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PROSPECTS

Rho-GTPases: New Members, New Pathways

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Abstract Proteins comprising the Rho family of GTPases mediate reorganization of the actin cytoskeleton as well as transcription of genes. Recent findings from genome sequencing efforts, genetic screens, and signal transduction research have revealed that the Rho family contains several new, hitherto unrecognized members. In this review, we focus on these newly discovered Rho-GTPases and discuss their role in signaling to the cytoskeleton and the nucleus. J. Cell. Biochem. 94: 225–231, 2005. © 2004 Wiley-Liss, Inc.

Key words: GTPases; Rho; Cdc42; Rac; cytoskeleton; effectors; transcription

Small GTPases comprise a family of 20–25 kDa monomeric signal proteins that plays a major role in a host of cell functions, including growth, differentiation, motility, cytokinesis, and intracellular transport. Small GTPases cycle between an active, GTP-bound state, which is able to bind effector proteins, and an inactive, GDP-bound state [Erickson and Cerione, 2004]. Cycling between the GTP- and GDP-bound states is regulated by: (1) guanine nucleotide exchange factors (GEFs) that stimulate the replacement of GDP by GTP; (2) GTPase activating proteins (GAPs) that stimulate the intrinsic GTP hydrolyzing activity of GTPases; and (3) guanine nucleotide dissociation inhibitors (GDIs) that act most often as negative-regulators by blocking GDP-dissociation and hence nucleotide exchange of GTPases.

Of the five major families of small GTPases (Ras, Rho, Arf, Rab, and Ran), the Rho family plays a special role in regulating the organization of the actin cytoskeleton and thereby the morphogenesis of the cell, as well as the ability of cells to migrate and invade surrounding tissues. In addition, Rho-GTPases regulate gene transcription through effects on mitogen activated protein kinase (MAPK) cascades, activation of Nuclear Factor κB (NFκB), and other signaling modules. These actions of Rho-GTPases are mediated by a wide variety of downstream effectors. Importantly, the expression or activation of Rho-GTPases and/or their effectors is often altered in cells derived from human malignancies.

The mammalian Rho family can be divided into eight subgroups comprising 22 members: Cdc42 (Cdc42, TC10, TCL, Chp, Wrch-1); Rac (Rac1-3, RhoG); Rho (RhoA-C); Rnd (Rnd1, Rnd2, Rnd3/RhoE); RhoD (RhoD and Rif); RhoH/TTF; RhoBTB (RhoBTB1 and RhoBTB2); and Miro (Miro-1 and Miro-2) (Fig. 1). These enzymes all contain at least one GTPase domain located in the N-terminal half of the protein and, in almost all cases, a C-terminal sequence that is modified by the addition of lipid groups. In this article, we focus on the regulation and function of newly discovered members of this family, with particular emphasis on novel features found in these GTPases.

EXPANDING Cdc42 SUBGROUP

Wrch-1 (Wnt-1 responsive Cdc42 homolog) was identified in a screen for transcripts induced by Wnt-1 [Tao et al., 2001]. Like most Rho family proteins, Wrch-1 contains a C-terminal CAAX lipid modification signal, although neither the nature of the potential attached lipid, nor its role in intracellular localization of Wrch-1 are known. Wrch-1 also possesses an N-termini domain that differs from other Cdc42, containing several putative PXXP SH3-binding motifs [Tao et al., 2001]. Recently, Tao et al. [2001] showed that Wrch-1 is expressed

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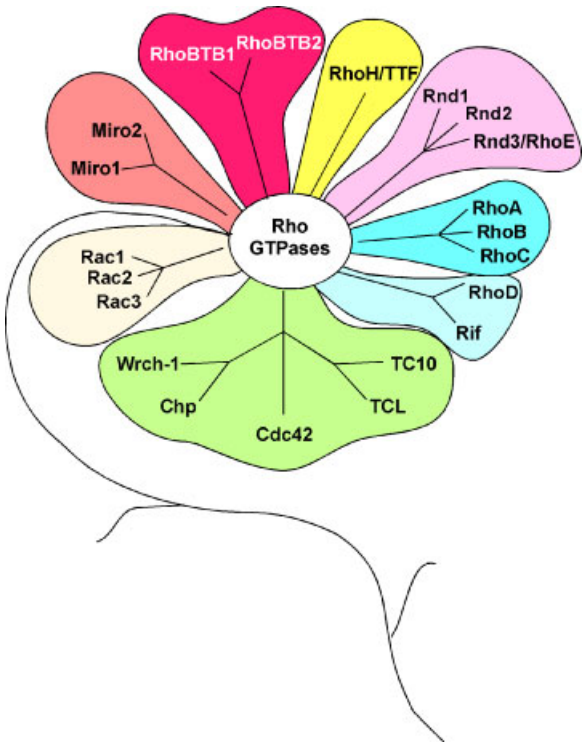

