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## REVIEW

# Rho GTPases and signaling networks

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The Rho GTPases form a subgroup of the Ras superfamily of 20- to 30-kD GTP-binding proteins that have been shown to regulate a wide spectrum of cellular functions. These proteins are ubiquitously expressed across the species, from yeast to man. The mammalian Rho-like GTPases comprise at least 10 distinct proteins: RhoA, B, C, D, and E; Rac1 and 2; RacE; Cdc42Hs, and TC10. A comparison of the amino acid sequences of the Rho proteins from various species has revealed that they are conserved in primary structure and are 50%–55% homologous to each other. Like all members of the Ras superfamily, the Rho GTPases function as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state. Until recently, members of the Rho subfamily were believed to be involved primarily in the regulation of cytoskeletal organization in response to extracellular growth factors. However, research from a number of laboratories over the past few years has revealed that the Rho GTPases play crucial roles in diverse cellular events such as membrane trafficking, transcriptional regulation, cell growth control, and development. Consequently, a major challenge has been to unravel the underlying molecular mechanisms by which the Rho GTPases mediate these various activities. Many targets of the Rho GTPases have now been identified and further characterization of some of them has provided major insights toward our understanding of Rho GTPase function at the molecular level. This review aims to summarize the general established principles about the Rho GTPases and some of the more recent exciting findings, hinting at novel, unanticipated functions of the Rho GTPases.

## Regulators of the Rho GTPases

Like all members of the Ras superfamily, the activity of the Rho GTPases is determined by the ratio of their GTP/GDP-bound forms in the cell (Boguski and McCormick 1993). The ratio of the two forms is regulated by the opposing effects of guanine nucleotide exchange factors (GEFs), which enhance the exchange of bound GDP for GTP, and the GTPase-activating proteins (GAPs), which increase the intrinsic rate of hydrolysis of bound

GTP. In addition, the Rho-like GTPases are regulated further by guanine nucleotide dissociation inhibitors (GDIs), which can inhibit both the exchange of GTP and the hydrolysis of bound GTP.

## GEFs

GEFs for Rho-like GTPases belong to a rapidly growing family of proteins that share a common motif, designated the Dbl-homology (DH) domain for which the Dbl oncogene product is the prototype (Cerione and Zheng 1996). The *Dbl* oncogene was originally discovered by its ability to induce focus formation and tumorigenicity when expressed in NIH-3T3 cells (Eva and Aaronson 1985). The first clue to Dbl's function as a GEF came from the observation that it contains 29% sequence identity with the *Saccharomyces cerevisiae* cell division cycle protein Cdc24, which by genetic analysis was placed upstream of the yeast small GTP-binding protein Cdc42 in the bud assembly pathway (Ron et al. 1991). Biochemical analysis has shown that Dbl is indeed able to release GDP from the human homolog of Cdc42 in vitro. Furthermore, deletion analysis of the Dbl protein demonstrated that the DH domain was essential and sufficient for this activity and that this domain was also necessary to induce oncogenicity (Hart et al. 1991; Ron et al. 1991; Hart and Roberts 1994). In addition to the DH domain, Dbl and the yeast Cdc24 share a pleckstrin homology (PH) domain, which is essential for proper cellular localization (Zheng et al. 1996).

Since the discovery of Dbl, a growing list of mammalian proteins containing both a DH and a PH domain has been assembled (for review, see Cerione and Zheng 1996). The majority have been identified as oncogenes in transfection assays (see Table 1). Tiam, however, was first identified as an invasion-inducing gene using proviral tagging in combination with in vitro selection for invasiveness (Habets et al. 1994). Two other members of the DH protein family, Fgd1 and Vav, have been shown to be essential for normal embryonic development (Pasteris et al. 1994; Tarakhovskiy 1995; Zhang et al. 1995a). Proteins containing a PH and a DH domain have also been identified in *Drosophila melanogaster* and *Caenorhabditis elegans* (Benian et al. 1996; Sone et al. 1997).

It has been generally assumed that all proteins that contain a DH and PH domain in tandem will be GEFs for Rho subtype proteins. This appears to be true for some

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**Table 1.** Mammalian GEFs for the Rho subfamily of GTPases

DH/PH-containing proteins	GEF specificity for Rho GTPases	Biological properties	Tissue distribution	References
Dbl	Cdc42, Rho	oncogenic	brain, adrenal glands, gonads	Hart et al. (1991)
Lbc	Rho	oncogenic	heart, lung, skeletal muscle	Zheng et al. (1995)
Lfc	Rho	oncogenic	hematopoietic cells, kidney, lung	Glaven et al. (1996)
Lsc	Rho	oncogenic	hematopoietic cells, kidney lung	Glaven et al. (1996); Aasheim et al. (1997)
Dbs	?	oncogenic	kidney lung predominantly brain	Whitehead et al. (1995)
Tiam	Rac	metastatic and oncogenic	brain, testis	Habets et al. (1994)
Vav	Rac, Cdc42, Rho	oncogenic, implicated in lymphocytic proliferation and lymphopenia	hematopoietic cells	Crespo et al. (1997); Han et al. (1997)
FGD1	Cdc42	implicated in faciogenital dysplasia	brain, heart, lung, kidney	Olson et al. (1996); Zheng et al. (1996)
Trio	Rac and Rho	cell migration?	ubiquitous	Debant et al. (1996)
Ost	Rho, Cdc42 (binds to Rac-GTP)	oncogenic	brain, heart, lung, liver	Horii et al. (1994)
Bcr	Rac, Cdc42, Rho (GAP for Rac)	implicated in leukemia	predominantly brain	Chuang et al. (1995)
Abr	Rac, Cdc42, Rho (GAP for Cdc42 and Rac)	?	predominantly brain	Chuang et al. (1995)
Ect-2	?	oncogenic	testis, kidney, liver, spleen	Miki et al. (1993)
Tim	?	oncogenic	kidney, liver, pancreas, lung, placenta	Chan et al. (1994)
NET1	?	oncogenic	ubiquitous	Chan et al. (1996)
SOS	?		ubiquitous	Bowtell et al. (1992); Chardin et al. (1993)
RasGEF	?		brain	Shou et al. (1992)

but not all DH/PH-containing proteins (see Table 1). Moreover, some members of the DH protein family (such as Dbl) have been shown to exhibit exchange activity in vitro for a broad range of Rho-like GTPases, whereas others appear to be more specific. Lbc, for example, and the more recently discovered oncoproteins Lfc and Lsc, are specific for Rho, whereas Fgd1 is specific for Cdc42 (Glaven et al. 1996; Zheng 1996). Although Vav previously was reported to be an activator of Ras (Gulbins et al. 1993), it has been demonstrated more recently to function as a GEF for members of the Rho family (Crespo et al. 1997; Han et al. 1997).

Further studies will be required to gain more insight into the determinants defining the selectivity of GEFs for specific Rho family members. Recently, however, several studies have been performed to determine whether the proteins that serve as Rho GEFs in vitro can perform similar functions in vivo. Microinjection experiments have demonstrated that Lbc induced stress fiber formation and Fgd1 elicited filopodial extensions selec-

tively in response to Rho and Cdc42, respectively (Olson et al. 1996). Furthermore, Fgd1 has been shown to activate the stress-activated protein kinase/c-Jun amino-terminal kinase (SAPK/JNK) signal transduction pathway, an activity mediated by Cdc42 and/or Rac (Olson et al. 1996). Both Dbl and Vav have been shown to trigger the formation of filopodia, lamellipodia, and stress fibers mediated by Cdc42, Rac, and Rho, respectively. Dbl and Vav also stimulated SAPK/JNK activity (Olson et al. 1996). Overexpression of Tiam in fibroblasts elicited the formation of membrane ruffles and activation of JNK in a Rac-dependent manner (Michiels et al. 1995, 1997). Furthermore, Michiels et al. (1997) demonstrated that an intact amino-terminal PH domain was essential for these activities.

In addition to the PH and DH domains, many of the exchange factors have other domains that are commonly found in signaling molecules, such as a Src homology (SH3) domain and a diacylglycerol-binding zinc butterfly motif, suggesting that they may have additional func-

tions (Cerione and Zheng 1996). The Ras GEF, Sos, has been shown to bind the adaptor molecule GRB2, which in turn binds to the platelet-derived growth factor (PDGF) receptor in response to growth factors (Downward 1996). However, little is known about the signaling cascades coupling the Rho GEFs to elements that function upstream of the GTPases (see below).

### GAPs

A prototype GAP protein specific for the Rho family GTPases was purified by biochemical analysis of cell extracts using recombinant Rho. This protein, designated p50Rho-GAP, was shown to have GAP activity toward Rho, Cdc42, and Rac in vitro (Hall 1990; Lancaster et al. 1994). Since then, additional proteins that exhibit GAP activity for the Rho GTPases have been identified in mammalian cells (Table 1). Also several Rho GAP-containing proteins have been discovered in *S. cerevisiae*, *Drosophila*, and *C. elegans* (Agnel et al. 1992; Chen et al. 1994, 1996; Zheng et al. 1994; Stevenson et al. 1995; Schmidt et al. 1997). These proteins all share a related GAP domain that spans 140 amino acids of the protein but bears no significant resemblance to Ras GAP. The substrate specificity of the Rho GAPs toward members of the Rho subfamily varies with each GAP protein (Table 2). Although some of these proteins exhibit GAP activity for several Rho GTPases in cell-free assays, their substrate specificities in vivo appear to be more restricted. For example, the substrate spectrum of p50Rho-GAP in vitro encompasses Cdc42, Rac, and Rho; however, in vivo, it appears to be restricted to Rho only (Ridley et al. 1993). The p190GAP, although first identified as a tyrosine-phosphorylated Ras GAP-associated protein in Src-transformed cells and in growth factor-treated

cells, was later shown to possess GAP activity for the Rho GTPases (Ellis et al. 1990; Settleman et al. 1992). Microinjection of p190GAP in fibroblasts resulted in an inhibition of Rho-mediated stress fiber formation but not Rac-induced membrane ruffling (Ridley et al. 1993). The biological implications of a direct link between Ras and Rho-mediated pathways for signaling remain unclear. Recently, two tyrosine-containing peptides in p190 have been shown to bind simultaneously to the SH2 domains of Ras GAP upon tyrosine phosphorylation of p190. This interaction appears to induce a conformational change in Ras GAP, resulting in an increased accessibility of the target binding surface of its SH3 domain. Thus, a role for p190 in the Ras GAP signaling complex may be to promote Ras GAP interactions via its SH3 domain (Hu and Settleman 1997).

Rho GAPs, in addition to accelerating the hydrolysis of GTP, may mediate other downstream functions of the Rho proteins in mammalian systems. A role for p190 in regulating Rho function in cells undergoing cytoskeletal rearrangements has been suggested (Chang et al. 1995). The N- and  $\beta$ -chimerins have been demonstrated to exhibit GAP activity toward the Rac GTPase, and microinjection of the chimerin GAP domain into fibroblasts prevented Rac- and Cdc42-induced cytoskeletal rearrangements (Diekmann et al. 1991; Leung et al. 1993; Manser et al. 1995; Kozma et al. 1996). Unexpectedly, microinjection of full-length N-chimerin as well as a chimerin mutant lacking GAP activity, resulted in the induction of lamellipodia and filopodia formation. Furthermore, the formation of the latter structures could be inhibited by dominant-negative Rac and Cdc42 mutants, suggesting that N-chimerin, in addition to functioning as a GAP, may also function as an effector (Kozma et al. 1996).

**Table 2.** Mammalian GAPs for the Rho subfamily of GTPases

Rho-GAP-containing proteins	GAP specificity for Rho GTPases <sup>a</sup>	Tissue distribution	References
p50 Rho-GAP	Cdc42*, Rac, Rho	ubiquitous	Barfod et al. (1993); Lancaster et al. (1994)
Bcr	Rac*, Cdc42	predominantly brain	Diekmann et al. (1991)
Abr	Rac, Cdc42	predominantly brain	Tan et al. (1993)
N-Chimerin	Rac	brain	Diekmann et al. (1991)
$\beta$ -chimerin	Rac	testis	Leung et al. (1993)
p190GAP	Rho*, Rac, Cdc42	ubiquitous	Settleman et al. (1992)
p85 $\alpha$	?	ubiquitous	Otsu et al. (1991)
p85 $\beta$	?	ubiquitous	Otsu et al. (1991)
3BP-1	Rac, Cdc42	spleen, kidney, lung, brain, heart	Cicchetti et al., (1992, 1995)
p122	Rho		Homma and Emori (1995)
Myr5	Rho*, Cdc42	ubiquitous	Reinhard et al. (1995)
RalBP1/RLIP76/RIP1	Cdc42*, Rac	ubiquitous	Cantor et al. (1995); Jullien-Flores et al. (1995); Park and Weinberg (1995)
Graf	Rho, Cdc42	ubiquitous; abundant in brain and liver	Hildebrand et al. (1996)

<sup>a</sup>(\*) Preferred GTPase for GAP.

