al., 2002)

Table 1. Rho GTPases and Effectors That Act on MTs

Although we have organized the chapter by the specific GTPases, it should be borne it mind that in a number of cases, the Rho effectors that interact with MTs or MT associated proteins, are regulated by more than one GTPases. Also for some effectors, there are multiple isoforms and each

isoform may have a distinct profile of activation by the individual GTPases There is also increasing evidence that some of the less-well studied Rho GTPases (for example, Rho G and Rnd2) regulate MTs, raising the possibility that there will be even more Rho family members involved in MTs function (in addition to Rho(A,B,C), Rac(1,2,3) and Cdc42, there are ~15 additional Rho GTPases). Finally, MTs themselves affect the activity of Rho GTPases and this may be one way that MTs regulate the actin cytoskeleton, modulate their own activity or that of other cellular processes. Rho GTP exchange factors (GEFs) and Rho GTPase activating proteins (GAPs), which activate and inactivation Rho GTPases respectively, are known to interact with MTs either directly or indirectly and in some cases this interaction regulates their activity (see Table 2). We consider these GEFs and GAPs and the known molecular mechanisms through which they are regulated by MTs to affect Rho GTPase signaling.

Regulator	Effect of MTs	GTPase	Ref
Rho GEF-H1 /Lfc	Binds MTs, release activates (RhoGEF-H1)	↑Rho	(Ren et al., 1998; Glaven et al., 1999; Krendel et al., 2002)
p190RhoGEF	Binds to MTs	?	(van Horck et al., 2001)
RhoG	MTs, kinectin and kinesin required for Cdc42/Rac activation	↑Cdc42/Rac	(Gauthier-Rouviere et al., 1998; Vignal et al., 2001)
ASEF	Kif3 mediated transport on MTs	↑Rac (local)	(Jimbo, 2002; Kawasaki et al., 2003)
Vav	Interacts with MTs	?	(Huby et al., 1995; Fernandez et al., 1999)
Mgc-RacGAP Cyk-4 RacGAP50C	Kinesin mediated transport on MTs	↓Rac (local)	(Adams et al., 1998; Jantsch-Plunger et al., 2000; Hirose et al., 2001; Kuriyama et al., 2002; Mishima et al., 2002; Somers and Saint, 2003)

Table 2. Connections between MTs and Rho GTPases

2. SITES OF RHO GTPASES ACTION ON MTS

2.1 MT Dynamics and the effect of MT interacting proteins

MTs are comprised of α/β tubulin heterodimers that have the capacity to self-assembly and exhibit dynamic instability. Dynamic instability refers to the capability of MTs to undergo periods of growth and shrinkage punctuated by infrequent transitions between these states. The transitions are referred to as catastrophe if the MT begins to shrink and as rescue if the MTs begins to grow. In cells where the minus end of the MT is typically attached to nucleation sites, such as the centrosome, a shrinking MT may disassemble completely exposing its nucleation site and allowing a new MT to be nucleated. Thus, dynamic instability contributes to the dramatic turnover of MTs in cells; the half-life of typical interphase MTs is 5-10 min, whereas the half-life of typical mitotic spindle MTs in 15 sec (Salmon et al., 1984; Saxton et al., 1984). Other factors, such as severing (McNally and Thomas, 1998), breakage (Waterman-Storer and Salmon, 1997), and centrosome release (Keating et al., 1997) may also contribute to MT turnover.

Tubulin is a GTPase and hydrolyzes GTP upon assembly into MTs, although usually with a lag. The presence of a cap of GTP tubulin subunits at the end of the MTs promotes subunit addition and MT assembly, whereas loss of this cap and exposure of the core of GDP subunits promotes MT disassembly. The intrinsic dynamic instability of tubulin is modified by a number of cellular factors, nonetheless, the prominent behavior of MTs in cells is dynamic instability. Classic MT associated proteins (MAPs), such as tau, MAP2, MAP4, and XM215 bind to the side of MTs and dampen MT dynamic instability by reducing MT catastrophe and stabilizing MT growth (Howard, 2003). Rho GTPases can affect MAPs by stimulating kinases that phosphorylate these proteins and alter their interaction with MTs (Kawamata et al., 1998; Taniguchi et al., 2001)(Figure 1). Other proteins, particularly members of the kinesin family of motor proteins, stimulate MT catastrophe (Howard, 2003; Ovechkina and Wordeman, 2003), however, these proteins are not known to be regulated by Rho GTPases.

Tubulin monomer binding proteins such as stathmin/Op18 and SCG10 can also regulate the dynamics of MTs by regulating the availability of tubulin monomers for polymerization (Cassimeris, 2002). Stathmin has also been proposed to regulate the catastrophes of MTs by binding to their ends (Belmont and Mitchison, 1996). Stathmin is a target of a Rac signaling pathway that regulates its ability to interact with tubulin (Fig. 1) (see below).

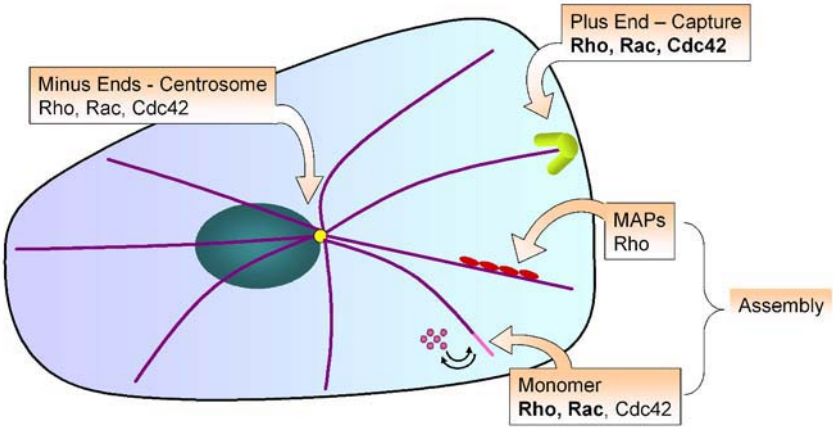

Figure 1. Sites of Rho GTPase action on MTs. GTPases in bold indicate principle factors working at each site.

Another protein that functions by binding tubulin monomers is CRMP-2 (collapsing response mediator protein-2) (Fukata et al., 2002). CRMP-2 stimulates MT polymerization and this activity may be regulated during growth cone collapse by Rho signaling (see below).