Fig. 1. Small GTPases of the Rho family. Rho family members can be grouped in eight subgroups: Cdc42 (Cdc42, TC10, TCL, Chp, and Wrch-1); Rac (Rac1-3, RhoG); Rho (RhoA-C); Rnd (Rnd1, Rnd2, and Rnd3/RhoE); RhoD (RhoD and Rif); RhoH/TTF; RhoBTB (RhoBTB1 and RhoBTB2); and Miro (Miro-1 and Miro-2). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

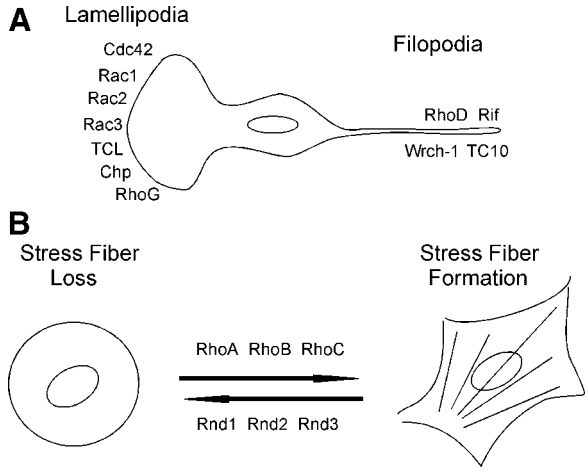

Fig. 2. Cytoskeletal effects of Rho-GTPases. Rho-GTPases affect cytoskeleton organization in characteristic ways. Many Rho-GTPases induce the formation of lamellipodia and/or filopodia (**A**). The Rho and Rnd subgroup have dramatic, and opposed, effects on stress fiber formation (**B**). Other Rho proteins, such as those in the RhoBTB and Miro-subfamilies, have no obvious cytoskeletal effects.

highly in brain, skeletal muscle, and placenta, moderately in liver, lung, and heart, and at low levels in colon, spleen, kidney, and small intestine. Wrch-1 mRNA was found to be significantly upregulated in primary tumors of the kidney, colon, stomach, breast, ovary, and uterus, although only a small number of cases were analyzed, and Wrch-1 mRNA was significantly downregulated in other tumors. While the signaling mechanisms that downregulate Wrch-1 expression are not well defined, it is known that expression of Wrch-1 mRNA is downregulated by beta-estradiol in MCF-7 cells [Kirikoshi and Katoh, 2002].

As with Cdc42, activated mutants of Wrch-1 induce formation of filopodia and dissolution of stress fibers (Fig. 2A). However, the long, thin, filopodia induced by Wrch-1 in Swiss 3T3 cells are distinct from the shorter, broader structures elicited by other members of the Cdc42 subfamily such as TC10 and Chp [Tao et al., 2001]. In porcine aortic endothelial cells, activated Wrch-1 induces a spiky phenotype, whereas Cdc42 induces small lamellipodia [Aspenstrom et al., 2004]. These morphologic differences suggest that Wrch-1 recruits a distinct set of effector proteins. Alternatively, differences in intracellular localization and/or activation kinetics might underlie the unique signaling properties of this GTPase, like Cdc42 and Rac1, constitutively active Wrch-1 is a strong activator of c-Jun Kinase (JNK). The signaling mechanism underlying this effect is not known, but might involve p21-activated kinases (Paks), as Pak1 binds to, and is activated by Wrch-1, and is a known activator of JNK [Bagrodia et al., 1995; Brown et al., 1996].