## GDI<sub>s</sub>

The first GDI identified for the members of the Rho family was the ubiquitously expressed protein Rho GDI, which was isolated as a cytosolic protein that preferentially associated with the GDP-bound form of RhoA and RhoB and thereby inhibited the dissociation of GDP (Fukumoto et al. 1990; Ueda et al. 1990). Subsequently, Rho GDI was found to be active on Cdc42 and Rac (Abo et al. 1991; Leonard et al. 1992). Further studies demonstrated that Rho GDI also associated weakly with the GTP-bound form of Rho, Rac, and Cdc42 (Hart et al. 1992; Chuang et al. 1993). This weak interaction resulted in an inhibition of the intrinsic and GAP-stimulated GTPase activity of the Rho GTPases. Thus, Rho GDI appears to be a molecule capable of blocking the GTP binding/GTPase cycle at two points: at the GDP/GTP exchange step and at the GTP hydrolytic step.

The Rho GDIs appear to have a crucial role in the translocation of the Rho GTPases between membranes and the cytoplasm. In resting cells, the Rho proteins are found in the cytosol as a complex with Rho GDIs, which inhibit their GTP/GDP exchange ratio, but are released from the GDI and translocated to the membranes during the course of cell activation (Takai et al. 1995). Until recently, the mechanism by which Rho is released from Rho GDI was largely unknown. However, studies from Takahashi et al. (1997) have provided a tentative model by which this may occur. Rho GDI was found to coimmunoprecipitate with moesin, which is a member of the ERM (ezrin, radixin, moesin) family (Tsukita et al. 1994, 1997; Hirao et al. 1996). ERM proteins have been shown to bind a membrane-spanning protein, CD44, and F-actin via their amino- and carboxy-terminal regions, respectively (Hirao et al. 1996). Takahashi et al. (1997) investigated whether binding of Rho GDI to ERM affected Rho GDI activity. They observed that the amino-terminal region of radixin inhibited Rho GDI activity. Moreover, they found that binding of the amino-terminal region of radixin to Rho/Rho GDI complex resulted in the release of Rho from Rho GDI, and that Rho GEF could stimulate the GDP/GTP exchange reaction of Rho complexed with Rho GDI when radixin was present. Similar activities were found in the presence of ezrin and moesin. These data suggest that members of the ERM family are involved in the activation of Rho. Interestingly, moesin has been identified from porcine brain cytosolic extracts as a component that could reconstitute the formation of stress fibers, focal adhesion, and cortical actin polymerization in response to activation of Rho and Rac in permeabilized fibroblasts (Mackay et al. 1997). These results clearly demonstrated the involvement of members of the ERM family in the formation of stress fibers and focal adhesions, as well as actin polymerization. Whether the mechanism by which moesin can reconstitute this response involves GDI is unclear, as GDI was not detected in the purified activity.

More recently, two novel GDIs have been isolated: D4-GDI (also called LY-GDI), which is expressed only in hematopoietic tissues (Lelias et al. 1993; Scherle et al.

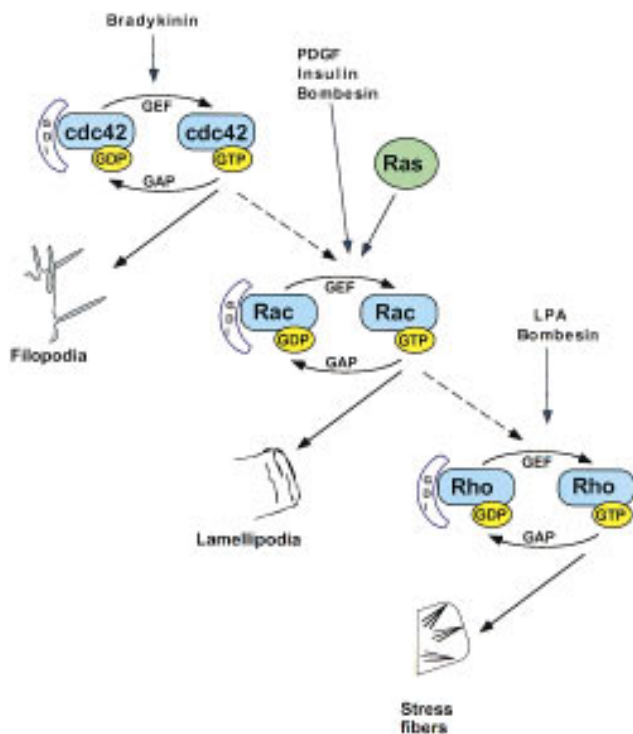
1993), and Rho GDI $\gamma$ , which is preferentially expressed in brain and pancreas (Adra et al. 1997). A murine homolog of Rho GDI $\gamma$ , designated Rho GDI3, has been cloned independently (Zalcman et al. 1996). In vitro studies showed that D4-GDI can function as a GDI for RhoA, Rac, and Cdc42 (Adra et al. 1993). The combined data of Rho GDI $\gamma$  and Rho GDI3 indicate that this third member of GDI proteins binds and functions as GDI for Cdc42, RhoB, and RhoG and possibly RhoA. Deletion and mutant analysis demonstrated that the last six amino acids and, more importantly, one residue near the carboxyl terminus of the GDI molecule, is critical for the interaction with and specificity for the GTPase. A single change at residue 174 of D4-GDI to the corresponding residue of Rho GDI imparted nearly full GDI activity on the D4 molecule (Platko et al. 1995).

Little is known about the physiological function of the Rho GDIs in vivo. Microinjection studies have shown that Rho GDI inhibits several downstream functions of Rho (Nishiyama et al. 1994; Coso et al. 1995). Deletion of the D4-GDI gene in embryonic stem (ES) cells resulted in only a subtle defect of superoxide production in D4-GDI $^{-/-}$  macrophages, a function mediated by Rac (Guillemot et al. 1996). This suggests that there may be considerable redundancy of function between the Rho GDIs. Recent findings by Na et al. (1996) showed that D4-GDI is a specific substrate of apoptotic proteases. The cleavage and inactivation of D4-GDI (and perhaps other GDIs) may be a crucial part of the process of programmed cell death and/or a mechanism for regulation of GDIs. Further studies in vivo will likely point out the necessity of Rho GDIs in several physiological events in the cell.

## Upstream signaling pathways

In Swiss 3T3 fibroblasts, Cdc42, Rac, and Rho have been placed in a hierarchical cascade wherein Cdc42 activates Rac, which in turn activates Rho (Nobes and Hall 1995) (Fig. 1). In addition, Ras has been shown to activate Rac (Ridley et al. 1992). The molecular links between these GTPases in mammalian cells are unknown. A similar cascade is believed to control bud formation and morphogenesis in the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*. In the latter organisms, a role for GEFs in linking these GTPases has been demonstrated (Chang et al. 1994; Chant and Stowers 1995; Bender et al. 1996; Matsui et al. 1996b); however, no such connections have yet emerged in mammalian cells.

Several lines of evidence indicate that the Rho-family GTPases link plasma membrane receptors to the assembly and organization of the actin cytoskeleton. In fibroblasts, extracellular stimuli have been shown to activate the Rho GTPase cascade at different points. Addition of LPA (lysophosphatidic acid) to quiescent fibroblasts induces the formation of actin stress fibers, a response that is completely blocked with prior microinjection of C3 transferase, a bacterial coenzyme that ribosylates ADP and inactivates Rho proteins (Ridley and Hall 1992). On the other hand, growth factors such as PDGF, insulin, and bombesin stimulate polymerization of actin at the



**Figure 1.** GTPase cascades involved in cytoskeleton organization in fibroblasts. Various extracellular stimuli trigger the activation of Cdc42, Rac, and Rho GTPases and elicit specific short-term responses such as the formation of filopodia, lamellipodia, and stress fibers, respectively. Moreover, Cdc42 appears to activate Rac, which in turn activates Rho. The direct links between these GTPases remain to be clarified. The Rho GTPases can be activated independently by different agonists. The mechanism by which these agonists activate Rho GTPases may involve GEFs, GAPs, or GDIs.

plasma membrane of many cell types to induce lamellipodia formation and surface membrane ruffling (Ridley et al. 1992; Nobes et al. 1995). This ruffling response can be inhibited by the dominant-negative mutant of Rac, RacN17, thereby establishing a Rac-regulated signaling pathway linking growth factor receptors to the polymerization of actin at the plasma membrane. Furthermore, the activation of Cdc42 by bradykinin results in the formation of filopodia and the subsequent formation of lamellipodia. Filopodia formation was inhibited by a dominant-negative mutant of Cdc42, Cdc42N17, whereas RacN17 inhibited only membrane-ruffling formation (Kozma et al. 1995; Nobes and Hall 1995).

Progress has been made in establishing the connection between growth factor receptors and Rho-like GTPases. The bradykinin, LPA, and bombesin receptors belong to the seven-transmembrane-domain heterotrimeric G protein-coupled receptor family. Thus, the trimeric G proteins are likely to play a role in the activation of the respective GTPases. Recently, an activated mutant form of the  $\alpha$  subunit of the heterotrimeric G protein  $G_{12}$  has been demonstrated to stimulate JNK activity in a Ras-

and Rac-dependent manner (Collins et al. 1996). Furthermore, it has been shown that signaling from m1 and m2 muscaric receptors (mAChRs) to JNK involves  $\beta\gamma$  subunits of heterotrimeric G proteins (Coso et al. 1996). In *S. cerevisiae*, pheromone signaling is mediated by  $\beta\gamma$  subunits (encoded by *STE4* and *STE18*, respectively) (White-way et al. 1989). Epistasis analysis placed Cdc24 and Cdc42 as essential components downstream of Ste4 in the pheromone signaling pathway. Furthermore Ste4 has been shown to interact with Cdc24 in the yeast two-hybrid system (Zhao et al. 1995). Taken together, these results from yeast suggest that a GEF may link the trimeric G proteins to the low-molecular-weight GTPases.

Several lines of evidence have implicated the involvement of phosphoinositide 3 kinase (PI3 kinase) in PDGF- and insulin-induced cytoskeletal rearrangements. Treatment of fibroblasts with the drug PI3 kinase inhibitor wortmannin, inhibits membrane ruffling induced by PDGF, epidermal growth factor (EGF), and insulin, although not by microinjected Rac protein (Kotani et al. 1994; Wennstrom et al. 1994; Nobes et al. 1995). Furthermore, PDGF could stimulate the level of Rac GTP by increasing GEF activity in a PI3 kinase-dependent manner (Hawkins et al. 1995). Hence, PI3 kinase appears to function upstream of Rac for the induction of membrane ruffling in response to extracellular growth factors. Moreover, a constitutively active PI3 kinase mutant has been shown to trigger membrane ruffles and stress fibers in a Rac- and Rho-dependent manner (Reif et al. 1996). Interestingly, this active mutant failed to induce Rac/Rho signaling pathways that regulate gene transcription (Reif et al. 1996). A plausible explanation given for this observation is that the Rho GTPases are linked to different upstream regulatory proteins, which may determine the interaction with different GTPase effector pathways leading to the diverse biological activities. This may explain how the Rho GTPases regulate such a wide variety of biological activities (see below). The mechanism by which PI3 kinase activates Rac is unknown but may involve GEFs, GAPs, or GDIs. Interestingly, in *S. cerevisiae*, putative phosphatidylinositol kinase homologs have been identified. Among them, TOR2 is required for organization of the actin cytoskeleton (Schmidt et al. 1996) and activates the GTPases RHO1 and RHO2 via their exchange factor ROM2 (Schmidt et al. 1997).

As wortmannin does not inhibit the RasV12-induced membrane ruffling in Swiss fibroblast cells, it suggests that PI3 kinase is not involved in Ras-mediated membrane ruffling (Nobes et al. 1995). However, Downward and coworkers reported recently that wortmannin partially blocked RasV12-induced membrane ruffling and that the inhibition was complete when the RasV12,C40 mutant was used in another cell type (Rodriguez et al. 1997). RasV12,C40 is a Ras mutant that fails to bind the serine/threonine kinase Raf and RalGDS but can still bind PI3 kinase and AF6 (Van Aelst et al. 1994; Joneson et al. 1996b; Khosravi et al. 1996; Rodriguez et al. 1997). Furthermore, Downward and coworkers showed that a dominant-negative form of PI3 kinase completely blocked RasV12-induced membrane ruffling. It is pos-

sible that the pathways leading to the activation of PI3 kinase may vary in different cell types.

More recently, two groups have demonstrated the importance of tyrosine phosphorylation of the exchange factor Vav for its ability to activate members of the Rho family both *in vitro* and *in vivo* (Crespo et al. 1997; Han et al. 1997). Incubating Vav with Lck, a member of the Src family, resulted in an increased GDP/GTP exchange activity. Furthermore, coexpression of Lck with Vav enhanced Vav-transforming activity and its ability to induce JNK activation. The mechanism by which LPA activates Rho also appears to involve a tyrosine kinase, as the LPA (but not Rho)-induced stress fiber formation can be blocked by tyrphostin, a tyrosine kinase inhibitor (Nobes and Hall 1995). An alternate mechanism for the regulation of the GTP/GDP ratio of RhoA GTPase recently has been described in cytotoxic lymphocytes. Phosphorylation of RhoA by cAMP-dependent protein kinase A (PKA) increases the affinity of RhoA in its GTP-bound form for GDI, thereby translocating RhoA from the membrane to the cytoplasm (Lang et al. 1996).