A more recently discovered class of proteins, the MT tip proteins, also regulate the dynamics of MTs (Schuyler and Pellman, 2001; Howard, 2003; Mimori-Kiyosue and Tsukita, 2003). The first described member of this class was CLIP-170, but there are now many proteins that have been found to exhibit MT tip behavior (EB1 [Bim1 in S. cerevisiae; Mal3 in S.pombe], APC, CLASPs, Kar9 [S .cerevisiae], dynactin, Lis1, and dynein (in some cases), and the S. pombe proteins Tea1, Tea2, Tea4 and Tip1). These proteins reside primarily at the plus ends of growing MT, but in some cases have been observed on shrinking MTs (Carvalho et al., 2004) and stabilized, non-growing MTs (Wen et al., 2004). The mechanism responsible for their localization at the ends of MTs is not yet clear, although in yeast and mammalian cells there is evidence that they may be moved to MT ends by kinesin motors (Mimori-Kiyosue Y, 2000; Jimbo, 2002; Maekawa, 2003; Carvalho et al., 2004). Evidence that MT tip proteins regulate MT dynamics comes from knock out experiments in yeast (Tirnauer et al., 1999; Adames and Cooper, 2000), siRNA experiments in Drosophila S2 cells (Rogers et al., 2002) and from dominant negative studies in mammalian cells(Komarova et al., 2002). In each case, the loss of these proteins tends to alter the dynamic instability of MTs and sometimes, but not always, cause changes in MT length. Although MT tip proteins play a role in MT dynamics, this activity is not known to be regulated by Rho GTPases. Instead, many of the MT tip

proteins have been found to play an important role in MT capture, where they are thought to mediate the interaction between MTs and their cortical targets (see below).

2.2 Capture of MT Plus Ends

Dynamic instability of MTs was proposed by Kirschner & Mitchison (Kirschner and Mitchison, 1986) to allow MTs to search the three dimensional space of the cell for sites of stabilization. According to this "selective stabilization model", dynamic MTs that encountered cortical sites that had been activated by external signals, would be captured at the sites and be stabilized. This could lead to polarization of MT arrays by producing a subset of stabilized MTs at specific sites within the cell. Evidence for such selective stabilization of MTs has been obtained in many systems including yeast and mammalian cells (Bulinski and Gundersen, 1991; Gundersen, 2002). As MTs are principal tracks for transport of vesicles and other factors, this could lead to localized delivery of the trafficked components to sites originally responding to the signal.

The selective stabilization model hypothesized that there would be cortical factors, regulated by external signals, that could capture MTs. It has taken a long time to identify such factors, but in the last few years there has been an explosion in the number of proteins implicated in cortical capture of MTs. Strikingly, almost all of these factors are regulated by Rho GTPases. Another surprise has been the complexity of cortical capture events. Depending on the factors involved, cortical capture of MTs can lead to stabilization of MTs, but with widely varying extents of stabilization, and also to the generation of forces, principally pulling forces, on captured MTs. Cortical capture of MTs contributes to polarizing arrays of MTs in migrating cells, to positioning of the MTOC toward target cells in T cells and to spindle positioning in many cells. Recent work suggests that the capture of MTs at kinetochores on chromosomes during mitosis may also involve Rho GTPases (Yasuda et al., 2004). Thus, the repertoire of MT capture is extensive and plays a role in many cellular contexts.

2.3 Centrosome and MT Minus Ends

In most cells, MTs are nucleated from specific sites, centrosomes in mammalian cells, spindle pole bodies in yeast, and so their minus ends are initially anchored. While most minus ends remain attached to their nucleating sites, there are some cases where MTs will be released. In fibroblasts, MT release is infrequent and the released MTs depolymerize. In

contrast, MTs are frequently released in epithelial and neuronal cells and this contributes to the formation of the specialized arrays in these cells (Yu et al., 1993; Keating et al., 1997). MTs are also released from spindle centrosomes but are kept near the centrosomes by a variety of motor proteins (Keating and Borisy, 1999). In epithelial and neuronal cells the minus ends are stabilized by as yet unidentified factors. Rho GTPases have been implicated in MT interactions with centrosomes and this is considered below.

3. RHO A

3.1 Regulation of MT Stabilization Through Formins

The existence of stabilized subsets of MTs in cells has been known for decades (for an early review, (Bulinski and Gundersen, 1991)), but it was not until recently that the signals leading to their formation have been identified. Such stable MTs coexist in a common cytoplasm with dynamic MTs, and their number increases in highly polarized and differentiated cells where they tend to lie along the axis of polarization. Thus, the formation of stable subsets of MTs is likely to play an important role in cell polarization. Unlike dynamic MTs, stabilized MTs persist for many hours (Webster et al., 1987), are resistant to breakdown by MT antagonists (Khawaja et al., 1988; Cook, 1998) and in many cells (but perhaps not yeast), tubulin comprising stabilized MTs becomes posttranslationally modified by detyrosination, acetylation, glutamylation, or phosphorylation (Westermann and Weber, 2003). Such modifications may serve as another layer of regulation of MT function since some factors, including kinesin, selectively recognize the modified MTs (Larcher et al., 1996; Liao, 1998; Kreitzer et al., 1999).

Selectively stabilized MTs arise from the action of Rho GTPases working on the ends of MTs to regulate capture. There is growing evidence for multiple levels of MT stability and different members of the Rho GTPase family appear to be involved in regulating MTs with different degrees of stability. The best characterized of these selectively stabilized MTs are those in mammalian cells that are regulated by Rho and exhibit long term stability. Their stability derives from the fact that their ends are captured and capped and this renders them unable to add or loose subunits (Infante et al., 2000). Classic MT associated proteins (MAPs) such as tau, MAP2 and MAP4 bind along the length of MTs and contribute to the stability of MTs by decreasing the off rate of tubulin from MTs, rather than regulating capture of MTs. These MAPs are particularly abundant in neuronal cells

and there is evidence in some cases that their interaction with MTs may be regulated by Rho GTPases (see below).

Evidence that long-lived stable MTs are regulated by Rho came from the observation that serum stimulated the formation of stable MTs in starved 3T3 cells and that the serum factor responsible was lysophosphatidic acid (LPA) (Cook, 1998). LPA acts through G protein coupled receptors of the Edg family (Contos et al., 2000) and is known to stimulate a number of other signaling pathways including Rho GTP formation through p115RhoGEF(Hart et al., 1998; Kozasa et al., 1998). However, Rho alone was found to be necessary and sufficient for the formation of stable MTs in starved 3T3 cells. In wounded monolayers of 3T3 cells, serum, LPA and active Rho selectively stabilize MTs in the leading lamella, showing that Rho signaling generates a polarized response in the MT array in the context of cells at the edge of a wound. The induction of MT stability occurs within 5 minutes of LPA treatment and time lapse movies of MT in LPA-treated cells showed that Rho activation affected the stability of a subset of MTs near the cell cortex without changing the parameters of dynamic instability (Cook, 1998). Thus, Rho regulates the stability of MTs without altering dynamic instability. Subsequent studies have shown that Rho also regulates the stability of MTs in peripheral blood lymphocytes (Vicente-Manzanares et al., 2002) and primary endothelial cells (Lee and Gotlieb, 2002).