Given its discovery as a Wnt-1-regulated gene, it is assumed that Wrch-1 plays some role in Wnt-1 signaling. The Wnt/Wingless signaling pathway is known to regulate cell morphology and polarity, and cell-cell adhesion, and to specify cell fate and control cell proliferation [Cadigan and Nusse, 1997]. In mammals, the Wnt family proteins initiate signaling by binding to their cognate receptor, Frizzled. The canonical Wnt-Frizzled pathway activates the cytoplasmic protein disheveled (Dsh), which in turn inhibits the kinase activity of glycogen synthase kinase-3β (GSK-3β), resulting in stabilization and accumulation of β-catenin in the cytoplasm [Polakis, 2000]. The elevated levels of β-catenin promote its oligomerization with TCF/LEF transcription factors, which activate

the transcription of downstream genes, such as c-Myc, c-Myb, and Cyclin D, which affect cell proliferation and differentiation, as well as matrilysin and matrix metalloprotease 7, which promote cell motility [Giles et al., 2003] (http://www.stanford.edu/~rnusse/pathways/targets.html). In addition to this canonical model, Wnt can also control the expression of other genes in a β-catenin-independent, but protein kinase C sensitive manner [Ziemer et al., 2001]. Wnt-1 regulates Wrch-1 expression in a β-catenin-independent manner, implying the involvement of a non-canonical pathway [Tao et al., 2001]. Irrespective of the mechanism of its induction, the role of Wrch-1 in Wnt-1 signaling is not established.

Chp (a.k.a. Wrch-2) was first identified as a novel protein interacting with the regulatory domain of another Pak kinase, Pak2 [Aronheim et al., 1998]. Chp is most similar to Wrch-1 (55.4% total-amino-acid identity) and Cdc42 (43.5% total-amino-acid identity). Like Wrch-1, Chp contains an N-terminal proline-rich domain that is absent in Cdc42 [Katoh, 2002]. At its C-terminus, Chp lacks a classic CAAX box, but instead contains a unique 32 amino acid extension that does not share sequence homology to known proteins [Aronheim et al., 1998].

Chp is highly expressed in pancreas, placenta, brain, and testis; lower amounts are detected in spleen and lung [Aronheim et al., 1998]. Chp is abundant in a number of cancer cell lines, and it has been suggested that Chp over-expression in human cancer cells might lead to a more malignant phenotype [Aronheim et al., 1998]. As with Rac and Cdc42 [Coso et al., 1995], active forms of Chp stimulate Pak and JNK; however, Chp fails to activate p38 kinase and shows no significant Erk2 activation [Aronheim et al., 1998]. Further, expression of a dominant negative form of Chp blocks JNK activation by Src and Cdc42, as well as by tumor necrosis factor-α (TNF-α), confirming that Chp interacts with key JNK regulators. Based on these data, Aronheim et al. have proposed that Chp acts as a selective activator of the JNK MAPK cascade but not of the Erk and p38 pathways.

Like active forms of Rac and Cdc42 [Sells and Chernoff, 1997] Chp interacts with the regulatory (N-terminal) domain of Paks in a GTP-dependent manner [Aronheim et al., 1998]. Interestingly, a C-terminal deletion mutant of Chp is unable to bind Pak2 in a two-hybrid system, whereas an N-terminal deletion has no significant affect on Pak2 binding. These findings are unusual, because similar enzymes such as Cdc42 and Rac bind to Paks by means of the GTPase effector domain, which is located near the N-terminus of these proteins. These data suggest that the extended C-terminus of Chp is either directly involved in binding Pak or that the loss of this domain results in a conformational change in Chp that prevents Pak binding. In either case, these findings suggest an unusual and unprecedented mode of interaction between this small GTPase and one of its effectors.

Expression of activated Chp, like its cousins Cdc42 and Wrch-1, induces reorganization of the cytoskeleton. Cells expressing an activated form of Chp show morphological changes, chiefly induction of lamellipodia (Fig. 2A). A substantial portion of activated Chp localizes to the Golgi apparatus, as has been reported for its putative effector Pak2 [Huang et al., 2003]. It will be interesting to determine if Chp is required for Pak2 localization to this organelle.

TCL (TC10-like) is preferentially expressed in heart and shares 85% and 78% amino acid similarity with TC10 and Cdc42, respectively [Vignal et al., 2000]. Like TC10, TCL contains an N-terminal extension compared with canonical small GTPases such as Cdc42 and Rac. At its C-terminus, TCL contains a two-cysteine, CCAAX box, a potential substrate for both geranylgeranyl- and farnesyltransferases.