The identification of additional upstream signaling molecules will be required to gain more insight into the signaling pathways that lead to the activation of the Rho-like GTPases in response to extracellular stimuli.

### Multiple functions mediated by Rho GTPases

Although Rho was initially shown to have a role in cytoskeletal remodeling, it is now known that Rho GTPases are involved in several other cellular processes such as membrane trafficking, transcriptional activation, and cell growth control. The signal transduction pathways mediating these biological phenomena appear to be complex and interwoven. The involvement of the Rho family members in the various fundamental cellular processes as well as the recent progress made toward a better understanding of the biochemical nature of the pathways mediating these events is discussed below.

### Rho GTPases and cytoskeleton organization

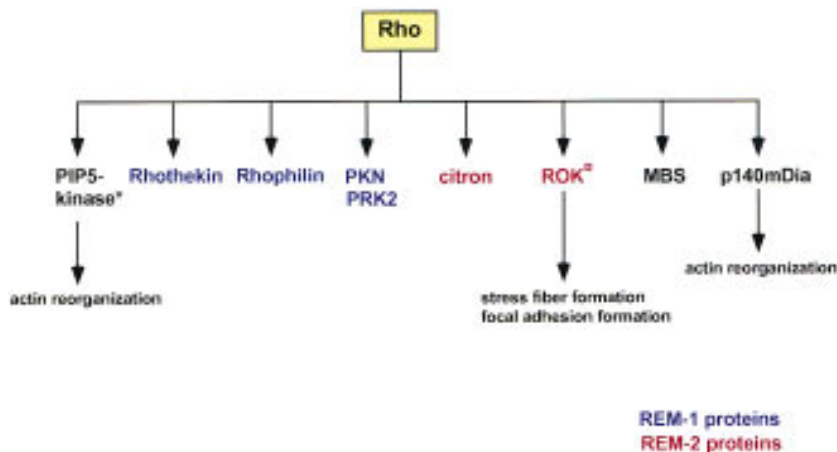
The ability of a eukaryotic cell to maintain or change its shape and its degree of attachment to the substratum in response to extracellular signals is largely dependent on rearrangements of the actin cytoskeleton. Cytoskeletal rearrangements play a crucial role in processes such as cell motility, cytokinesis, and phagocytosis. The actin cytoskeleton of animal cells is composed of actin filaments and many specialized actin-binding proteins (Stossel 1993; Small 1994; Zigmond 1996). Filamentous actin is generally organized into a number of discrete structures: (1) filopodia—finger-like protrusions that contain a tight bundle of long actin filaments in the direction of the protrusion. They are found primarily in motile cells and neuronal growth cones. (2) lamellipodia—thin protrusive actin sheets that dominate the edges of cultured fibroblasts and many motile cells. Membrane ruffles observed at the leading edge of the cell result from lamellipodia that lift up off the substrate and

fold backward. (3) actin stress fibers—bundles of actin filaments that traverse the cell and are linked to the extracellular matrix (ECM) through focal adhesions. It is important, therefore, that the polymerization of cortical actin is tightly regulated. This regulation of actin polymerization, for the most part, is orchestrated by Rho GTPases.

To date, the GTPase Rho has been shown to be required for many actin-dependent cellular processes, such as platelet aggregation, lymphocyte and fibroblast adhesion, cell motility, contraction, and cytokinesis (Narumiya et al. 1997). Direct evidence for the involvement of Rho in stress fiber formation was obtained from microinjection experiments using an activated mutant form of Rho, RhoV14. Expression of RhoV14 in quiescent fibroblasts resulted in the induction of stress fibers and the appearance of focal adhesions (Ridley and Hall 1992). Focal adhesions are the regions where stress fibers are anchored to the plasma membrane and where the cell adheres most tightly to the substratum. The cytoplasmic components of focal adhesions include cytoskeletal proteins such as  $\alpha$ -actinin, vinculin, and talin and signaling molecules such as the focal adhesion kinase, FAK (Burrige and Chrzanowska 1996). As mentioned above, LPA-induced stress fiber formation is mediated by Rho (Ridley and Hall 1992). Thus, these observations indicate that Rho regulates signal transduction pathways linking extracellular stimuli to the reorganization of the actin cytoskeleton.

Until recently, little was known about the molecular mechanism(s) by which Rho affects the cytoskeleton. However, over the past year, numerous proteins that bind Rho in a GTP-dependent manner have been identified (Fig. 2). Characterization of some of these proteins has provided major insights toward our understanding of Rho action at the molecular level. Of particular interest has been the serine/threonine kinases, ROK $\alpha$ /Rho kinase and its close relative, p160ROCK (also known as ROCKII or ROK $\beta$ ) (Leung et al. 1995, 1996; Ishizaki et al. 1996; Matsui et al. 1996a; Nakagawa et al. 1996). ROK $\alpha$  and Rho kinase differ only at their amino termini, where Rho kinase is nine amino acids longer. The interaction of Rho with p160ROCK or Rho kinase resulted in a modest increase of the kinase activity (Leung et al. 1995; Ishizaki et al. 1996; Matsui et al. 1996a). Clues to their functions came from two lines of experiments. First, Leung et al. (1995) found that expression of full-length ROK $\alpha$  and its amino-terminal half promotes the formation of stress fibers and focal adhesions. Kinase activity, but not the Rho-binding domain, was required for this response. Furthermore, expression of a kinase-dead mutant or the carboxy-terminal half of the protein resulted in the disassembly of stress fibers and focal adhesions (Leung et al. 1996). These results indicate a role for ROK $\alpha$  in the formation of stress fibers and focal adhesions. Similar observations were obtained for Rho kinase in other cell types (Amano et al. 1997). Interestingly, Ishizaki et al. (1997) demonstrated that although a kinase-negative, Rho binding-defective mutant blocked Rho-induced formation of stress fibers and focal adhesions in HeLa cells,

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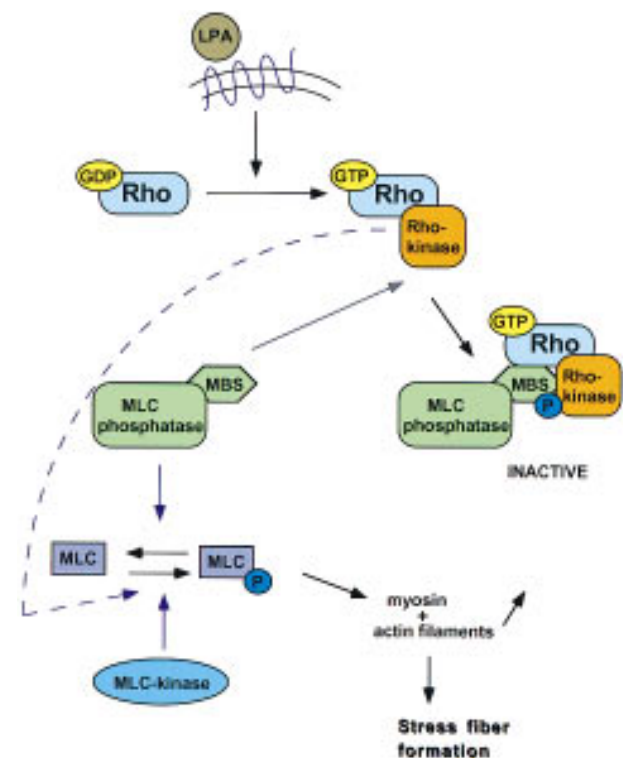


**Figure 2.** Mammalian targets of RhoA. The kinases PKN and PRK2, and the nonkinases Rhothekin and Rhoophilin (REM-1 proteins) contain a homologous Rho-binding motif, whereas ROK<sup>α</sup> (Rho kinase/ROK<sup>α</sup> and p160ROCK/ROK<sup>β</sup>/ROCKII) and citron share a distinct Rho-binding motif (REM-2). MBS (myosin-binding subunit of myosin light chain phosphatase). (\*) The PIP5 kinase interaction may not be direct.

it was still capable of eliciting an enhancement of actin polymerization. These results suggest that the pathways leading to actin polymerization and the formation of stress fibers and focal adhesions are mediated by distinct effectors. A second contribution toward the elucidation of Rho-kinase function came from the findings that the myosin-binding subunit (MBS) of myosin light-chain (MLC) phosphatase was a substrate for Rho kinase in vitro (Kimura et al. 1996; Matsui et al. 1996). It was subsequently shown that phosphorylation of MBS led to a decrease in MLC phosphatase activity, resulting in an accumulation of the phosphorylated form of MLC (Kimura et al. 1996). Phosphorylation of MLC has been shown to induce a conformational change in myosin, thereby increasing its binding to actin filaments and subsequently the formation of stress fibers (Tan et al. 1992; Chrzanowska and Burridge 1996). More recently, evidence has been provided that Rho kinase stoichiometrically phosphorylates MLC at the same site that is phosphorylated by MLC kinase (Amano et al. 1996a). A molecular model proposed for Rho-induced stress fiber formation is depicted in Figure 3. Notably, it was found that overexpression of constitutively activated Rho in NIH-3T3 cells resulted in an increase in MLC phosphorylation and stress fiber formation (Kimura et al. 1996). Also, Rho-stimulated contraction of fibroblasts could be blocked by the MLC kinase inhibitor KT5926, which resulted in a decrease of MLC phosphorylation and the loss of stress fibers and focal adhesions (Chrzanowska and Burridge 1996). It is likely that a cascade of events, as shown in Figure 3, may partly account for the mechanism by which Rho regulates cytokinesis, motility, or smooth muscle contraction.

As described above, Rho elicits the formation of focal adhesions. However, it is unknown whether the stress fiber and focal adhesion formation are separate or interdependent events. In a model proposed by Burridge and Chrzanowski 1996, Rho-mediated stress fiber formation generates tension, thereby inducing aggregation of the integrins on the ventral surface of the cells, which in turn stimulates the formation of focal adhesions and tyrosine phosphorylation of focal adhesion proteins. A sec-

ond line of investigation, using pharmacological inhibitors, demonstrated that the formations of focal adhesion



**Figure 3.** A potential mechanism for Rho-induced stress fiber formation. Extracellular factors, such as LPA, trigger the activation of Rho, which then binds to and activates Rho kinase. Rho GTP also interacts with MBS; however, the relevance of the interaction between Rho and MBS remains unclear. Activated Rho kinase phosphorylates the MBS of the myosin light chain (MLC) phosphatase, leading to the inactivation of the phosphatase and subsequently an accumulation of the phosphorylated form of MLC. Direct phosphorylation of MLC by Rho kinase has also been reported. This increase in the phosphorylated state of MLC enhances binding of myosin-actin filaments and, subsequently, stress fiber formation.



and stress fibers are separate events (Nobes and Hall 1995). In accordance with the latter, Chihara et al. (1997) reported that microinjection of a constitutively active Rho kinase into fibroblasts induced the formation of focal adhesions under conditions wherein actin stress fibers were disrupted.

The involvement of phosphoinositide kinases in Rho signaling has been reported. Rho (and Rac) have been shown to stimulate the synthesis of phosphatidylinositol bisphosphate (PIP<sub>2</sub>) (Chong et al. 1994; Hartwig et al. 1995). The exact mechanism by which Rho induces this event is not yet known because opposing results have been reported as to whether phosphatidylinositol 4-phosphate-5 kinase (PIP<sub>5</sub> kinase), which catalyzes the phosphorylation of PI4P to PIP<sub>2</sub>, interacts directly with Rho (Tolias et al. 1995; Ren et al. 1996). The observation that PIP<sub>2</sub> binds to and is thought to regulate the function of many actin-associated proteins, led to the hypothesis that Rho-stimulated PIP<sub>2</sub> synthesis may induce actin rearrangements. In light of this, Gilmore and Burridge (1996) reported that the association of PIP<sub>2</sub> with vinculin induces a conformation change in vinculin, allowing it to interact with talin, which binds actin. Furthermore, they showed that injection of anti-PIP<sub>2</sub> antibodies into fibroblasts inhibited LPA/Rho-induced stress fiber and focal adhesion formation, suggesting a role for PIP<sub>2</sub> in focal adhesion and stress fiber assembly.