By use of a Rho effector screen, the formin mDia was identified as the Rho effector sufficient for promoting this selective microtubule stabilization in 3T3 cells (Palazzo, 2001a). Constitutively active, truncated forms of mDia2 are sufficient for activating MT stabilization in starved cells in the absence Rho (Palazzo, 2001a; Wen et al., 2004). In addition, activation of endogenous mDia1 with the Dia autoinhibitory domain (DAD) (Alberts, 2001) also induces stable MTs in fibroblasts (Palazzo, 2001a), and endodermal cells (Kodama et al., 2003). Although mDia collaborates with another Rho effector, Rho kinase, to regulate actin stress fibers (Watanabe et al., 1999), stable MT formation does not require Rho kinase activity (Palazzo, 2001a) Rho kinase may regulate MTs in other ways in neurons (see below). Also, while both Rho and mDia induce polymerization of actin filaments in vivo, and mDia and other formins are now known to directly nucleate actin filaments (Pruyne et al., 2002; Sagot et al., 2002; Li and Higgs, 2003), blocking actin filament formation with cytochalasin D or with dominant negative Rho kinase does not interfere with mDia's ability to induce stable MT formation. Although this does not prove that mDia has an independent activity toward MTs, the observation that mDia is localized on MTs in some cases in vivo (Kato et al., 2001; Palazzo, 2001a) and can bind MTs in vitro (Palazzo, 2001a) hints that it may.

Additional support for the idea that mDia directly affects MTs comes from the observation that mDia1 and mDia2 directly interact with the MT tip proteins EB1 and adenomatous polyposis coli protein (APC) (Wen et al., 2004). The mammalian proteins EB1 and APC were identified as candidates for functioning downstream of mDia, since homologs of these proteins function in a Rho-formin pathway mediating MT capture and stabilization in yeast (Fig. 3, see below). EB1 is essential for formation of stable MTs in 3T3 cells as siRNA knockdown of EB1 and a C-terminal construct of EB1 (EB1C) that binds to APC acts as a dominant negative construct and blocks LPA and mDia-induced stable MT formation (Wen et al., 2004) The dominant negative EB1C also inhibits cell migration into the wound, providing the first functional evidence implicating stable MTs in cell migration. EB1 or APC overexpression induce the formation of stable MTs, whereas a mutant of EB1 incapable of binding APC does not. Thus, both EB1 and APC function downstream of Rho and mDia and together comprise a signaling pathway that regulates MTs (Fig. 2).

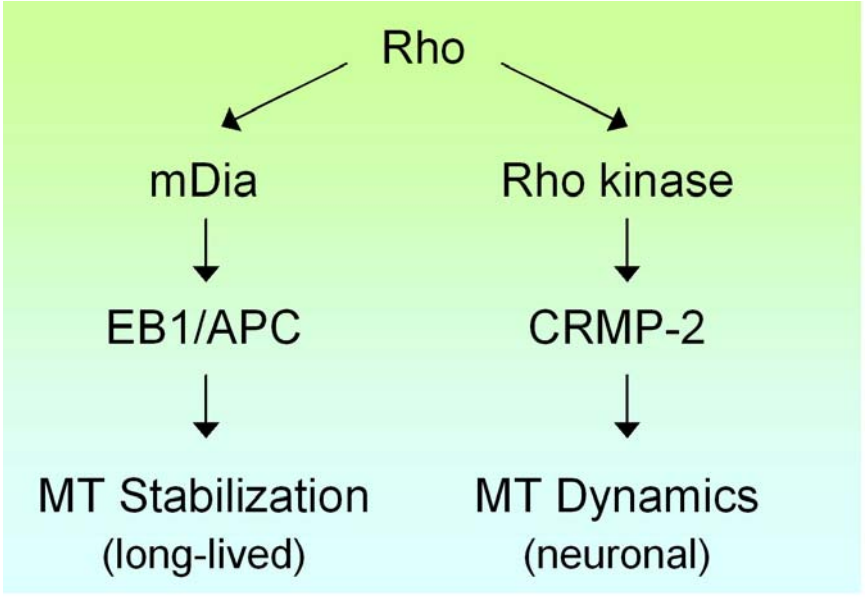

Figure 2. Defined Rho signaling pathways regulating MTs. Activation of RhoA leads to long-term MT stabilization through its effector mDia and the MT tip proteins EB1 and APC. In neurons, Rho also activates Rho kinase which regulates MT dynamics through CRMP-2.

As EB1 and APC bind to each other through domains distinct from those used to bind mDia, it is possible that the three proteins form a complex to cap and stabilize MTs. Consistent with this model, APC and EB1, like

mDia, are localized on the ends of stable MTs (Wen et al., 2004). However, only a subset of the stable MTs displayed EB1, APC or mDia at their ends. Thus, there may be additional molecules that function in this pathway to cap and stabilize MTs. One such protein is the MT-actin crosslinking protein ACF7/MACF. ACF7/MACF has a MT interacting domain, which stabilizes MTs when overexpressed (Sun et al., 2001; Kodama et al., 2003). ACF7 -/- endodermal cells are unable to generate stable MTs in response to LPA or to expression of active mDia (Kodama et al., 2003). ACF7/MACF appears to be involved in this pathway, but it is not yet know how it contributes to MT stabilization.

While the pathway shown in Fig. 2 regulates MT stabilization, a separate integrin signaling pathways arrears to be responsible for limiting the formation of stable MTs to the lamella of migrating fibroblasts (Palazzo et al., 2004). Integrin engagement activates focal adhesion kinase (FAK) which regulates the Rho-mDia MT stabilization pathway indirectly by controlling the distribution of specific ganglioside GM1 lipid domains ("lipid rafts"). GM1 lipid rafts are formed near the leading edge in an integrin and FAK-dependent manner and in some way limit the ability of Rho to activate mDia to these sites (Palazzo et al., 2004).

In budding yeast, there are two genetically separable pathways that involve MT capture and contribute to the proper orientation and positioning of the nucleus and spindle during cell division. One of these pathways is homologous to the mammalian MT stabilization pathway described above (Fig. 3). Like the mammalian pathway, this yeast pathway is regulated by Rho GTPases and involves a formin, Bni1 (Lee et al., 1999), and the tip proteins Bim1 (an ortholog of EB1) and Kar9 (Miller and Rose, 1998; Korinek et al., 2000; Lee et al., 2000; Miller et al., 2000), which may be a functional homolog of APC. This pathway regulates the stabilization of MTs at bud sites, although unlike the mammalian pathway, the MTs are only transiently stabilized and undergo a controlled shrinkage while remaining attached to the bud (Adames and Cooper, 2000). The other pathway involves dynein, dynactin, Pac1 (LIS1 homolog) (Lee et al., 2003; Sheeman, 2003), Num1 (Heil-Chapdelaine et al., 2000; Carvalho et al., 2004) and Bik1 (a CLIP170 ortholog) (Carvalho et al., 2004) and regulates capture and sliding of MTs along the bud cortex (Carminati and Stearns, 1997; Adames and Cooper, 2000; Carvalho et al., 2004). This pathway is not known to be regulated by Rho GTPases.

Although Rho GTPases regulate the activity of Bni1 in yeast (Kohno et al., 1996; Evangelista et al., 1997; Dong et al., 2003), there is no evidence yet that Bni1 is directly involved in MT capture as in mammalian cells. Instead, cytoplasmic MTs use actin cables that are nucleated by Rho stimulation of Bni1 in the bud as tracks to guide the MTs toward the bud.