In vitro, purified TCL shows ~40% more rapid GDP/GTP exchange than its close relative TC10. These findings suggest that TCL exists predominantly in the activated, GTP-bound form in vivo. TCL binds many of the same effectors as TC10 and Cdc42,such as Pak and Wiskott-Aldrich Syndrome Protein (WASP), and these interactions appear to be GTP-dependent. Despite these similarities, expression of constitutively active TCL elicits the formation of distinctive cytoskeletal structures in fibroblasts. In one study, these were characterized as lamellipodia [Aspenstrom et al., 2004], (Fig. 2A) in another, as a limited number of ruffle-like protrusions on the dorsal cell membrane associated with large intracytoplasmic vesicles [Vignal et al., 2000]. Interestingly, this phenotype was found to be blocked by expression of dominant negative Rac1 or Cdc42, suggesting that either

these GTPases act downstream of TCL in a cytoskeletal signaling pathway or that they compete for a common pool of effectors.

TCL localizes to the plasma and intracellular membranes through its C-terminal polybasic and CCAAX box regions [de Toledo et al., 2003]. Deletion of the CCAAX box causes TCL to relocate to the cytoplasm. TCL significantly co-localizes with early endosome antigen-1, a marker of the compartment, and the transferrin receptor, which cycle between the plasma membrane and recycling endosome compartments. siRNA-mediated knock-down methods have been used to establish that TCL is required for clathrin-dependent endocytosed receptors to enter the early/sorting endosomes. These findings are supported by overexpression studies, in which expression of activated TCL promotes direct recycling of transferrin from early/sorting endosomes [de Toledo et al., 2003].

TCL might also play an important role in differentiation in some cell types. Nishizuka [2003] has reported that TCL expression is induced during the differentiation of 3T3-L1 cells to adipocytes. Antisense-induced loss of TCL expression inhibits differentiation, whereas constitutive expression of TCL in NIH-3T3 cells, which do not normally differentiate into adipocytes, causes these cells to accumulate fat droplets when the cells are grown in the presence of the ligand for peroxisome proliferator-activated receptor gamma (PPARγ). These results indicate that TCL plays a key role in the early stage of adipocyte differentiation, probably linked to the PPARγ pathway.

Rif

The Rif GTPase, a 211 amino acid protein, was identified as a novel Rho family GTPase from a partial cDNA sequence in the human expressed sequence tag (EST) database [Ellis and Mellor, 2000]. Rif shares between 32%–49% identity with other Rho-GTPases, showing the greatest degree of identity with Rac2 (49%), RhoD (48%), RhoA (47%), and Cdc42 (43%). Rif is widely expressed in human tissues, with the highest levels of expression in colon, stomach, and spleen.

A constitutively active form of Rif localizes entirely to the plasma membrane, and, like Cdc42, causes the formation of numerous long, highly dynamic, actin-rich filopodia structures, (Fig. 2A) with a modest increase in actin stress fiber formation [Ellis and Mellor, 2000] [Aspenstrom et al., 2004]. However, unlike Cdc42-induced filopodia, which contain vinculin-rich focal complexes at their tips, Rif-induced filopodia contain vinculin-rich focal complexes at their base. Rif also differs from Cdc42 in its use of effectors. Rif lacks key residues that are required for interaction with CRIB-containing proteins such as Pak and WASP, and is unlikely to bind such proteins. In the case of WASP, this idea has been tested directly by coimmunoprecipitation assays, which confirm that Rif and WASP do not interact in cells [Ellis and Mellor, 2000].

Miro-1, Miro-2

Miro-1 and Miro-2 (mitochondrial Rho) are unusual members of the Rho-GTPase family [Fransson et al., 2003], by virtue of their size (~70 kDa), their unique domain organization, and their mitochondrial location. The N-terminal regions of Miro-1 and -2 encode a typical Rho-related, GTPase domains, followed by two EF hands (a domain that confers binding to calcium ions). The C-terminus of each Miro-protein contains a second potential GTP-binding domain, but in this case without a homology to GTP-binding domains found in other Rho-GTPases. In addition, Miro-1 and -2 lack C-terminal CAAX motifs. The unusual domain organization of Miro suggests that these proteins are regulated and function in a manner distinct from classical Rho-GTPases. Because genes encoding Miro-like proteins are found in diverse eukaryotic organisms from *Saccaromyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster* to mammals, it is likely that these genes evolved early during evolution and serve an important function. Miro-mRNA is also present in most human tissues and is abundant in heart, liver, skeletal muscle, kidney, and pancreas [Fransson et al., 2003].