Recently, a potential target of the *S. cerevisiae* Rho1 protein, designated Bni1p, was isolated and shown to play a role in cytoskeleton reorganization (Kohno et al. 1996). *RHO1* is a homolog of the mammalian RhoA gene and is essential for the budding process (Yamochi et al. 1994). Bni1p belongs to a family of formin-related proteins, which includes *Drosophila diaphanous* and *cap-puchino*, FigA in *Aspergillus*, and *fus1* in *S. pombe* (Castillon and Wasserman 1994; Emmons et al. 1995; Marhoul and Adams 1995). These proteins have been shown to be involved in cytokinesis, cell polarity, and cell morphology and share two formin homology domains, FH1 and FH2. In an elegant study, Imamura et al. (1997) demonstrated that Bni1p and its more recent isolated related member, Bnr1p, are mediators of the Rho GTPase effects on actin cytoskeleton reorganization. These investigators observed that these proteins interacted with profilin via the FH domains. Although originally thought to function in the sequestration of unpolymerized actin in the cytoplasm, profilin recently has been shown to have a promoting effect on actin polymerization (Nishida 1985; Goldschmidt et al. 1991; Cao et al. 1992). Taken together with the above results, Bni1p and Bnr1p are likely to mediate Rho GTPase effects on the cytoskeleton through a mechanism involving profilin. More recently, a mammalian homolog of Bni1p, p140mDia, was shown to selectively interact with mammalian Rho in a GTP-dependent manner and also with profilin (Watanabe et al. 1997). Moreover, p140mDia colocalized with profilin in polarized and nonpolarized fibroblasts and in phagocytic cells, and overexpression of p140mDia in Cos cells enhanced actin filament assembly. Based on these findings, a model was proposed in which activated Rho

recruits p140mDia/profilin to a specific site beneath the plasma membrane, which results in a locally increased concentration of profilin. This, in turn, may lead to actin polymerization (Narumiya et al. 1997; Watanabe et al. 1997). Such a model points toward a role for p140mDia in mediating the effect of Rho on cytokinesis in mammalian cells.

In addition to Rho kinase, MBS, and p140mDia, five other mammalian proteins have been identified as potential Rho targets (Fig. 2). PKN and its homolog PRK2 encode leucine zipper-bearing proteins containing a serine/threonine kinase-domain that is highly related to that of protein kinase C (PKC) (Amano et al. 1996b; Quilliam et al. 1996; Watanabe et al. 1996). Although it was reported previously that PKN and PRK2 interact specifically with GTP-bound Rho, it was also reported that the interaction of PRK2 with Rho is nucleotide-independent and that PRK2 is able to interact with Rac in a GTP-dependent manner (Vincent and Settleman 1997). These investigators also showed that a kinase-defective form of PRK2 disrupts actin stress fibers, suggesting a role for PRK2 in actin cytoskeletal organization. A *S. cerevisiae* PKC homolog, Pkc1, has been isolated and shown to interact directly with Rho1 (Nonaka et al. 1995). Furthermore, Pkc1 regulates cell wall integrity through the activation of the mitogen-activated protein kinase (MAPK). Another Rho1 target, glucan synthase (GS), contributes to cell wall remodeling, although in a distinct pathway (Arellano et al. 1996; Drgonova et al. 1996; Qadota et al. 1996). Whereas Rophilin (Watanabe et al. 1996) and Rhothekin (Reid et al. 1996) share homology with PKN in their Rho binding domain, citron (Madaule et al. 1995) has homology with Rho kinase in this domain. None of the three proteins contains an obvious catalytic domain and their roles in Rho-mediated cytoskeletal rearrangements remain to be established.

### Rac, Cdc42, and the cytoskeleton

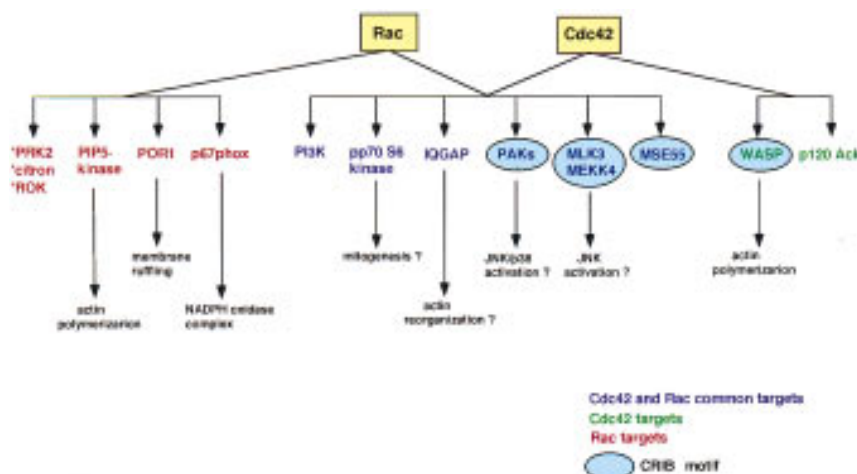
In fibroblasts, Rac has been shown to be a key control element in the reorganization of the actin cytoskeleton induced by growth factors and RasV12 (Ridley et al. 1992). Injection of RacV12 is sufficient to induce lamellipodia and membrane ruffles and subsequent stress fiber formation, whereas microinjection of RacN17 prior to the addition of growth factors or together with RasV12 abolishes these effects (Ridley et al. 1992). Furthermore, as described above, a role for yet another member of the Rho subfamily, Cdc42, in actin remodeling has been established. Most of our prior understanding of the function of Cdc42 came from studies in the yeast *S. cerevisiae*. Much of this work has been reviewed recently by Herskowitz (1995) and hence will not be discussed here. Injection of mammalian Cdc42 into fibroblasts revealed a third distinct signaling pathway linking plasma membrane receptors to actin cytoskeletal organization. Expression of Cdc42Hs triggered the formation of filopodial protrusions at the cell periphery followed by the formation of lamellipodia and membrane ruffling (Kozma et al. 1995). Both Rac and Cdc42 have also been shown to in-

duce the assembly of multimolecular focal complexes at the plasma membrane of fibroblasts (Nobes and Hall 1995). These complexes, which are most apparent as punctate spots of vinculin and phosphotyrosine around the leading edge of the lamellipodia and at the tips of filopodia, are morphologically distinct from the Rho-regulated focal adhesions, yet the protein components of the FACs appear similar to those in Rho-triggered focal adhesions. In addition, Ridley et al. (1995) demonstrated that Rac regulates hepatocyte growth factor (HGF)- and RasV12-induced motility in MDCK cells. In T cells, a role for Cdc42 in the polymerization of both actin and microtubules toward antigen-presenting cells has been provided (Stowers et al. 1995). Furthermore, the involvement of Cdc42 in the control of cytokinesis in fibroblasts, HeLa cells, and *Xenopus* embryos has been reported (Dutartre et al. 1996; Drechsel et al. 1997; Qiu et al. 1997).

To date, several potential targets of Cdc42 and Rac have been identified (Fig. 4). Interestingly, several of these proteins associate with both Rac and Cdc42. Among them, the family of serine/threonine kinases known as PAKs have attracted a lot of attention. At least three mammalian PAK isoforms have been isolated: rat p65PAK/h-PAK-1, h-PAK-2, and mPAK-3 (Bagrodia et al. 1995b; Knaus et al. 1995; Manser et al. 1995; Martin et al. 1995; Brown et al. 1996). All isoforms bind Rac and Cdc42 in a GTP-dependent manner, and this binding stimulates the activity of the kinase. Homologs of the mammalian PAKs have been identified in *S. cerevisiae* (Ste20 and Cla4) (Cvrckova et al. 1995), *S. pombe* (pak1, also called shk1) (Marcus et al. 1995; Otilie et al. 1995), *Drosophila* (PAK1) (Harden et al. 1996), and *C. elegans* (Ste20) (Chen et al. 1996). A role for the yeast PAK homologs in morphogenesis has been well documented and appears to involve the formation of a multimolecular complex (Sells and Chernoff 1997). For example, in *S. cerevisiae* Cdc42p interacts with Ste20p, which in turn associates with Ste5p and Bem1p, and the latter interacts with actin (Leeuw et al. 1995; Zheng et al. 1995). *Drosophila* PAK1 has been shown to play a role in dorsal

closure (see below). Thus, in these organisms it is likely that PAK proteins are involved in mediating the effects of the Rho-like GTPases on the cytoskeleton. In mammalian cells, the role of PAK remains unclear. Expression of mutant forms of Rac or Cdc42 that are unable to bind and activate PAKs can still induce the formation of membrane ruffles and lamellipodia (Joneson et al. 1996a; Lamarche et al. 1996). These studies indicate that PAK is not required for Rac-elicited membrane ruffling and lamellipodia formation or for Cdc42-triggered filopodia formation. This does not exclude the possibility, however, that PAK itself may play a role in cytoskeletal rearrangements by inducing actin reorganization independently of the Rho GTPases. Alternatively, PAK may mediate effects on the cytoskeleton triggered by Rac or Cdc42, that are different from the above-described short-term actin reorganization. In this regard, two groups reported that expression of activated PAK alleles resulted in actin reorganization at the plasma membrane and that this effect appears to be mediated by an SH3-binding motif located at the amino terminus of PAK1 (Manser et al. 1997; Sells et al. 1997). Notably, the actin structures elicited by activated PAK mutants do not completely mimic those induced by activated mutants of Rac or Cdc42 (Sells et al. 1997).

All PAK proteins identified to date share a similar motif stretching over 18 amino acids that mediates the interaction with Rac or Cdc42 and is referred to as CRIB (Cdc42/Rac interactive binding). Moreover, a computer-assisted search for proteins containing a CRIB homology domain led to the identification of >25 proteins from a wide range of species; among them were three mammalian proteins, MSE55, MLK2/3, and WASP (Burbelo et al. 1995) (Fig. 4). WASP, the Wiskott-Aldrich syndrome protein, may link Cdc42 to the actin cytoskeleton. WASP binds to Cdc42 in a GTP-dependent manner and has been shown to associate with Nck, an SH3 domain-containing adaptor protein (Aspenstrom et al. 1996; Symons et al. 1996). Furthermore, expression of WASP in normal rat kidney (NRK) epithelial cells or Jurkat cells induced actin cluster formation. This induction of ectopic actin



**Figure 4.** Mammalian targets of Rac and Cdc42: Rac and Cdc42 interact with a variety of common targets. The serine/threonine kinases belonging to the PAK family, MLK3, MEKK4, MSE55, and WASP share a common Rac/Cdc42 binding motif (CRIB); (POR1) partner of Rac; (IQGAP) GAP-containing Ile-Gln motifs. 'PRK2, 'citron, and 'ROK also interact with Rho.

polymerization could be prevented by coinjection of a dominant-negative mutant form of Cdc42, suggesting that WASP-elicited cytoskeletal rearrangements are controlled by Cdc42 (Symons et al. 1996). Although WASP has been reported to bind weakly to Rac, expression of dominant-negative Rac mutants do not interfere with WASP-induced actin clustering and therefore WASP is unlikely to be a physiological target of Rac. Whether WASP mediates Cdc42-induced filopodial protrusions remains to be clarified. One caveat, however, is that WASP expression is restricted to cells of hematopoietic origin and therefore cannot account for the ability of Cdc42 to elicit filopodia in fibroblasts. However, it cannot be excluded that isoforms of WASP in other cells exist. Supporting evidence for a role of WASP in cytoskeletal organization came from the observation that disruption mutants of the *S. cerevisiae* WASP homolog, BEE1, exhibited a striking change in the organization of actin filaments, resulting in defects in budding and cytokinesis (Li 1997). In yeast, another potential link between Cdc42 and the actin cytoskeleton involves the protein Bni1p. Bni1p interacts also with Cdc42p and actin, as well as two actin-associated proteins, profilin and Bud6p, and during mating response, *bni1* mutants showed defects in cell polarization and in organization of the actin cytoskeleton (Evangelista et al. 1997).

In addition to the CRIB motif-containing proteins, several other proteins have been isolated that bind to Rac and/or Cdc42. Among them, a 34-kD protein, POR1 (partner of Rac), was isolated in a two-hybrid screen and shown to play a role in Rac-mediated membrane ruffling (Van Aelst et al. 1996). POR1 interacts specifically with Rac in a GTP-dependent manner. Deletion mutants of POR1 inhibited the induction of membrane ruffles by RacV12, whereas a synergistic effect of wild-type POR1 with RasV12 was observed for the induction of membrane ruffling. Consistent with a role of POR in membrane ruffling was the observation that a mutant Rac that failed to bind POR also failed to induce membrane ruffling (Joneson et al. 1996a). Interestingly, POR1 also interacts with the GTPase ARF6 (D'Souza-Schorey et al. 1997). ARF6 is the least conserved member of the ARF family of GTPases (Tsuchiya et al. 1991). In addition to its role in regulating peripheral membrane trafficking (D'Souza-Schorey et al. 1995; Peters et al. 1995), ARF6 and its activated mutant ARF6(Q67L) have been shown to elicit cytoskeletal rearrangements at the cell surface (Radhakrishna et al. 1996; D'Souza-Schorey et al. 1997). Cytoskeletal rearrangements induced by ARF6(Q67L) could be inhibited by coexpression deletion mutants of POR1 but not with the dominant-negative Rac mutant Rac(S17N) (D'Souza-Schorey et al. 1997). These findings indicate that ARF6 and Rac function on distinct signaling pathways to mediate cytoskeletal reorganization and suggest a role for POR1 as an important regulatory element in orchestrating cytoskeletal rearrangements at the cell periphery induced by ARF6 and Rac. It is possible that depending on the nature of the extracellular stimuli, POR1 could interact with either ARF6 or Rac1 or both, to establish highly specified pat-

terns of cytoskeletal rearrangements at the plasma membrane.

Another protein with a potential role in cytoskeletal organization is IQGAP (Brill et al. 1996; Kuroda et al. 1996; McCallum et al. 1996). IQGAP interacts with both Rac and Cdc42 and localizes to membrane ruffles. Although IQGAP contains some interesting motifs found in signaling molecules, such as WW domain, SH3-binding domain, a calmodulin-binding domain and, somewhat surprisingly, a RasGAP-like motif, its function remains to be established.