This process is mediated by the type V myosin, Myo2, which orients MTs by interacting with the MT tip protein Kar9 (Beach et al., 2000; Yin et al., 2000; Liakopoulos, 2003). Kar9's location on MT tips is dependent on another tip protein Bim1 (Beach et al., 2000; Liakopoulos, 2003; Maekawa, 2003). Kar9 is specifically loaded onto MTs at the spindle pole body destined for the daughter cell and may be transported to MT ends by a kinesin (Liakopoulos, 2003; Maekawa, 2003). There maybe further interactions of MTs that arrive in the bud, since this is where MTs appear to be anchored while they shrink. It is not yet known whether there is a type V myosin involved in mammalian MT stabilization pathway (Fig. 3).

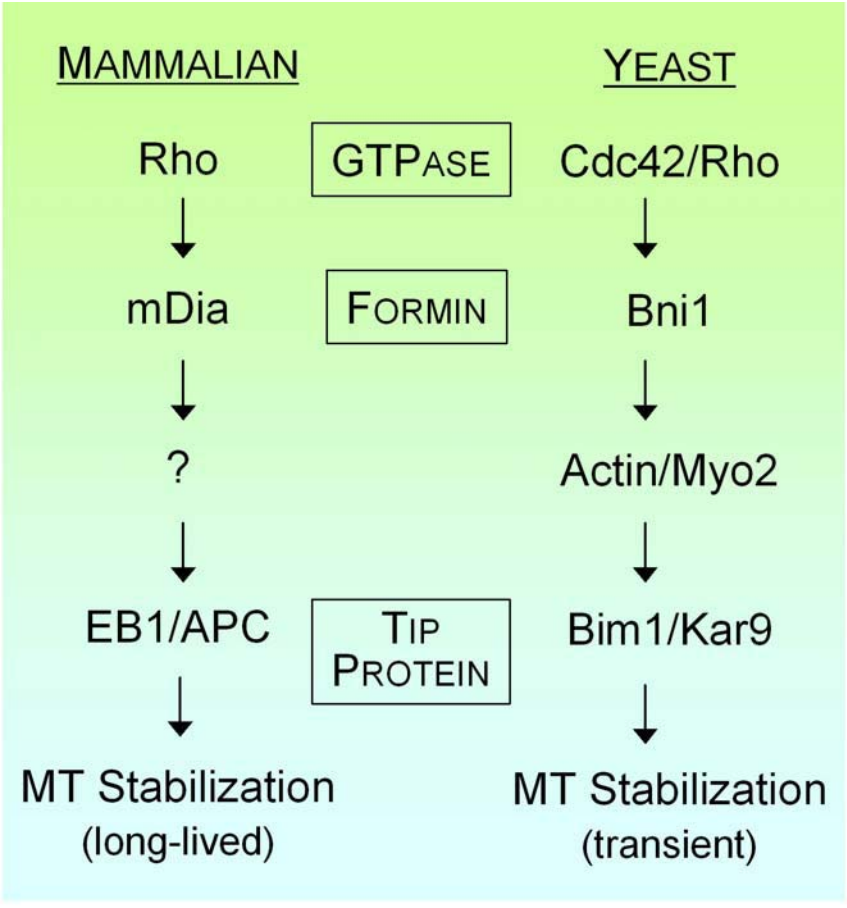

Figure 3. Evolutionary conservation of Rho-mediated MT stabilization pathways. In both mammals and yeast, Rho regulates homologous formins and MT tip proteins to stabilize MTs.

3.2 Regulation of MT Dynamics by Rho Kinase

Rho activation promotes MT stabilization in many cells types (fibroblasts, endothelial cells, endodermal cells and lymphocytes), but may have a contradictory effect on MT stability in neuronal cells. Activation of Rho causes collapse of the neuronal growth cone and inhibits neurite outgrowth (Luo, 2000; Nikolic, 2002). At least in some cases, growth cone collapse depends on MTs, as addition of taxol prevents the collapse (McNeil et al., 1999). Thus, in neurons, Rho may actually inhibit MT stabilization, correlating with the observation that LPA promotes a decrease in detyrosinated MTs, a marker of stable MTs, in neuronal cells (Sayas et al., 2002).

The downstream effectors mediating Rho's affects on neurons have not been definitively identified, although possible candidates include Rho kinase/ROK/ROCK and the PKC-related kinase, PKN. Inhibition of Rho kinase blocks LPA-triggered growth cone collapse (Arimura et al., 2000) and stimulates neurite outgrowth (Bito et al., 2000; Schmidt et al., 2002). Possible MT targets of Rho kinase include the tubulin binding protein CRMP-2 and the MAPs, tau and MAP2. Rho kinase phosphorylates CRMP-2 and this decreases its affinity for tubulin (Fukata et al., 2002). CRMP-2 stimulates MT polymerization in vitro and is enriched in the growth cone (Fukata et al., 2002; Arimura et al., 2004). How the phosphorylation CRMP-2 affects its MT polymerizing activity has not been studied directly, but since phosphorylated CRMP-2 binds tubulin weakly, it may decrease it. Nonetheless, Rho kinase modulation of CRMP-2 activity constitutes another signaling pathway by which Rho can affect MTs, although it is likely to be restricted to neurons where CRMP-2 is expressed (Fig. 2). CRMP-2 may also affect Rho itself, since it interacts with the Rho GEF LARG (Arimura et al., 2004).

Rho kinase also affects the neuronal specific MAPs tau and MAP2, although this is less well-studied than CRMP-2. Rho kinase phosphorylates tau and MAP2 in vitro (Amano et al., 2003), and phosphorylation of these proteins decreases their affinity for MTs. However, it is not yet clear whether tau or MAP2 are phosphorylated during growth cone collapse. PKN is another Rho effector that is capable of phosphorylating tau and interfering with its binding to MTs (Kawamata et al., 1998; Taniguchi et al., 2001).

3.3 Rho Kinase and Centrosomes

Rho kinase has been demonstrated to have effects on the minus-end of MTs. A centrosome-specific monoclonal antibody was found to react with Rho

kinase, and it localized to the mother centriole and a linker structure between the centrioles. Inhibition of Rho kinase by treatment with Y-27632, expression of dominant negative Rho kinase, or siRNA knockdown of Rho kinase, caused separation of centrioles in cultured cells (Chevrier et al., 2002). Exposure of dividing cells to the drug did not affect centrosomal movements during mitosis, but caused the mother centriole to remain in motion after the completion of mitosis. What targets Rho kinase to centrioles and what it does there are unknown.