Unlike other Rho-GTPases, overexpression of activated Miro-1 and -2 has no effect on the organization of the actin filament system or microtubules (Fig. 2C). Instead, expression of an activated form of Miro-1 induces a collapse of the mitochondrial network, suggesting that Miro-1 is important for the integrity of mitochondrial function. The exact mechanism of these effects remains to be studied. It is interesting to note, however, that cells expressing activated Miro-1 are prone to apoptosis, a phenotype one might expect in the presence of

compromised mitochondrial function [Parone et al., 2002].

RhoH/TTF

RhoH is a hematopoietic-specific GTPase with several unusual properties. First, the protein appears to be locked in the active, GTP-bound state, due to the presence of alternate amino acids at two critical positions that confer resistance to the action of GAPs [Li et al., 2002]. Regulation of RhoH may instead occur through modulation of transcription. Second, the gene encoding this protein is frequently involved in translocations and point mutations on lymphoma cells, making it one of the few examples of a Rho-GTPase that is mutated in human cancer [Dallery et al., 1995]. RhoH does not affect the actin cytoskeleton; however, it antagonizes the activation of p38 and NFκB by other Rho-GTPases [Li et al., 2002]. Reduction of RhoH levels in T-cells augments the response to Rac activation, suggesting that these GTPases act in opposition in cells.

RhoBTB

Members of the RhoBTB subfamily of Rho-GTPases are present in vertebrates, *Drosophila* and *Dictyostelium* [Rivero et al., 2001]. The RhoBTB family consists of three members. RhoBTB1 and RhoBTB2 have no obvious role in the organization of actin filament system (Fig. 2C) [Ramos et al., 2002]. RhoBTB1 and RhoBTB2 are localized at vesicular structures in the cytoplasm that are not lysosomes or Golgi apparatus [Aspenstrom et al., 2004]. Like RhoH, these GTPases are thought to function as constitutively GTP-bound proteins since they contain amino acid residues in positions critical for GTP hydrolysis. The GTPase domain of these proteins is followed by a proline rich region, tandem BTB (Broad-Complex, Tramtrack, and Bric-a-Brac), and a C-terminal region of unknown function. The BTB domain, also known as a POZ (poxvirus and zinc finger) domain, is an evolutionary conserved domain involved in protein-protein interactions, participating in homomeric and heteromeric associations with other BTB domains.

The gene encoding RhoBTB2 is located on human chromosome 8p21, a region commonly deleted in cancer. Rivero et al. [2001] have recently reported that RhoBTB2 is a substrate for a Cul3-based ubiquitin ligase complex. Further, a RhoBTB2 missense mutant identified in a lung cancer cell line is neither able to bind Cul3, nor regulated by the ubiquitin/proteasome system, resulting in increased RhoBTB2 protein levels in vivo. These findings suggest that RhoBTB2 might function as a tumor suppressor by recruiting proteins to a Cul3 ubiquitin ligase complex for degradation.

RhoBTB1 and RhoBTB3 are ubiquitously expressed, but they display different patterns of expression [Ramos et al., 2002]. RhoBTB1 is highly expressed in stomach, skeletal muscle, placenta, kidney, and testis, whereas RhoBTB3 is highly expressed in neuronal and cardiac tissues, pancreas, placenta, and testis. RhoBTB2 is expressed at lower levels than RhoBTB1 or RhoBTB3, and mostly in neuronal tissues. RhoBTB expression appears to be upregulated in some cancer cell lines, suggesting that RhoBTB proteins participate in the pathogenesis of particular tumors.

Rnd

The Rnd family comprises the closely related Rnd1, Rnd2, and Rnd3 (a.k.a. RhoE) proteins (Fig. 1). Rnd proteins share 54%–63% identity pairwise, ~45%–49% identity with Rho, and slightly less identity with Rac and Cdc42 [Nobes et al., 1998]. Rnd1 and Rnd3 have N- and C-terminal extensions of ~30 amino acids relative to Cdc42, giving these proteins an apparent molecular mass of ~32 kDa. Unlike canonical members of the Rho family, Rnd proteins in the basal state are not cytosolic, but are predominantly associated with membranes, suggesting that they are not associated with GDIs [Nobes et al., 1998]. Also, unlike Cdc42, Rac, or Rho, Rnd proteins are predominantly farnesylated rather than geranyl-geranylated.