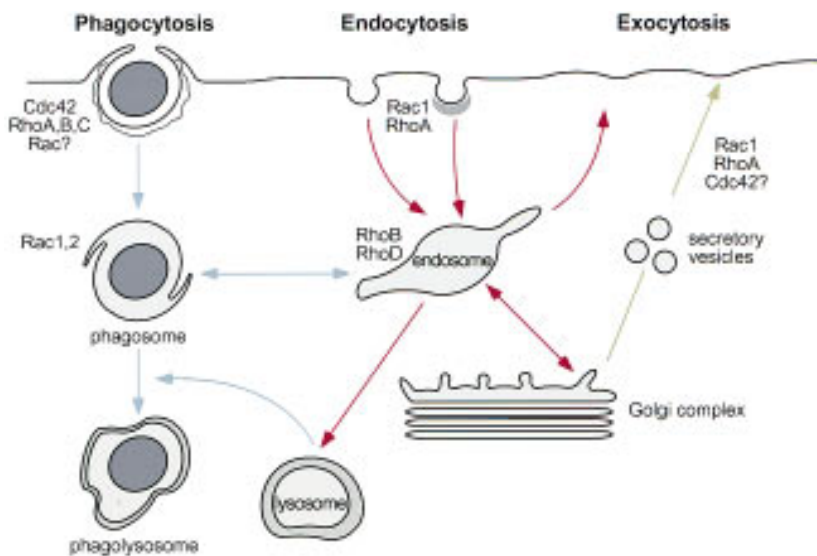
As was suggested for Rho, a potential link to the cytoskeleton may involve phospholipid metabolism. In a model proposed by Hartwig et al. (1995), in blood platelets Rac-induced PIP<sub>2</sub> generation leads to an enhancement of actin polymerization by inducing the uncapping of actin filaments, thereby increasing the number of free barbed ends. It has been demonstrated that Rac affinity columns can bind PIP5 kinase. Thus it is likely that PIP5 kinase may be the responsible kinase mediating Rac-stimulated PIP<sub>2</sub> generation.

Although a number of potential targets of Rho family members have been identified, a major task in the future will be to determine the physiological relevance of these proteins in Rho-mediated cytoskeletal remodeling.

### Rho GTPases in membrane trafficking

Vesicular transport along the biosynthetic and secretory pathways is essential for the biogenesis and maintenance of subcellular organelle integrity and for the trafficking of proteins and lipids within the cell, as well as between the cell and its extracellular environment. A number of cellular processes such as secretion, endocytosis, phagocytosis, and antigen presentation involve the vectorial transport of intracellular membrane vesicles that may be accompanied by the remodeling of the actin cytoskeleton. Therefore, it is not unexpected that over the past few years, a number of studies have implicated the members of the Rho family in various membrane-trafficking processes (Fig. 5).

Cytoskeletal rearrangements are intimately coupled to the onset of phagocytosis. The phagocytic process is initiated by the attachment of a particle or microorganism to cell surface receptors, followed by subsequent ingestion into a phagosome (Allen and Aderem 1996). Ingestion is accompanied by actin polymerization at a localized region of the plasma membrane (Swanson and Baer 1995). Subsequent to ingestion, a series of vesicle budding and fusion events ensue, which allow phagosome maturation and the delivery of hydrolases, proteases, and antimicrobial enzymes, such as superoxide-generating NADPH oxidase and myeloperoxidase, into the phagocytic vesicle (Beron et al. 1995; Edwards 1996). The proper orchestration of these events results in pathogen destruction. Several studies have demonstrated that the Rho-family GTPases are implicated in one or more steps of the phagocytic response. The three isoforms of the Rho GTPases have been implicated in *Shigella* entry of epithelial cells (Adam et al. 1996). In a model proposed



**Figure 5.** Schematic representation of intracellular membrane trafficking in eukaryotic cells and steps where a role for Rho family GTPases have been implicated.

by Adam and coworkers, *Shigella* invasion begins with actin nucleation and RhoA-induced actin polymerization. This is followed by continued actin polymerization around membrane-bound protrusions that fold over the bacterium, coalesce, and engulf it. The observations of a complete inhibition of *Shigella*-induced membrane folding by C3 transferase suggests that actin polymerization is essential for the generation of the surface extensions. Further studies will be required to examine the role of Rho in the induction of membrane alterations that accompany surface remodeling during *Shigella* entry and the interaction of the membrane and the cytoskeleton in this process. Although Rac has been shown to induce actin polymerization at the cell periphery, Rac-induced lamellipodia formation and membrane ruffling have not been directly linked to the internalization step of phagocytosis. The observation that *Salmonella* invasion of epithelial cells leads to the activation of EGF receptors (Galan et al. 1992), a known mechanism for induction of Rac-dependent ruffling (Ridley et al. 1992), suggests a role for Rac in this process. However, there have been reports that *Salmonella* phagocytosis occurs independent of Rho and Rac (Jones et al. 1993). Interestingly, Cdc42 has been shown to play a direct role in *Salmonella* internalization (Chen et al. 1996). Although cells expressing an activated mutant of Cdc42 were capable of phagocytosing an invasion-defective *Salmonella* mutant, the dominant-negative Cdc42 prevented bacterial internalization. On the other hand, Rac in its GTP-bound form, in addition to two other cytosolic proteins, p47<sup>phox</sup> and p67<sup>phox</sup> has been shown to be required for the activation of NADPH oxidase of phagocytic cells (Abo et al. 1992; Diekmann et al. 1994; Bokoch 1995). Rac antisense oligonucleotides and expression of RacN17 inhibit superoxide generation (Dorseuil et al. 1995; Gabig et al. 1995). Bcr, a Rac GAP, has been shown to down-regulate Rac-induced activation of NADPH oxidase in neutrophils, thereby preventing excess tissue

damage (Voncken et al. 1995). Whether these distinct functions of Rac, such as NADPH oxidase activation and actin reorganization, are coordinately or sequentially regulated awaits further investigation.

Rac and Rho have also been implicated in the regulation of endocytosis. In mammalian cells, expression of constitutively activated mutants of Rac or Rho decreased the efficiency of receptor-mediated endocytosis of the transferrin receptor (Lamaze et al. 1996). In a cell-free assay these mutant proteins have been shown to inhibit the formation of clathrin-coated vesicles (Lamaze et al. 1996). It has been proposed that the activation of PLD and/or PIP5 kinase might control coated pit assembly, consistent with the contention that alterations in membrane lipids might trigger the recruitment of components required to initiate vesicle budding. In contrast, microinjection of either wild-type or activated Rho into *Xenopus* oocytes enhanced constitutive endocytosis (Schmalzing et al. 1995). Furthermore, injection of C3 transferase or ADP-ribosylated RhoA into oocytes prevented the uptake of surface sodium pumps and caused the formation of large membranous folds at the cell surface. Interestingly, the dense cortical layer of filamentous actin beneath the plasma membrane in oocytes was insensitive to C3 treatment. These data suggest a direct involvement of Rho in endocytic membrane trafficking in *Xenopus*, independent of its effect on the actin cytoskeleton. RhoB is localized to early endosomes in rat-2 cells, suggesting a potential role for this GTPase in endocytic trafficking in mammalian systems (Adamson et al. 1992). RhoD, another member of the Rho family, has been identified and also localizes on early endosomes at the cell surface (Murphy et al. 1996). Upon overexpression, RhoD induced the formation of long thin F-actin-containing membrane processes and a disassembly of stress fibers and focal adhesions along the cell periphery. This cytoskeletal remodeling was accompanied by an increase in endosome fission and a scattering of vesicles

throughout the cell. Time-lapse video microscopy revealed that cells expressing the RhoD mutant exhibited decreased organelle motility that impeded movement and fusion of endosomes. Thus, the ability of RhoD to cycle between its GTP- and GDP-bound forms appears to be critical for the regulation of endosomal motility and for maintaining the equilibrium between endosomal fusion and fission (Murphy et al. 1996).

The role for the Rac GTPase in pinocytosis is still unclear. Membrane ruffling has long been thought to be linked to an increase in pinocytosis. In Swiss 3T3 cells, expression of the activated mutants of Rac stimulated pinocytosis (Ridley et al. 1992). However, Li et al. (1997) demonstrated more recently that the expression of the activated Rac mutant had no effect on pinocytosis in baby hamster kidney fibroblasts. The latter study also demonstrated that H-Ras-induced enhancement of pinocytosis could not be blocked by a dominant-negative mutant of Rac but was effectively blocked by the dominant interfering mutant of Rab5. The latter is a member of the Rab subfamily and has been shown to promote fusion among early endosomes (Bucci et al. 1992; Barbieri et al. 1994). Whether these discrepancies reflect differences in pinocytic routes among various cell types remains to be explored.

In addition to a role in endocytic trafficking, Rac and Rho have also been implicated in the regulation of secretory vesicle transport. In mast cells, recombinant Rac and Rho proteins stimulated the exocytosis of secretory granules, whereas C3 transferase and the dominant-negative mutants of Rac or Rho inhibited secretion induced by GTP $\gamma$ S (Norman et al. 1996). Although the secretory granule exocytosis is accompanied by a redistribution and polymerization of F-actin, the latter is selectively inhibited by cytochalasin D, whereas membrane secretion remains unaffected. Furthermore, constitutively activated mutants of Rac and Rho enhance secretion in the presence of cytochalasin D. Therefore, the signaling pathways controlling outward membrane flow and cytoskeletal rearrangements in mast cells are divergent and act in parallel rather than in concert. Consistent with the above findings, it was independently demonstrated that Rho GDI inhibited GTP $\gamma$ S-stimulated exocytosis in mast cells (Mariot et al. 1996). In *S. cerevisiae*, the RhoA homolog Rho1 has been localized to the Golgi and post-Golgi vesicles, supporting the contention that Rho may be involved in the secretory response in yeast (McCaffery et al. 1991). On the other hand, yeast Cdc42 has been shown to be localized at the plasma membrane in the vicinity of secretory vesicles that are found at the site of bud emergence (Ziman et al. 1993). In mammalian cells however, wild-type Cdc42 has been localized to the Golgi apparatus, and it has been proposed that activation of Cdc42 and the formation of filopodia at the cell periphery may be coupled to vesicular transport from the trans-Golgi network to the plasma membrane (Erickson et al. 1996). Interestingly in this regard, Cdc42 has been shown to regulate polarization of T cells toward antigen-presenting cells, which may indeed be coupled to targeted lymphokine secretion toward the appropriate

antigen-presenting cell (Stowers et al. 1995). Further studies will be required to address the role of Cdc42 on lymphokine secretion in T cells.

Although it is now clear that a number of vesicular transport processes require coordinated interactions between the membrane and the cytoskeleton, the biochemical mechanisms that integrate these cellular processes are largely unknown. It is possible that the effects of Rho GTPases on phospholipid metabolism may provide a point of intersection between the coordinated control of membrane flow and cytoskeletal organization. There is mounting evidence that polyphosphoinositides are implicated in the regulation of vesicular traffic in a variety of systems. PIP2 has been shown to have a role in Ca<sup>2+</sup>-regulated exocytosis in adrenal chromaffin cells (Eberhard et al. 1990). In addition, enzymes involved in PIP2 biosynthesis are required for the priming of secretory granules during exocytosis in PC12 cells (Hay and Martin 1993). Mutations in the *SEC14* gene of *S. cerevisiae*, which codes for PITP (phosphatidylinositol transfer protein), results in defective post-Golgi secretory traffic (Bankaitis et al. 1990; Novick et al. 1980). Moreover, wortmannin has been shown to affect fluid-phase endocytosis and receptor recycling (Clague et al. 1995; Li et al. 1995; Shepherd et al. 1995; Martys et al. 1996). Also, mutations in the PDGF receptor that inhibit its association with PI3 kinase result in a defect in postendosomal sorting of the receptor (Kapeller et al. 1993). As described above, polyphosphoinositides such as PIP2 and the kinases that regulate their turnover have been shown to serve as a link between the Rho GTPases and the actin cytoskeleton. Thus, it is tempting to speculate that local changes in membrane phospholipids induce changes in the actin-based cytoskeleton and regulate vesicle-target interactions. Further studies are required to determine if and how the Rho/Rac-induced effects on phospholipid metabolism bridge vesicular transport with the actin cytoskeleton.