4. CDC42 EFFECTS

4.1 Centrosome Positioning Through Modulation of Dynein

Another well-characterized effect of Rho GTPases on MT arrays is the Cdc42 regulation of centrosome or MTOC (MT organizing center) position. In many migrating interphase cells this is manifest as a reorientation of the centrosome to a specific position between the nucleus and the leading edge. In T cells interacting with their targets, a similar reorientation of the centrosome occurs, but here the centrosome is positioned so that it lies between the nucleus and the site of target cell interaction. In asymmetrically dividing C. elegans embryos, the centrosomes of the spindle are positioned asymmetrically rather than symmetrically and this involves Cdc42. In all these cases, the specific positioning of the centrosome with respect to other cellular structures gives the cell polarity. Common to most of these systems is the Cdc42 effector Par6 in a complex with atypical PKC (aPKC) and the motor protein dynein (Fig. 4).

The involvement of Cdc42 in centrosome positioning was first observed in T-cells, where dominant negative and constitutively active Cdc42 inhibited the positioning of the centrosome towards the antigen-presenting cell (Stowers et al., 1995). Further information about the pathway regulated by Cdc42 has come from studies of wounded monolayer systems and from asymmetrically dividing C. elegans embryos. In astrocytes and fibroblasts wounding combined with serum factors such as LPA, lead to a rise in Cdc42 GTP levels, triggering centrosome reorientation (Etienne-Manneville, 2001; Palazzo, 2001b). Wounding alone does not appear to be sufficient to induce centrosome reorientation as serum-starved fibroblasts do not reorient their centrosomes until serum or LPA is added (Palazzo, 2001b). Cdc42 is

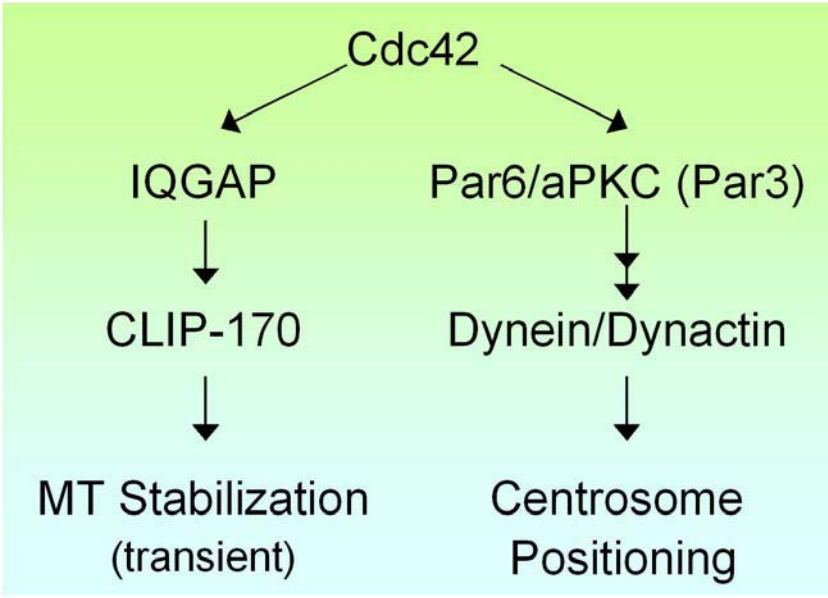

Figure 4. Defined Cdc42 signaling pathways regulating MTs. Activation of Cdc42 leads to transient MT stabilization through its effector IQGAP and the MT tip protein CLIP-170. Activated Cdc42 also regulates centrosome positioning via its effector Par6, aPKC, dynein and dynactin. In *C. elegans*, additional proteins (e.g., Par3) function with Par6 and aPKC to mediate asymmetric spindle positioning.

activated by shear stress in endothelial cells and this stimulates centrosome reorientation (Tzima et al., 2003).

Par6, a polarity protein initially discovered in a screen for proteins that affect the first asymmetric division in C. elegans embryos, is one of the Cdc42 effectors involved in centrosome reorientation in astrocytes, endothelial cells and fibroblasts (Etienne-Manneville, 2001; Tzima et al., 2003; Gomes, 2004). Par6 forms a complex with aPKC and aPKC activity is necessary for centrosome reorientation in all three cell types. It is not known how the Par6-aPKC complex might regulate the proximal MT targets of this pathway, which are presumably dynein or its regulator dynactin (Fig. 4) (also see below). The Par6-aPKC complex is capable of phosphorylating and inactivating GSK3β and this appears to be important for centrosome reorientation in astrocytes (Etienne-Manneville, 2003), but not in fibroblasts, where GSK3β regulates MT stability, but has no effect on centrosome reorientation (C.H.E and G.G.G., unpublished observations). Indeed, in fibroblasts, the Rho-mDia-EB1-APC pathway regulating MT stability and the Cdc42-Par6-aPKC-dynein pathway regulating centrosome reorientation

function independently of each other, indicating that MT stabilization is not required for centrosome reorientation and vice versa (Palazzo, 2001b; Wen et al., 2004).

One potential clue for how Par6-aPKC may regulate dynein and dynactin comes from localization studies. In astrocytes, Cdc42, Par6 and aPKC are localized at the leading edge (Etienne-Manneville, 2003). In fibroblasts, dynein and dynactin also accumulate at the leading edge (Dujardin et al., 2003). Some of the dynein and dynactin appear as puncta on the ends of MTs, suggesting that dynein and dynactin may capture MTs and pull on them to reorient the centrosome (Dujardin et al., 2003). Although there is evidence for cortical capture and pulling on MTs by dynein in budding yeast (Adames and Cooper, 2000), and pulling forces have been studied in C. elegans(Grill et al., 2001; Colombo et al., 2003; Grill et al., 2003), there is no direct evidence in fibroblasts that the centrosome is moved to the front of the cell by pulling forces. In fact, imaging experiments now indicate that it is the movement of the nucleus rearward that is responsible for orienting the centrosome in fibroblasts, rather than a forward movement of the centrosome (see below).

By directly imaging the centrosome during LPA-induced centrosome reorientation, Gomes et al., found that centrosome reorientation in fibroblasts occurs through rearward nuclear movement, rather than a movement of the centrosome toward the front of the cell (Gomes, 2004). Nuclear movement is also regulated by Cdc42 but utilizes a second effector known as myotonic dystrophy related kinase Cdc42 binding kinase (MRCK) (Leung et al., 1998). These in turn activate actin-myosin centripetal flow and move the nucleus rearward (Gomes, 2004). This study also showed that the Par6-aPKC, dynein and dynactin pathway does not participate in nuclear movement, but instead functions to keep the centrosome at the cell centroid. This dynein activity is critical for proper centrosome reorientation as it prevents the centrosome from moving rearward with the nucleus (Gomes, 2004). It is not yet known whether rearward nuclear movement is responsible for centrosome reorientation in other cell types. In T cells, the centrosome does move toward the target cell during reorientation, although it is not known whether this is mediated by the same Cdc42 effectors (Kuhn and Poenie, 2002).