Because Rnd proteins have low affinity to GDP and no detectable GTPase activity, even in the presence of RhoGAP, it has been suggested that Rnd proteins are constitutively activated. If true, regulation of Rnds proteins might be controlled exclusively by the balance between transcription/translation and degradation, and/or by post-translational modifications such as phosphorylation, rather than by GEFs, GAPs, and GDIs [Chardin, 2003].

Like many other members of Rho family, Rnd1 and Rnd3 proteins affect the cytoskeleton (Fig. 2B). These effects are characterized by disruption of focal adhesions and stress fibers,

accompanied by cell rounding, suggesting that Rnd proteins antagonize Rho function [Nobes et al., 1998; Aspenstrom et al., 2004]. This antagonistic activity is achieved at several levels. First, Rnd3 has been shown to activate Rho GAPs, leading to inactivation of Rho. In fibroblasts, [Wennerberg et al. [2003]. showed that Rnd3 binds and activates p190 RhoGAP, and that loss of p190 RhoGAP prevents Rnd3-mediated Rho inhibition [Wennerberg et al., 2003]. During cytokinesis in germ cells, Rnd2 interacts and co-localizes with male-germ-cell Rac GTPase-activating protein (MgcRacGAP), which is found at the midzone/midbody during the late stages of cell division [Naud et al., 2003]. MgcRacGAP has been proposed to downregulate RhoA, which is also localized to the cleavage and midbody during cytokinesis. Although the effects of Rnd2 on cytokinesis have not been reported, it is attractive to speculate that Rnd2 might regulate Rho activity via MgcRacGAP. Second, Rnd proteins also antagonize Rho-signaling by inhibiting downstream effectors such as ROCK I. Rnd3 binds to the amino-terminal region of ROCK I encompassing the kinase domain, at the site distant from the C-terminal RhoA-bounding site [Riento et al., 2003]. Since Rnd3 overexpression inhibits ROCK I-induced stress fiber formation and phosphorylation of the ROCK I target myosin light chain phosphatase, it is likely that Rnd3 induces stress fiber disassembly by binding to ROCK I and inhibiting it from phosphorylating downstream targets. Finally, it is probable that other proteins also participate in mediating the effects of Rnd GTPases on cytoskeletal structure. For example, Rnd1 and Rnd3 bind to a Ultrabithorax domain-containing protein known as Socius, which, when artificially tethered to the plasma membrane, induces loss of stress fibers and cell rounding [Katoh et al., 2002]. Rnd1 and Rnd3 cause the translocation of Socius to the cell periphery, inducing a dramatic loss of stress fibers and rounding up the cell body. These findings suggest that Socius acts at the plasma membrane, downstream of Rnd1 and Rnd3, and contributes to the cell rounding effect, although the mechanism by which Socius induces these changes is not known.

In cells transformed by oncogenic Ras or Raf, changes in the actin cytoskeleton include a loss of actin stress fibers and enhanced cortical actin. These effects might, at least in part, be mediated by Rnd. Hansen et al. [2000] reported that the loss of actin stress fibers in Raf-transformed cells is preceded by Raf-induced expression of Rnd3, which, as discussed above, is known to inhibit Rho function. Enforced expression of Rnd3 alone led to substantial loss of actin stress fibers, while cells expressing activated RhoA failed to respond to Raf. Interestingly, activation of MAPK/extracellular signal-regulated kinase (MEK) was able to prevent these biological and biochemical changes. These data suggest that Rnd3 acts as downstream effector of the Raf-MEK-extracellular signal-regulated kinase pathway, leading to inhibition of Rho and subsequently, loss of stress fibers.

SUMMARY

The Rho-GTPase family has expanded to include a total of 22 members. Many of these new members display novel properties, such as constitutive membrane association, and lack of GTPase activity and/or resistance to the effects of GAPs. These findings indicate that unusual regulatory systems act upon these new GTPases, such as transcriptional regulation and different types of post-translational modifications. In addition, these findings have expanded the purview of small GTPases to include organelles such as mitochondria. Finally, at least one of these small GTPases is known to be involved in human tumorigenesis, suggesting that some of these enzymes affect key proliferative signaling pathways. The challenge now is to discern the regulatory properties of these new GTPases and to determine their effector proteins, which in most cases appear not to overlap with Cdc42, Rac, or Rho. We can be sure that we will be in for some surprises.

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