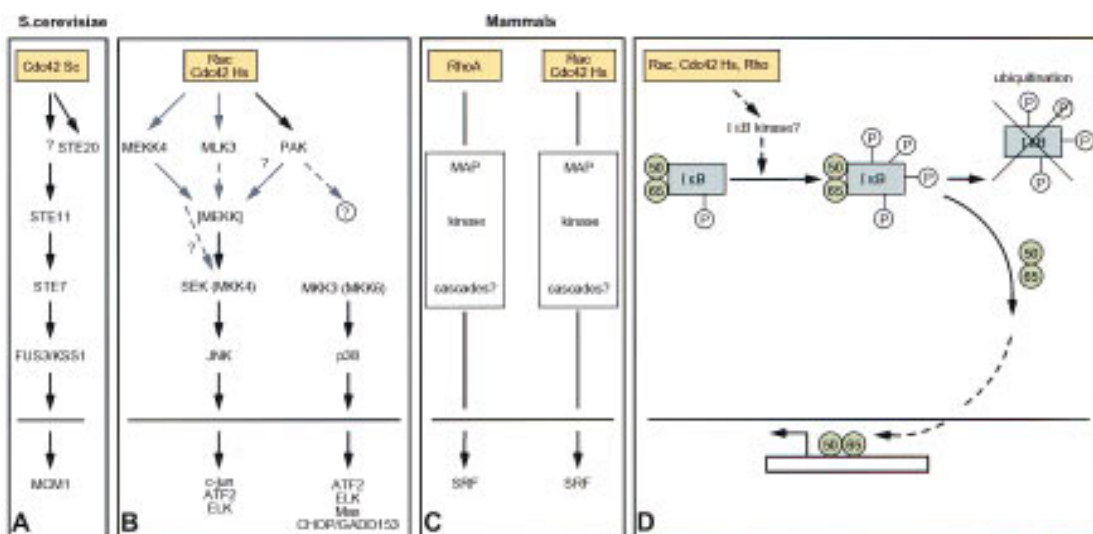
### Rho GTPases and transcriptional activation

Accumulating data points to the involvement of Rho family members in regulating nuclear signaling. Whereas Ras has been shown to control the activation of the p42/44 MAPK cascade, several groups demonstrated that in certain cell types, Rac and Cdc42 (but not Rho) regulate the activation of JNK and the reactivating kinase p38RK (Seger and Krebs 1995). Expression of constitutively active mutants of Rac and Cdc42 in HeLa, NIH-3T3, and Cos cells resulted in a stimulation of JNK and p38 activity (Coso et al. 1995; Minden et al. 1995). Furthermore, the same effects were obtained with oncogenic GEFs for these Rho proteins (Minden et al. 1995). However, Teramoto et al. (1996b) reported that in human kidney 293 T cells, Cdc42 and the Rho protein, but not Rac, can induce activation of JNK. In contrast to the p44/42 MAPKs, the JNKs and p38 are poorly activated by mitogens but strongly activated by inflammatory cytokines, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and a diverse array of cellular stresses such as

heat shock, UV, and ionizing radiation (Kyriakis and Avruch 1996). Until now, only a few of these stimuli (e.g., IL-1 $\beta$ ) have been demonstrated to exert their effects through Rho GTPases (Bagrodia et al. 1995a; Zhang et al. 1995; Whitmarsh et al. 1997). Upon activation, JNKs and p38 translocate to the nucleus where they phosphorylate transcription factors. Substrates for JNKs include the amino terminus of c-Jun, ATF2, and Elk (Pulverer et al. 1991; Derijard et al. 1994; Gille et al. 1995; Gupta et al. 1995). Furthermore, Rac has been shown to stimulate the transcriptional activity of PEA3, a member of the Ets family, in a JNK-dependent fashion (O'Hagan et al. 1996). Activated p38 phosphorylates ATF2, Elk, Max (Zervos et al. 1995), and the cAMP response element-binding protein-homologous protein/growth arrest DNA damage 153 (CHOP/GADD153) (Wang and Ron 1996). In addition to the above transcription factors, MAPKAP-K2 and more recently 3pK have been identified as targets of p38 (Rouse et al. 1994; Ludwig et al. 1996). The latter is also activated by JNK and MAPK. It is important to note that the substrate specificities for these kinases appear to be cell-type dependent.

The identity of the molecules that link the Rac and Cdc42 GTPases to JNK and p38 is still not completely understood. Direct activators of JNK and p38 are either the dual specificity kinase SEK1 (also called MEKK4 or JNKK), which activates both JNK and p38 when overexpressed, or MKK3 and MKK6 which specifically activate p38 (Sanchez et al. 1994; Derijard et al. 1995; Han et al. 1996; Jiang et al. 1996; Raugeaud et al. 1996). Although originally identified as MEK1/2 kinases (Lange-Carter et al. 1993), MEKK1 has since been shown not to trigger activation of MEK and MAPK, but to stimulate SEK1 (Xu et al. 1995). Because MEKK1 activates SEK1 and JNK but not p38 in vivo, it is not clear whether SEK1 functions as

a physiological p38 activator. The family of serine/threonine kinases known as PAKs were proposed to act as the farthest upstream kinases connecting Rho GTPases to JNK and p38 as they bind Rac and Cdc42 in vitro in a GTP-dependent manner and become activated upon binding to the activated forms of these GTPases (Bagrodia et al. 1995b; Knaus et al. 1995; Manser et al. 1995; Martin et al. 1995). Furthermore, certain constitutively-activated forms of PAK can stimulate the activity of JNK and p38 (Bagrodia et al. 1995a; Knaus et al. 1995; Zhang et al. 1995b; Brown et al. 1996; Frost et al. 1996). By analogy with the Ste20 kinase cascade in *S. cerevisiae* (Fig. 6), it was suggested that PAK regulates the activity of MEKK. However, direct phosphorylation of any of the identified MEKKs by PAK has not been demonstrated thus far. Notably, the recent work of Peter et al. (1996) strongly suggests that Cdc42/Ste20 interaction is not required for the activation of the MAPK pathway triggered by mating pheromones. Also, some groups did not observe an increase in JNK activity upon coexpression of PAK1 with activated forms of Cdc42 or Rac (Teramoto et al. 1996a). Furthermore, an effector mutant of Rac, which fails to bind to PAK, remains a potent JNK activator (Westwick et al. 1997). Therefore, it is possible that other kinases, in addition to (or instead of) PAK, participate in signaling from the Rho-like GTPases to JNK. In support of this possibility, MLK3 (also called SPRK) and MEKK4 are regulated by Cdc42 and Rac and selectively activate the JNK pathway (Gallo et al. 1994; Teramoto et al. 1996a; Tibbles et al. 1996; Gerwins et al. 1997). Different groups reported the ability of Cdc42/Rac to bind to MLK3 both in vitro and in vivo and that coexpression of activated Cdc42/Rac mutant forms elevated the enzymatic activity of MLK3 in Cos-7 cells (Gallo et al. 1994; Teramoto et al. 1996b; Gerwins et al. 1997).



**Figure 6.** Signal transduction through members of the Rho subfamily leading to transcriptional activation in yeast and mammals. (A) Pheromone signaling pathway in *S. cerevisiae*; (B) the JNK and p38 signaling cascades in mammals; (C) the SRF signaling pathway; (D) the pathway leading to NF- $\kappa$ B activation in mammalian cells.

MEKK4 was demonstrated to bind Cdc42 and Rac in vivo, and kinase-inactive mutants of MEKK4 block Cdc42/Rac stimulation of the JNK pathway (Gerwins et al. 1997). Both MLK3 and MKK4 have been shown to activate SEK1. Although MLK3 and MEKK4 are attractive candidates as effectors of Rac/Cdc42-induced JNK activation, it remains to be proven whether direct binding of MLK3 to the activated GTPases results in its activation and whether MEKK4 binds directly to the activated forms of Rac and Cdc42. It is possible that different kinases may be involved in mediating the signal from the Rho GTPase to JNK (or p38), depending on the cell type and the extracellular stimulus.

In addition, Rac, Cdc42, and Rho stimulate the activity of the serum response factor (SRF) (Hill et al. 1995). SRF complexes with TCF/Elk proteins to stimulate transcription of genes with serum response elements (SREs) in their promoter enhancer regions (e.g., the Fos promoter) (Treisman 1990, 1994). Rho was shown to be required for LPA-, serum-, and AIF4-induced transcriptional activation by SRF. The activation of SRF by Cdc42 and Rac is not Rho-dependent, suggesting that at least two signaling pathways controlled by Rho GTPases lead to SRF activation (Hill et al. 1995). The identity of these signaling pathways remains unknown. It is clear, however, that neither the JNK nor p38 MAPK cascades are sufficient to mediate SRF-linked signaling pathways (Fig. 6). Interestingly, Chihara et al. (1997) recently observed that expression of constitutively active Rho kinase stimulated the transcriptional activity of c-fos SRE.

More recently, the Rho-like GTPases have been implicated in the activation of the nuclear transcription factor- $\kappa$ B (NF- $\kappa$ B) (Sulciner et al. 1996; Perona et al. 1997). In unstimulated cells, NF- $\kappa$ B consists of a cytoplasmic complex of homo- or heterodimeric Rel-related proteins bound to a member of the I $\kappa$ B family of inhibitor proteins. One mechanism described for the activation of NF- $\kappa$ B in response to a variety of agents, including cytokines, phorbol esters, UV light, and TNF $\alpha$ , depends on the phosphorylation and subsequent degradation of the inhibitory I $\kappa$ B subunits (Verma et al. 1995). Evidence has been provided that the Rho-like GTPases, including Cdc42, Rac, and Rho, mediate phosphorylation of I $\kappa$ B and cause translocation of the dimers to the nucleus. The precise mechanism by which Rho GTPases trigger activation of NF- $\kappa$ B remains to be elucidated. Finkel and coworkers provided evidence that Rac regulates cytokine-stimulated NF- $\kappa$ B activation via a redox-dependent pathway (Sulciner et al. 1996). It was demonstrated previously that agents stimulating NF- $\kappa$ B also trigger an increase in intracellular reactive oxygen species (ROS) (Schreck et al. 1991; Lo and Cruz 1995). Expression of RacV12 in HeLa cells resulted in a significant increase in intracellular ROS, whereas dominant-negative Rac markedly reduced the increase in ROS as well as the activation of NF- $\kappa$ B after cytokinin stimulation (Sulciner et al. 1996). How Rac regulates the level of ROS is currently unclear. Interestingly in this regard, as mentioned earlier, Rac has been shown to be essential for the NADPH oxidase-catalyzed superoxide formation by

phagocytic cells for the purpose of killing microorganisms (Bokoch 1995). Superoxide can be generated in a cell-free system by combining the membrane-bound cytochrome, cytosolic factors p47<sup>phox</sup> and p67<sup>phox</sup>, and the activated GTP-bound Rac (Abo et al. 1992; Diekmann et al. 1994). It has been postulated that a NADPH oxidase enzyme complex may exist in nonphagocytic cells and that Rac may directly activate protein-protein interaction to organize the ROS system (Sulciner et al. 1996), similar to what occurs in phagocytic cells.

Compiling data for the involvement of the above-mentioned transcription factors in multiple aspects of cell growth have been provided. However, it remains to be clarified whether the Cdc42/Rac/Rho-mediated activation of transcription factors contributes to their ability to influence cell growth (see below).

### Rho GTPases and cell growth control

Over the past few years, several lines of evidence have been provided that indicate Rho family members play a critical role in several aspects of cell growth. A potential role for Rho GTPases in cell growth control and mitogenesis was initially suggested by the rapidly expanding number of oncogenes that encode exchange factors for Rho family members (Table 1). More recently, it has been shown that injection of Cdc42, Rac, and Rho proteins in Swiss 3T3 fibroblasts stimulated cell cycle progression through the G<sub>1</sub> phase and subsequent DNA synthesis, whereas injection of dominant-negative forms of these GTPases blocked stimulation of DNA synthesis in response to serum (Olson et al. 1995). Rac is required for v-Abl to activate a mitogenic program in a subclone of NIH-3T3 cells (Renshaw et al. 1996). Furthermore, Rac, Rho, and more recently Cdc42 have been demonstrated to have transforming and oncogenic potential in some cell lines. Cells expressing constitutively active mutants of Rac and Rho displayed enhanced growth in low serum, were anchorage independent, and caused tumor formation when inoculated into nude mice (Khosravi et al. 1995). The observation that Tiam, a GEF for Rac, can also transform NIH-3T3 cells strengthens a role for Rac in transformation (van Leeuwen et al. 1995). Whereas expression of constitutively activated Rac was sufficient to cause malignant transformation of rodent fibroblasts, this was not the case for Rho, suggesting that the growth-promoting effects of the Rho-related proteins are cell type specific (Qiu et al. 1995a,b). In addition, Rac and Rho have been shown to be essential for transformation by Ras. Two groups independently demonstrated that RacN17 inhibits focus formation caused by oncogenic Ras but not by a constitutively active form of Raf, Raf-CAAX (a cRaf1 kinase targeted to the plasma membrane by a carboxy-terminal lipid modification signal from H-ras) in NIH-3T3 cells (Khosravi et al. 1995; Qiu et al. 1995a). They also showed that RacV12 synergizes strongly with Raf-CAAX in focus-formation assays. These data are consistent with a model in which Ras activates both the Raf/MAPK and the Rac signaling pathways independent of each other to cause full trans-

formation. In light of this, it was previously well documented that multiple pathways contribute to Ras transformation (White et al. 1995; Khosravi et al. 1996). The same groups also demonstrated that a dominant-negative form of Rho, RhoN19, inhibits the transformation of oncogenic Ras and that an activated mutant form of Rho, RhoV14, cooperates with Raf-CAAX for the induction of foci, consistent with a role for Rho in Ras transformation (Khosravi et al. 1995; Qiu 1995b). Somewhat puzzling was the observation that focus formation induced by Raf-CAAX was inhibited by coexpression with RhoN19, as RhoV14 failed to activate MAPK in several different cell systems (Qiu 1995b). A model in which Rho mediates an autocrine loop driven by Raf-CAAX was postulated to explain this phenomenon (Symons 1995). By analogy to the previous studies using the reorganization of the actin cytoskeleton as readout, it was assumed that Rho acts downstream of Rac in mediating oncogenic Ras-induced transformation. However, Qiu et al. (1997) observed a synergistic effect between the activated mutant forms of Rac and Rho for the induction of foci, suggesting that Rac and Rho may act independently of each other and downstream of Ras.