Cdc42 also contributes to the regulation of the spindle centrosome (pole) position in the first, asymmetric cell division of C. elegans embryos and certain aspects of this resemble centrosome positioning in migrating cells. As with centrosome positioning in migrating cells, asymmetric spindle pole position involves Par6 and aPKC, although there are addition Par proteins (for partitioning defective) involved and these are not known to be involved in migrating cells (Kemphues, 2000; Ahringer, 2003; Schneider and

Bowerman, 2003; Cowan and Hyman, 2004). The first division produces a slightly larger daughter cell (termed AB) that is the progenitor for the anterior of the animal, while the smaller daughter cell (termed P1) gives rise to the posterior of the animal. This asymmetric division results from the displacement of the posterior spindle pole toward the posterior of the embryo. Par6-aPKC and Par3, which interacts with Par6, are localized in a Cdc42 dependent manner on the anterior pole of the embryo (Gotta et al., 2001; Kay and Hunter, 2001). Other Par proteins (Par1 and Par2) localize to the posterior pole. The polarized localization of Par proteins generates unequal pulling forces on either side of the spindle, causing the spindle to be pulled towards the posterior side of the embryo (Grill et al., 2001). It is likely that dynein is involved in this process, although this has not been shown directly. Dynein and its regulator dynactin are known to be involved in earlier aspects of spindle alignment in C. elegans (Skop and White, 1998; Gonczy et al., 1999). The unequal distribution of the Par proteins also affects the dynamics of astral MTs, as MTs in the anterior cortex persist ~15% longer that those in the posterior cortex (Labbe et al., 2003). Whether these differences contribute to or reflect the asymmetric pulling forces is unknown.

An interesting new twist on this story is that heterotrimeric G proteins are involved in regulating the pulling forces. The activity of the Gα subunit appears to be regulated by asymmetrically localized G protein regulators, GPR1 and GPR2 (Colombo et al., 2003). These proteins work downstream of the Par proteins as knocking them down by RNAi blocks pulling forces and asymmetric division without altering the polarized distribution of the Par proteins (Colombo et al., 2003; Grill et al., 2003). It will be interesting to see how the Cdc42 regulated events are coordinated with the heterotrimeric G proteins in this system.

4.2 Capture by IQGAP and mDia3

Another instance of MT capture regulated by Cdc42 in mammalian cells is through the interaction of the tip protein CLIP-170 and the cortical actin binding protein IQGAP (Fig. 4). IQGAP is an effector of both Rac and Cdc42, and binds actin through its N-terminal calponin homology domain. Fukata et al. used a 2-hybrid screen to identify CLIP-170 as an IQGAP binding protein (Fukata, 2002). CLIP-170 promoted the binding of IQGAP to MTs in vitro, and this was enhanced by active Rac1 and Cdc42. The CLIP-170 binding domain of IQGAP on the C-terminus (IQGAP-CT) caused the loss of CLIP-170 from the ends of microtubules, which subsequently led to an alteration in the entire MT array. Finally, microinjection of activated Rac or Cdc42 led to pausing of CLIP-170 spots

(and presumably MTs) for up to 2 min at Rac-induced lamellipodia or at the base of Cdc42-induced filopodia. These data provide evidence for a signaling pathway in which Rac and Cdc42 stimulate the interaction of IQGAP with CLIP-170-bound MTs at the cell cortex and stabilize them for up to 2 minutes (Fukata, 2002). Although the stabilization is transient, this may contribute to MT accumulation at sites such as the leading edge where IQGAP is abundant.

The relationship between these transiently stabilized MTs produced by Cdc42-IQGAP-CLIP170 and the long-lived stable MTs produced by Rho-mDia-EB1-APC is not known, although interfering with Rac or Cdc42 does not inhibit the formation of long-lived MTs (Palazzo, 2001b). This suggests that the transient stabilization induced by Rac and Cdc42 is not a precursor to the long-lived stable MTs produced by Rho-mDia.

While Rho regulates mDia1 and mDia2 to induce MT capture and stability in interphase cells, the third member of this family, mDia3, appears to promote MT capture at the kinetochore during mitosis and this is regulated by Cdc42 (Yasuda et al., 2004) Unlike mDia1, which is activated specifically by Rho, mDia3 can be activated by either Rho, Rac or Cdc42 (Yasuda et al., 2004). In mitosis, it is Cdc42 that appears to be responsible for mDia3's effects on the spindle (Yasuda et al., 2004). The kinetochore protein CENP-A and heterochromatin protein 1 bind to mDia3 in a 2-hybrid assay and partially co-localize with mDia at kinetochores. Knockdown of mDia3, but not mDia1, with siRNA results in a higher mitotic index with most cells in prometaphase. The arrested cells display defects in chromosome alignment, suggesting that mDia3 is required for proper spindle function (Yasuda et al., 2004). As mDia1 and mDia2 bind EB1 and APC (Wen et al., 2004), and both of these proteins localize to kinetochores, it would be interesting to examine whether mDia3 also binds to these plus-end binding proteins and whether together these contribute to MT capture at the kinetochore.

5. RAC AND MICROTUBULE DYNAMICS

5.1 Dynamics via Stathmin

In the lamella of PtK epithelial cells there is a subset of MTs, which have been termed "pioneer MTs", that are characterized by their proximity to the leading edge, reduced catastrophe frequency, and increased growth rate (Wittmann et al., 2003). It is not clear whether these pioneer MTs are themselves biochemically distinct, but expression of activated Rac1 in PtK

epithelial cells causes nearly all the MTs to exhibit behavior similar to pioneer MTs with a net overall increase in growth rate (Wittmann et al., 2003). Expression of dominant negative Rac1 eliminates pioneer MT behavior. The Rac1 effector Pak1 appears to be at least partially responsible for the effect of Rac on MTs, although activation of Pak did not completely mimic the effect of active Rac (Wittmann et al., 2003).

One way in which Rac causes changes in dynamics is through the phosphorylation of the microtubule-destabilizing protein stathmin (Fig. 5). By MALDI-MS analysis, Daub et al identified stathmin as a protein whose phosphorylation was induced by EGF but inhibited by inactivating Rac and Cdc42 (Daub et al., 2001). Pak1 phosphorylates stathmin at Ser16 in vitro (Wittmann et al., 2004), and dominant negative Pak1 blocks EGF-stimulated phosphorylation of stathmin in vivo (Daub et al., 2001). Phosphorylation at this site blocked the ability of stathmin to inhibit MT polymerization and promote catastrophe in vitro. While expression of active Rac was sufficient to phosphorylate stathmin in vivo, expression of constitutively active Pak1 was not, suggesting that other factors are necessary to mediate the phosphorylation of stathmin in vivo and regulate MT behavior (Wittmann et al., 2004). Nonetheless, these results establish a novel signaling pathway through which Rac and perhaps Cdc42, which also binds Pak1, can regulate MT dynamics (Fig. 5).

Stathmin interacts with tubulin in a gradient in motile cells with the lowest level of interaction near the leading edge (Niethammer et al., 2004). This is consistent with observations that Rac and Pak1 are more active toward the leading edge (Kraynov et al., 2000; Sells et al., 2000). The formation of a gradient of stathmin-tubulin interaction may be another way that Rho GTPases affect MTs.

5.2 Pak1 and regulation of MTs at the centrosome

Effectors of Rho GTPases are also localized at the centrosome and may function there. One such case is that of Pak1, where it can regulate astral microtubules at the centrosome in addition to regulating stathmin/Op18 as mentioned above. Pak1 localizes to the centrosome during interphase and the spindle during mitosis, but only when it is phosphorylated at T212 (Banerjee et al., 2002). This site is phosphorylated by the cell-cycle promoting kinase cyclin B1/cdc2. Phospho-peptides mimicking the T212 site caused disorganization and elongation of the MT array upon nocodazole growback, and during mitosis, enhanced the number and length of astral MTs. These results suggest that pT212-Pak1 may act to destabilize MTs at the centrosome, and that interfering with its function results in elongated MTs.

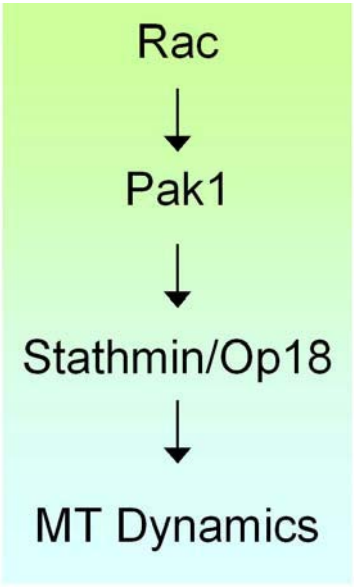

Figure 5. Defined Rac signaling pathway regulating MTs. The Rac effector Pak1 phosphorylates stathmin/Op18, a microtubule destabilizing protein, which alters MTs dynamics.

Whether this activity of Pak1 is regulated by Rac or Cdc42 or requires the kinase activity of Pak1 is unknown, as is the centrosomal protein(s) that interacts with T212-Pak1.

5.3 MT Regulation by Novel PAKs

In addition to the effects of Pak1 on stathmin and centrosomes, a novel Pak identified in Xenopus, X-PAK5, has been demonstrated to alternate between MTs and actin structures (Cau et al., 2001). X-PAK5 is most closely homologous to the human Pak4 isoform. This protein colocalizes with all cytoskeletal components, and colocalization of X-PAK5 with MTs increases during cell cycle progression, culminating during mitosis, where X-PAK5 localizes to spindle but not astral microtubules. Interestingly, kinase-dead X-PAK5 binds to MTs, whereas the active protein has a higher affinity for actin structures. In response to Rac1 or Cdc42 expression, X-PAK5 relocalizes to actin without a significant change in the kinase activity. Whether mammalian Pak4 exhibits such behavior is not known. Although numerous proteins have been identified that can bind to both actin and microtubules, X-PAK5 is an interesting example where GTPase interaction

appears to alter the affinity of an effector for one cytoskeletal element versus another.

6. ATYPICAL RHO GTPASES AND REGULATION OF MTS

While most of the evidence for Rho GTPase regulation of MTs comes from studies of the "typical" Rho GTPases (Rho A, B, C, Rac1, 2 and 3, and Cdc42), there is recent evidence that other members of the family may contribute to MT regulation. To date there are only a few examples of regulation of MTs by "atypical" Rho GTPases, yet this subgroup includes at least 15 additional GTPases (TC10, TCL, CHP(1,2), Rho G, Rnd (1,2,3) (Rnd3 is also known as RhoE), Rho BTB (1,2), Rho D, Rif, Rho H/TTF and Miro 1 and 2) (Wherlock and Mellor, 2002), so it is likely there will be more examples in the future.

Rho G, which has been implicated in regulating the actin cytoskeleton by activating Rac and Cdc42 (Gauthier-Rouviere et al., 1998), may also affect MTs through the pathways described above. RhoG also interacts with the proposed kinesin linker protein kinectin (Vignal et al., 2001). Rho G is not known to regulate MTs, but through kinectin may regulate kinesin dependent motor activities. Indeed, in cells expressing either active G12VRhoG or kinectin, lysosome motility was stimulated (Vignal et al., 2001). RhoG may also affect the activation of Rac and Cdc42 through kinectin and kinesin (see below).

Rnd2 is another Rho GTPase that has been implicated in regulating MTs. Rnd2 is primarily expressed in the nervous system, whereas Rnd1 and Rnd3 are more widely expressed (Foster et al., 1996; Nobes et al., 1998). Rapostlin is a recently identified Rnd2 specific effector that is very similar to the previously described human formin-binding protein 17 (FBP17) (Fujita et al., 2002). Rapostlin interacts with MTs though an N-terminal Fer-Cip4 homology domain (Fer-CHD) and coexpression with Rnd2 in PC-12 cells induces neurite branching, suggesting that it may affect MTs or activities associated with MTs (Fujita et al., 2002). Cip4 was the first protein identified with a Fer-CHD domain (Tian et al., 2000). It also contains an N-terminal Fer-CHD that binds MTs but additionally binds WASP through a region near its C-terminus. Overexpression of CIP4 leads to WASP recruitment to MTs, although it is not yet known whether Cip4 regulates MTs (Tian et al., 2000).

7. MT REGULATION OF RHO GTPASES

7.1 Regulation of Rho

The initial evidence that MTs may regulate Rho activation came from the observation that drug-induced breakdown of MTs stimulated cell contractility (Danowski, 1989). Subsequent work showed that colchine/nocodazole stimulated the formation of actin stress fibers and focal adhesions and that this was blocked by inhibitors of Rho (Bershadsky et al., 1996; Enomoto, 1996; Liu et al., 1998). When pull-down assays were developed to measure Rho GTP levels in cells, Schwartz and colleagues showed that colchicine treatment directly led to increased Rho GTP levels in cells (Ren et al., 1999). The activation of Rho by colchicine leads to levels of Rho GTP that are as high as those observed with growth factor treatments (e.g., LPA) and persist for hours.

The mechanism by which MT breakdown leads to Rho activation has been mysterious, although with the discovery of Rho GTP exchange factors (GEFs) that bind to MTs (see Table 2), attention has focused on these molecules as possible candidates. At least three separate Rho GEFs have been shown to interact with MTs in vivo: the human Rho GEF-H1 (Ren et al., 1998; Krendel et al., 2002), its murine ortholog, Lfc (Glaven et al., 1999) and p190Rho GEF(van Horck et al., 2001). All three of these proteins have the typical Dbl and PH domains found in Rho GEFs and have been shown to activate Rho by stimulating the exchange of GDP for GTP. Their GEF activity may be specific for Rho, although can both bind Rac (Ren et al., 1998; Glaven et al., 1999).