More recently, evidence for a role of Cdc42 in cell growth has been provided by two groups. Qui et al. (1997) demonstrated that fibroblasts expressing constitutively active Cdc42 were anchorage independent and proliferated in nude mice. Surprisingly, in contrast to RacV12-expressing Rat1 fibroblasts, Cdc42V12 cell lines failed to show enhanced growth in low serum. Lin et al. (1997) established a role for Cdc42 in transformation, by making use of a Cdc42 mutant, Cdc42(F28L), which can undergo GTP-GDP exchange in the absence of GEF. They reported that cells stably transfected with Cdc42(F28L) did not only exhibited anchorage-independent growth but also lower dependence on serum for growth. In their initial experiments using the GTPase defective mutant Cdc42(Q61L), Lin et al. (1997) observed that expression of the latter mutant gave rise to pronounced growth inhibition. Thus, their results suggest that complete cycling is necessary for Cdc42 growth-promoting signal. The discrepancy between the observations made by the two groups may be attributable to different levels of expression, induced by the Cdc42V12 and Cdc42(F28L) mutants, with the latter being higher expressed. An alternative explanation may be the differences in cell lines and conditions used. Also, a role for Cdc42 in Ras transformation has been established (Qiu et al. 1997). Coexpression of a dominant-negative mutant of Cdc42, Cdc42N17, with oncogenic Ras in Rat1 fibroblasts resulted in an inhibition of RasV12-induced focus formation and anchorage-independent growth and reversed the morphology of RasV12-transformed cells. Although RacN17 was able to suppress growth on soft agar induced by RasV12, it did not inhibit Cdc42-induced anchorage independency. Furthermore, expression of RacN17 caused inhibition of Ras-induced low serum growth, whereas Cdc42N17 had only a small effect. On the other hand, RacN17 had only a minor effect on the morphology of RasV12 expressing Rat1 fibroblasts (Qiu et al.

1997). These studies suggest that the pathways mediated by Rac and Cdc42 in the control of cell growth and Ras transformation are independent of each other and that the Rho GTPases may have differential effects on cell growth and Ras transformation. Further experiments will be required to establish the relative contribution of each of the Rho GTPases to cell growth control.

A role for Rac in invasiveness was suggested by the observation that the tumor invasiveness gene Tiam1 encodes a GEF for Rac (Michiels et al. 1995). As mentioned above, Tiam1 was identified by combining retroviral insertional mutagenesis with efficient *in vitro* selection for invasive T lymphoma cell variants (Habets et al. 1994). Invasiveness was acquired either by truncation of Tiam1 or by amplification of the normal Tiam1 protein. Furthermore, the cell clones that were invasive *in vitro* produced experimental metastasis in nude mice. Recently, activated Rac, when expressed in lymphoma cells, has been shown to confer an invasive phenotype and potential metastatic potential to cells that could not otherwise penetrate into surrounding fibroblasts (Michiels et al. 1995).

Despite the evidence that Rho GTPases have an important role in processes such as mitogenesis, proliferation, and invasiveness, the mechanism by which they function is not understood. One possible mechanism is that Rho GTPases, like Ras, might trigger changes in gene expression by modulating the activities of various transcription factors. As discussed above, Cdc42, Rac, and Rho have been shown to activate several transcription activators. However, studies employing Rac and Cdc42 effector loop mutants, which separate the ability of Rac and Cdc42 to interact with different downstream effectors, indicate that the effects of Rac and Cdc42 on the JNK-MAPK cascade and cell proliferation are mediated by distinct effector pathways that diverge at the level of Rac or Cdc42 itself (Joneson et al. 1996a; Lamarque et al. 1996). Mouse or rat embryo fibroblasts expressing Cdc42 or Rac mutants that fail to activate PAK and JNK nevertheless retain the ability to stimulate DNA synthesis or to mediate transformation. On the other hand, Cdc42 and Rac mutants, although able to activate PAK and JNK, failed to stimulate DNA synthesis or to mediate transformation. Using the same genetic approach, Westwick et al. (1997) demonstrated that the Rac-induced SRF activation is dispensable for Rac growth-promoting activity. As described above, Rac regulates the production of ROS by phagocytic and non-phagocytic cells, and in the latter this was correlated with NF- $\kappa$ B activation (Sulciner et al. 1996). The mitogenic activity of Ras-transformed NIH-3T3 cells is inhibited by agents that block ROS production (Irani et al. 1997). Evidence was provided that the superoxide production in response to Ras was independent of the MAPK pathway but involved a Rac-mediated signaling pathway. Therefore, a model was proposed in which the Rac-dependent ROS pathway cooperates with the MAPK pathway downstream of Ras to mediate Ras-induced mitogenic signaling. Another potential link to mitogenic signaling pathways may involve S6 kinase, shown to in-



teract with and be activated by Rac and Cdc42 (Chou and Blenis 1996). pp70<sup>S6K</sup> itself appears to play a role in cell cycle progression, in that microinjection of neutralizing antibodies against pp70<sup>S6K</sup> into the nucleus or cytoplasm inhibits DNA synthesis in response to growth factors (Proud 1996). However, it remains to be defined whether pp70<sup>S6K</sup> is a possible mediator of Rac- or Cdc42-induced mitogenesis. The observation that the promoters of most matrix metalloproteases contain binding sites for both AP-1 and PEA3 and that increased metalloprotease expression has been associated with malignant progression, combined with the fact that Rac has a role in the transcriptional activity of these factors, raises the question whether the latter contributes to the ability of Rac to trigger invasiveness (O'Hagan et al. 1996).

Because an altered morphological phenotype is a key feature of transformed cells, it has been postulated that the Rho GTPase signaling pathways that mediate cytoskeletal rearrangements contribute to the general phenomenon of transformation and invasiveness. However, the short-term induction of filopodia, lamellipodia, and stress fibers induced by the members of the Rho family appear not to correlate with their growth-promoting activity (Symons 1996). Whereas activated Rho induces stress fibers, the latter structures are inhibited in Ras-transformed cells (Qiu et al. 1995b). Coexpression of RacN17 abolishes the ability of RasV12-expressing cells to grow on soft agar but enhances filopodia formation (Nobes and Hall 1995). Furthermore, Tiam deletion mutants unable to induce membrane ruffles are still able to transform NIH-3T3 cells (van Leeuwen et al. 1995; Collard et al. 1996). Thus, the short-term induction of cytoskeletal rearrangements and the transformation effects mediated by Rho GTPases appear to be executed by distinct pathways. Although several observations (e.g., lamellipodia formation is a characteristic of motile cells) suggest a contribution of short-term cytoskeletal rearrangements to invasiveness and metastasis, this requires further investigation. As discussed above, a role for the Rho-like GTPases in integrin-mediated signaling pathways has been provided. Hints that integrins play a critical role in tumorigenesis and metastasis have been in evidence for a few years (Varner and Cheresch 1996). Of key interest in the future will be to investigate the interrelationships between these activities and to define the effectors involved in mediating Rho GTPases-induced mitogenesis, transformation, and invasiveness.

### Rho GTPases in development

During development, a hierarchy of biological phenomena leads to the generation of morphologically differentiated cells, many of which contain specialized actin-rich structures such as microvilli, neurites, or muscle spindles. The ability of the Rho family of GTPases in orchestrating actin-filament rearrangements raises the issue of their potential roles in morphogenesis. A number of genes coding for the Rho family of GTPases in *Drosophila* have been identified and their roles in various aspects of development have been investigated. The

current understanding on the role of the Rho GTPase family in development has been shaped from studies using *Drosophila* as a model system, although studies in other systems have also contributed significantly. In this section we have described the role of Rho GTPases in several aspects of *Drosophila* development.

The *Drosophila* homologs of Rac1, Rac2, and Cdc42, namely DRac1, DRac2, and DCdc42, respectively, have been shown to be highly expressed in the nervous system and in the mesoderm during neuronal and muscle development, respectively (Luo et al. 1994). Notably, in developing neurons, the expression of both constitutively activated and dominant-negative mutants of DRac1 perturbed the initiation and elongation of axonal outgrowth, whereas the development of dendrites appeared normal (Luo et al. 1994). These perturbations appear to result from defects in the actin cytoskeleton. In neurons of wild-type embryos relatively early in development, an enrichment of filamentous actin (F-actin) in dorsal neuronal clusters is observed that disappears during later stages in development. In the presence of a constitutively active mutant allele of Rac, DRacV12, the accumulation of actin in the dorsal neuronal clusters persisted through later stages of development. On the other hand, no F-actin accumulation at any stage was observed on expression of the dominant-negative mutant of DRac1. These observations suggest that the inhibition of axonal outgrowth induced on expression of the constitutively activated and dominant-negative mutants of DRac1, arises from different cytoskeletal defects in developing neurons and that oscillation of DRac1 between its GTP and GDP states is critical for the developing axon. Furthermore, these findings support the contention that DRac1 could participate in morphogenetic differentiation by regulating the establishment of neuronal asymmetry, that is, the initiation and elongation of axonal outgrowth without perturbing the formation of dendrites. Similar although not identical effects of Rac1 on developing neurons have been observed in mammalian systems. Transgenic mice expressing constitutively active human Rac1 in Purkinje cells were ataxic and exhibited a reduction of Purkinje cell axons, whereas dendrites branch out and appear normal (Luo et al. 1996). Interestingly, the dendritic spines in the developing and mature cerebella of the transgenic animals were reduced in size, were increased in number, and formed supernumerary synapses (Luo et al. 1996). It has been proposed that dendritic spines create microdomains for synaptic integration and memory storage (Harris and Kater 1994), although how they originate is still unclear. The differential effects resulting from perturbing Rac1 activity indicate that there may be distinct mechanisms in the generation and compartmentalization of axons, dendrites, and dendritic spines.

Expression of constitutively active DCdc42, DCdc42V12, in the neurons of the fly embryo results in a similar although qualitatively distinct effect on neuronal development as compared to DRac1V12. Neurons expressing DCdc42V12 exhibited incomplete axonal outgrowth, and the dorsal neuronal clusters appear to be

shorter and rounder, as opposed to the more elongated dorsal clusters seen in DRac1V12-expressing embryos (Luo et al. 1994). In addition, in DCdc42V12-expressing embryos a subset of dendrites (dorsal external sensory dendrites) are absent. Also, neuronal cell positioning is abnormal, which could have resulted from an abnormality in cell migration (Luo et al. 1994).

Little is known about the role of the Rho GTPase in neuronal development. The RhoA homolog in *C. elegans* has been isolated and CeRhoA was shown to be expressed uniformly throughout development with an enrichment at the larval stage in the nerve ring and the chemosensory and mechanosensory neuron at the tip of the head (Chen and Lim 1994). These observations suggest a role for CeRhoA in sensory signaling during embryonic development; however, this remains to be proven.

DRac1 and DCdc42 have also been implicated in muscle differentiation. Both GTPases have been found to be highly expressed in the mesoderm during muscle differentiation of developing flies. In *Drosophila*, studies on muscle development have led to the hypothesis that muscle fibers originate from a founder cell and that other fusion-competent myoblast cells fuse with the founder cell to generate patterned muscle fibers (Bate 1990; Bate and Rushton 1993). Thus, during muscle differentiation, myoblast fusions lead to the formation of muscle fibers. Expression of DRac1 mutants perturb muscle morphogenesis by perturbing myoblast fusion. Whereas expression of the constitutively activated mutant of Drac1 inhibited myoblast fusion, expression of the dominant-negative mutant generates excessively fused muscle fibers in later developmental stages (Luo et al. 1994). Myoblast fusions were unaffected by expression of mutant forms of DCdc42; however, the symmetry of the muscle fibers appeared abnormal. It has been proposed that Cdc42-induced muscle asymmetry may result from defective migration of myoblasts prior to fusion (Luo et al. 1994). Thus, Rac and Cdc42 regulate muscle development most likely by their effects on membrane fusion and the actin cytoskeleton, respectively. There has not been any report on the role of Rho in muscle differentiation; however, two genes isolated from *C. elegans*, *let-502* and *mel-11*, show homology to the mammalian Rho kinase and to the regulatory subunits of smooth muscle myosin-associated phosphatase, respectively. Both of these genes were found to be associated with cell shape changes during the elongation of the developing worm (Wissmann et al. 1997).