The interaction of Lfc with MTs may involve its pleckstrin homology (PH) domain, since a construct lacking this domain did not colocalize to MTs in vivo and an isolated PH domain could pulldown tubulin (Glaven et al., 1999). However, the PH domain was not tested for binding to polymerized MTs. Rho GEF-H1 appears to be the human ortholog of Lfc yet it does not appear to interact with MTs through its PH domain. Instead, its interaction with MTs requires a putative Zn-finger domain at its N-terminus and a coiled-coil domain at its C-terminus (Krendel et al., 2002). Rho GEF-H1 has not yet been demonstrated to interact directly with MTs and the interaction may require other proteins. The structurally related p190Rho GEF has been shown by both colocalization in vivo and sedimentation studies with taxol-stablized MTs in vitro to interact with MTs (van Horck et al., 2001). As with Rho GEF-H1, p190Rho GEF has an N-terminal Zn-finger and a C-terminal coiled-coil domain. Constructs lacking the N-terminus still bind to MTs, whereas those lacking the coiled-coil do

not and an isolated coiled-coil protein can interact directly with MTs in vitro (van Horck et al., 2001). These results suggest that at least for p190Rho GEF the interaction with MTs is direct.

Recent work has shown that the activity of Rho GEF-H1 is regulated by interaction with MTs (Krendel et al., 2002; Zenke et al., 2004). Compared to full length Rho GEF-H1, which localizes to MTs, mutant constructs that do not interact with MTs show increased Rho activity in vivo (Krendel et al., 2002). Since these constructs all showed equivalent Rho GEF activity in vitro, this suggests that interaction with MTs negatively regulates Rho GEF-H1 activity. Also, dominant negative forms of Rho GEF-H1 are able to block the nocodazole-induced increase in Rho activity (Krendel et al., 2002). Chimeras with MT binding defective mutants of Rho GEF-H1 and the MT-binding domain of MAP2c, restored the localization to MTs and the downregulation of Rho GEF-H1 activity (Zenke et al., 2004). How the interaction of Rho GEF-H1 with MTs inhibits the GEF activity is unknown and appears to require additional factors since MT inhibition of GEF activity cannot be reconstituted in vitro with MTs and purified Rho GEF-H1 (Zenke et al., 2004). Whether p190Rho GEF functions similarly is unknown, although given the similarity in structures it would not be surprising if it did.

These studies strongly suggest that MTs can negatively regulate activation of Rho by decreasing the activity of Rho GEFs. How this would affect the physiological regulation of Rho GTP levels is currently unclear. MTs could release Rho GEFs to activate Rho locally or could act as a sink and sequester Rho GEFs to down regulate Rho. Perhaps, in regions of more dynamic MTs, such as the leading edge, there would be relatively more release of Rho GEFs and this could enhance Rho activity relative to areas where there are more stabilized MTs that do not release the Rho GEFs.

7.2 Regulation of Rac

Rac GTP levels also appear to be regulated by MTs, although the molecular mechanisms have not been deciphered. Unlike the situation with Rho, MT growth seems to be responsible for activating Rac (Waterman-Storer et al., 1999). This conclusion was based upon the finding that regrowth of MTs following nocodazole wash-out, led to increased Rac GTP levels and that cells increased their ruffling. That MT regrowth stimulates Rac levels may reflect the reciprocal regulation of Rac and Rho activity in cells (Kjoller and Hall, 1999); as Rho GTP levels are high in cells depleted of MTs, allowing MT regrowth may lower Rho levels, which in turn would cause Rac levels to rise. Direct measurements of Rho and Rac activity in macrophage polykaryons has shown reciprocal changes in Rho and Rac GTP levels after nocodazole-treatment and washout (Ory et al., 2002).

There are more direct ways that MTs may affect Rac levels (see Table 2). APC, which is involved in MT capture (see above), also contains a binding site for ASEF, a Rac GEF (Kawasaki et al., 2003). The interaction of APC with ASEF stimulates the Rac exchange activity of ASEF (Kawasaki et al., 2003). Since APC travels along MTs via the kinesin, Kif3 (Jimbo, 2002), this may deliver ASEF to sites to activate Rac. Consistent with this, APC is observed in clusters at extending edges of epithelial cells (Nathke et al., 1996). Tubulin has also been reported to associate with the Rac GEF Vav in immune cells (Huby et al., 1995; Fernandez et al., 1999). Whether Vav interacts with polymerized MTs is unknown and the significance of the interaction of Vav with tubulin awaits further study. Nonetheless, Vav is another potential target of MT regulation of Rac.

During cytokinesis another type of Rac regulation may be mediated by MTs. In a number of organisms, the Rac GAP, MgcRacGAP (mammals (Hirose et al., 2001); Cyk-4, C. elegans, (Jantsch-Plunger et al., 2000) and RacGAP50C, Drosophila (Somers and Saint, 2003)) interact with the kinesin MLKP1/CHO1 (mammals (Kuriyama et al., 2002); Zen-4, C.elegans (Mishima et al., 2002) and Pavarotti, Drosophila (Adams et al., 1998)) and regulate the formation of cortical MTs important for the progression of cytokinesis. The Rac GAP and the kinesin contribute to bundled MTs in the spindle midzone and ultimately the midbody, a late cytokinesis structure, and a complex of the two proteins bundles MTs in vitro (Mishima et al., 2002). These proteins also contribute to the regulation of a Rho GEF localized in the furrow that is involved in the regulation of actin and myosin contractile ring formation (Mishima et al., 2002; Somers and Saint, 2003).

7.3 Regulation of Cdc42

The effects of MT antagonists on global Cdc42 activation have not been explored as they have for Rho and Rac. However, in a recent study examining the local activation of Cdc42, Hahn and colleagues observed that local activation of Cdc42 at the edges of cells was inhibited by nocodazole breakdown of MTs (Nalbant et al., 2004). As with Rac, the mechanism for MT regulation of Cdc42 is unclear. On potential candidate is RhoG. In its active form, RhoG dissolves stress fibers and enhances formation of peripheral ruffles in a Rac and Cdc42 dependent manner (Gauthier-Rouviere et al., 1998). These events appear to require MTs suggesting that RhoG may stimulate kinesins to deliver factors that affect Cdc42 and Rac activation. There is also abundant Cdc42 at the Gogli (Cerione, 2004) and MT delivery of membrane vesicles laden with Cdc42 may also contribute to the local activation of Cdc42.

8. CONCLUSIONS – COORDINATION OF THE MT AND ACTION CYTOSKELETONS

MTs are major targets of Rho GTPase regulation and numerous signaling pathways through which Rho GTPases affect MTs have been discovered. In at least one case, the Rho GTPase signaling pathway is conserved from yeast to mammals. Study of Rho GTPase regulation of MTs has contributed to the understanding of a new type of MT regulation involving the capture of MT plus ends. The complexity of MT capture and other MT processes regulated by Rho GTPases suggests there is still much to learn. For example, we have just begun to appreciate how Rho GTPase effectors affect events at the minus ends of MTs and how atypical Rho GTPases regulate MTs. It is now clearer that regulation of MTs by Rho GTPases provides one way that signaling pathways coordinate responses of multiple cytoskeletal systems during complex cell behaviors. MTs in turn modulate Rho GTPases and this provides the opportunity for additional regulation, including the possibility of positive and negative feedback. Further elucidation of the pathways through which Rho GTPases and MTs affect each other is an exciting prospect for future research.

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