DRac1 has also been shown to play a role during dorsal closure of the epidermis during fly development, a process in which the lateral epidermal cells migrate over the amnioserosa of the developing embryo and join at the dorsal midline (Martinez-Arias 1993). During this process, the shape of the epidermal cells in the dorsal region changes dramatically. It is believed that (1) actin-myosin interactions drive cell elongation at the lateral epidermis, and (2) the actin-myosin content at the leading edge of the lateral epidermal cells involved in closure and the degree of cell elongation are directly correlated (Young et

al. 1993). Microinjection of DRac1N17 into embryos resulted in a high frequency of embryos with defects in dorsal closure that was manifested as scabs or holes on the dorsal cuticle (Martinez 1993). These defects were characterized by morphological abnormalities in cells of the lateral epidermis. Cells expressing DRac1N17 exhibited a disruption in the accumulation of myosin and actin along the leading edge that is believed to drive epidermal cell shape (Harden et al. 1995). Such a closure defect is reminiscent of the observed phenotype in fly *zip* mutants, wherein dorsal holes likely caused by a lack of nonmuscle myosin at the leading edge of epidermal cells (Young et al. 1993). In addition, DRac1N17 expression also disrupted the localization of  $\alpha$ -spectrin at the leading edges of the cells. It was shown previously that during *Drosophila* embryogenesis  $\alpha$ -spectrin localized to areas of pre-existing actin accumulation (Pesacreta et al. 1989). Therefore, it has been proposed that DRac1 may regulate actin accumulation at the leading edge of cells and that the lack of actin accumulation may be responsible for the disrupted localization of  $\alpha$ -spectrin and myosin at the edges of DRac1N17-expressing epidermal cells. It remains to be seen whether the Rho GTPases are involved in other developmental processes that involve myosin-based motors. Interestingly, the *Drosophila* homolog of c-Jun kinase, DJNK, has been shown to play a role in dorsal closure; embryos lacking DJNK were defective in dorsal closure (Riesgo-Escovar et al. 1996; Sluss et al. 1996). Also the fly MAPK kinase HEP, shown to phosphorylate DJNK (Sluss et al. 1996), is required for expression of the *puckered* gene product and the latter is needed for dorsal closure to occur (Nüsslein-Volhard et al. 1984). Furthermore, *Drosophila* PAK homolog has been isolated and shown to bind the GTP-bound form of DRac1 and DCdc42 during embryogenesis (Harden et al. 1996). Interestingly, DPAK levels were elevated in focal complexes along the leading edges of epidermal cells. It has been proposed that DPAK may regulate the turnover of Rac1-dependent focal complexes at the leading edge. Therefore, it appears as though an analogous kinase cascade, as described for mammalian cells and yeast (Fig. 6), which includes DRac1, DPAK, HEP, and DJNK, could exist in *Drosophila* and may regulate the process of dorsal closure during fly development. It has been demonstrated that dorsal closure is also blocked by expression of the dominant-negative mutant of DCdc42 (Riesgo-Escovar et al. 1996). Therefore it is possible that, like their vertebrate counterparts, both DCdc42 and DRac1 function in the DJNK pathway.

A role for DRac1 and DCdc42 in the development of fly wing disc epithelium and wing hairs has also been demonstrated. Imaginal discs of the wing, derived from the embryonic ectoderm, are epithelial tissues that secrete the adult cuticle that generates the final shape of the adult. On pupal development, the disc unfolds and forms appendages (Poodry 1980). Expression of the dominant-negative alleles of DRac1 and DCdc42 in a subset of disc epithelial cells have revealed that these mutants have specific and nonoverlapping roles in disc development. It has been shown that DCdc42 controls epithelial

cell shape changes by modulating the basic actin cytoskeleton during pupal and larval development. Whereas normal wing disc cells increase in height at least twofold during the third instar, cells expressing dominant-negative DCdc42 remain short and unelongated. Expression of the negative mutant of DRac1 during disc development did not appear to affect cytoskeletal elements that regulate cell shape (Eaton et al. 1995). However, DRac1 activity appears to be required for actin assembly at the adherens junctions as the latter is disrupted on DRac1N17 expression (Eaton et al. 1995). Failure of adherens junction actin assembly was accompanied by increased cell death and consequently a reduction in wing size. Cell death may have resulted from DRac1N17 disrupting another vital cell function such as cytokinesis, or because adherens junction disassembly may itself be lethal. The defects caused by dominant-negative DRac1 or DCdc42 did not affect epithelial polarity per se, and apical and basolateral proteins remain correctly localized in cells expressing these mutants (Eaton et al. 1995).

The wing of *Drosophila* is covered by an array of distally pointing hairs. The wing epithelial cells form wing hairs by extending a single process from the apical membranes of the hairs (Mitchell et al. 1983; Wong and Adler 1993; Fristrom et al. 1994). This latter process is preceded by the accumulation of actin on the distal side of the cell and a bundling of microtubules that extend into the wing hair throughout its elongation. It has been shown that cells expressing the dominant-negative DCdc42 lack wing hairs and that at lower levels of mutant protein expression deformed and stunted hairs were formed (Eaton et al. 1996). In cells that did not extend hairs, actin failed to accumulate distally. In hair extensions, Cdc42 has been shown to be localized at the distal end, which may be its site of action. On the other hand, in cells expressing DRac1N17, multiple, but structurally normal, wing hairs were formed (Eaton et al. 1996). This failure to restrict hair outgrowth to a single site suggests that DRac1 might function to select the site for hair formation. Whether this function may be related to its role in organizing junctional actin is unclear. The DRac1N17 expressing cells also exhibited a disorganized microtubule network in the apical region of the cell. Thus, the site selection for hair outgrowth may require an intact microtubule network. It has been proposed that the regulation of adherens junction assembly and microtubule organization in response to Rac1 activity may be directly coupled to the suppression of inappropriate hair growth (Eaton et al. 1996). Notably, a multiple wing hair phenotype is typical of tissue polarity mutants such as *multiple wing hair (mwh)* and *intwined (in)*. (Adler 1992). Interestingly in this regard, hypomorphic alleles of RhoA in the fly wing exhibit abnormal hair polarity and a multiple wing hair phenotype. Expression of a dominant-negative form of RhoA in part of the wing resulted in a polarity defect typical of *frizzled (fz)*, a serpentine receptor-like transmembrane protein, and *dishevelled (dsh)*, which acts downstream of *fz* (Strutt et al. 1997). These cells exhibit a wing hair polarity defect.

The above findings suggest that RhoA is required for the generation of polarity in fly epithelial cells.

Studies investigating the effect of Rho in fly eye morphogenesis have also been conducted. These studies have shown that expression of a Rho transgene in the eye of *Drosophila* resulted in a dose-dependent disruption of normal eye development (Hariharan et al. 1995). Such flies exhibited a severe rough eye phenotype characterized by missing secondary and tertiary pigment cells, a substantial reduction in the number of photoreceptor cells, and a grossly abnormal morphology of rhabdomeres. These defects became manifest when cells were undergoing rapid changes in cell shape. Cells such as the primary pigment cells, which do not span the thickness of the retina and display fewer morphological changes, were more resistant to the effects of Rho expression. It has been suggested that Rho-induced actin polymerization could hinder normal development of cells undergoing dramatic shape changes. More recently, isolation of several loss-of-function alleles of RhoA in *Drosophila*, has revealed that RhoA may be critical for the establishment of tissue polarity in the developing eye. Homozygous mutant clones carrying null alleles in the imaginal disc of the eye fail to proliferate, indicating a role for RhoA in cell growth and/or viability, whereas clones expressing hypomorphic alleles of RhoA give rise to ommatida that are incorrectly rotated, resulting in a disruption of planar polarity (Strutt et al. 1997). Such a phenotype is similar to that seen in overexpression of tissue polarity genes such as *fz* and *dsh*. These studies have also shown that RhoA may be part of a signaling pathway downstream of *fz* and *dsh*. Furthermore, mutations in the *basket (bsk)* gene suppress the effects of *fz* and *dsh*, suggesting a role for JNK/SAPK-like kinases in the aforementioned Rho-mediated signaling pathway (Strutt et al. 1997).

*Drosophila* oogenesis provides an excellent system for investigating processes such as cell migration, membrane fusion, and cytoskeletal remodeling during development. *Drosophila* egg chambers harbor 15 polyploid nurse cells and 1 oocyte, and all 16 cells remain connected to each other by actin-rich bridges as a result of incomplete cytokinesis. Oogenesis involves a number of processes wherein the actin cytoskeleton undergoes dramatic changes (Murphy and Montell 1996). Early in oogenesis, germ-line clusters become surrounded by an epithelial monolayer of somatic follicle cells. Many of the follicle cells elongate to form columnar epithelial cells, whereas some assume a squamous shape, and yet others migrate to the tip of the chamber. Late in oogenesis the transfer of nurse cell cytoplasm into the oocyte occurs and is preceded by actin polymerization around the nurse cell nuclei (Cooley et al. 1992; Peifer et al. 1993). Studies on the Rho-family proteins, namely Drac1, RhoL, a novel Rho homolog, and Cdc42, have revealed that they are required for multiple processes during oogenesis (Murphy and Montell 1996). These GTPases appear to play distinct roles in each cell type, although the activities of all three GTPases are required for transfer of nurse cell cytoplasm into the oocyte. During oogenesis,

DRac1 activity was found to be required for follicle cell migration. Follicle cells expressing the dominant-negative DRac1 mutant were impeded in their ability to migrate and lacked pseudopodia extensions that were seen in normal migrating cells. Border cell migration was specifically dependent on DRac1 and was independent of Cdc42 and Rho function. Increases or decreases of Cdc42 activity or a decrease of RhoL function caused nurse cells to collapse and fuse together. Interestingly, Rac1, but not Cdc42, has been implicated in the regulation of myoblast fusion during muscle development (Luo et al. 1994). Thus, the involvement of specific Rho proteins in similar processes appears to vary with tissue type. As mentioned above, the activities of all three GTPases, Rac, RhoL, and Cdc42, were required for transfer of nurse cell cytoplasm into the oocyte, an actin-myosin-based process referred to as "dumping". The expression of dominant-negative forms of RhoL, DRac, and DCdc42 impeded the polymerization of actin around cell nuclei, thereby preventing the nuclei from being dumped during the transfer process. As a result, ring canals were obstructed by nurse cell nuclei. Thus, as in other cells and tissue, the Rho proteins, via their effects on the actin cytoskeleton, play related although distinct functions in oogenesis.

A recent study has demonstrated a role for Rho in T-cell development. Transgenic mice that lacked Rho function by targeting of a transgene encoding C3 transferase to the thymus displayed maturational proliferative and cell survival defects (Henning et al. 1997). Thymi were strikingly smaller, and the generation of thymocytes and mature peripheral T cells was severely impaired. However, analysis of the maturation stages of the thymocytes lacking Rho function showed the existence of a subpopulation of thymocytes representing all stages of thymocyte development, although they were severely reduced in numbers. Thus, differentiation of progenitor cells to mature T cells could occur in the absence of Rho function. It has been proposed that the decrease in cellularity in thymi lacking Rho function could be attributable to the failure of thymocytes to traverse through the G<sub>1</sub> phase of the cell cycle (Henning et al. 1997). Thus, Rho signaling appears to be selectively required in early development for proliferative expansion and survival of thymocytes.

Based on the findings described above, it is evident that Rho proteins appear to have multiple essential functions during morphogenesis, and their effects in development, although related, vary from one tissue to another. Their activities are called upon for dynamic processes such as cellular outgrowths, cell migration, cell fusion, and cell elongation. In some cases, loss of protein function induced by expression of the dominant-negative alleles produces phenotypes similar to those seen by expression of the activated form of the protein, suggesting that tight control of wild-type protein function is crucial for normal development. Also, as described earlier, regulators and effector molecules of the Rho family have been shown to modulate morphogenetic and developmental processes in a number of systems. Although a

number of Rho effectors and regulators have been identified, the elucidation of the molecular mechanisms by which they function inside the cell to regulate and mediate various Rho-induced physiological processes is still at a preliminary stage. Genetically tractable organisms may provide a unique and interesting system to better understand the molecular mechanisms underlying Rho protein signaling in vivo.

### Concluding remarks

During the past few years, considerable progress has been made toward understanding the signaling pathways and the function of Rho family members. These studies have revealed that Rho GTPases are versatile signaling molecules that regulate a diverse set of cellular functions and are capable of interacting with a large number of proteins. Currently, the significance of many of these interactions remains enigmatic, and it will be a major challenge in the future to establish their physiological relevance and to dissect the diverse signaling pathways upon which they act. In this regard, a promising approach will be the generation of additional Rho GTPase mutants that selectively associate with only a subset of effector proteins. In addition, detailed biochemical analysis of the effector proteins, along with identification of additional proteins that interact with them, will unravel downstream signaling pathways of Rho GTPases. Furthermore, the discovery that many Rho family members, as well as their targets, appear to be fairly conserved from yeast to man implies that many of the signaling pathways and functions controlled by these GTPases also may be conserved throughout the eukaryotic kingdom. Thus, studies in genetically tractable systems such as *Drosophila*, *C. elegans*, and yeast should prove to be extremely useful for the precise definition of Rho-signaling networks and the specificity of their responses in vivo.

The forthcoming resolution of these issues will involve some fascinating biological insights and will most likely represent an area of active and exciting research over the next few years.

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