

Chapter 2

Rho Proteins in Cancer

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Introduction

The control of coordinated cell motility and the plasticity of cell polarity lie at the center of tissue development in embryonic life. Proteins homologous to those present in bacteria and yeast evolved in multicellular organisms to control these functions. In recent years, we have come to understand that when proteins which govern motility are dysregulated in adult tissues, major derangements of growth and of interactions between tissues ensue. In particular, the Ras homology proteins, beginning with Ras itself, play major and diverse roles in cancer initiation and progression. In this chapter, we review with broad brushstrokes the role of Rho proteins in cancer.

Traditionally, the Ras superfamily is divided into five different branches: Ras, Rho, Rab, Ran, and ARF families. Recent in silico analysis of the human genome has revealed the presence of several new Ras-superfamily pseudogenes for which transcriptional evidence does not currently exist, and the function of these pseudogenes is unknown.

Ras homologous (Rho)-family GTPases comprise the largest subfamily cluster of the Ras-homology superfamily of small GTPases (~21 kDa). Currently, 22 Rho family entries are annotated in the ENTREZ database, which can be divided into 10 different groups on the basis of their sequence homology to either Cdc42, Rac1, RhoA, RhoD, Rif/RhoF, Rnd3/RhoE, TTF/RhoH, Chp/RhoV, or RhoBTB.

Like most Ras homology proteins, Rho GTPases function as molecular switches where GTP-bound Rho proteins are active and GDP-bound Rho proteins are inactive. Thus, Rho-GTP binds and activates downstream effectors leading to a variety of signaling cascades, while Rho-GDP does not. Although the involvement of a guanine nucleotide is a prerequisite for members of the Rho family, GTPase activity itself is not, as members of the Rnd and RhoH subfamilies are GTPase deficient (Nobes et al. 1998; Li et al. 2002) or in the case of RhoE/Rnd3, constitutively GTP-bound (Foster

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et al. 1996; Wennerberg et al. 2003). This is just one of several examples of the diversity, and consequently controversy, of the proteins classified as Rho GTPases.

Cycling between GTP- and GDP-bound states is tightly controlled by three different classes of Rho regulatory proteins: GTPase-activating proteins (GAPs), guanine nucleotide dissociation inhibitors (GDIs), and guanine nucleotide exchange factors (GEFs). GAPs and GDIs inhibit Rho activity; GAPs by accelerating Rho protein hydrolysis of GTP into GDP and GDIs by sequestering Rho proteins to the cytoplasm and blocking GDP dissociation. GEFs activate Rho proteins by triggering the release of GDP from Rho-GDP, thereby permitting the Rho protein to bind GTP.

The activation of Rho-family proteins is mediated through a wide array of mechanisms including interactions with G-protein-coupled receptors (GPCRs) and other cell surface receptors, such as cytokine, tyrosine kinase, and adhesion receptors. Once initiated, Rho signaling leads to the activation of several different pathways depending on the precursor signal, the cellular context, and the crosstalk with other activated or repressed pathways. In fact, when Rho-family proteins were first brought into the limelight following landmark publications by Ridley, Hall, and their coworkers in 1992, the number of publications on the diversity of functions of Rho proteins rose exponentially (Ridley and Hall 1992; Ridley et al. 1992). In these papers, Ridley and Hall related the assembly of focal adhesions and actin stress fibers to growth factor signaling through Rho GTPase. Since then, Rho proteins have also been shown to play roles in adhesion, migration, phagocytosis, cytokinesis, neurite extension and retraction, cell morphogenesis and polarization, growth and cell survival (Chimini and Chavrier 2000; Etienne-Manneville and Hall 2002; Evers et al. 2000; Raftopoulou and Hall 2004). Additionally, as summarized in Table 2.1, aberrant overexpression of some Rho-family proteins has been shown to promote malignant transformation and metastasis [See chart refs].

Rho-Family Structure

Ras-superfamily proteins have a highly conserved G box GDP/GTP-binding motif element at the start of the N-terminus, allowing this family of proteins to have a very high affinity for both GDP and GTP nucleotides. Specifically, the conserved domain structure is G1, GXXXXGKS/T; G2, T; G3, DXXGQ/H/T; G4, T/NKXD; and G5, C/SAK/L/T (Bourne et al. 1991). These five conserved elements compose the G domain, which has conserved structure and function shared by all Ras-superfamily proteins. Although Ras-superfamily proteins retain an overall similar conformation in the GDP or GTP bound states, two regions - Switch 1 and Switch 2 - undergo a significant shift relative to the GTPase core, causing a change in affinity for GAP or GEF regulatory proteins and downstream effectors (Bishop and Hall 2000; Repasky et al. 2004). Consequently, regulatory proteins and effectors most frequently bind to the Switch 1 and Switch 2 regions of Ras-superfamily GTPases.

A second important feature of Ras-superfamily and Rho-family proteins is the inclusion of a C-terminal CAAX amino acid motif, which undergoes a post-translational addition of a prenyl group through the transfer of either a farnesyl or a geranylgeranyl moiety to the cysteine in this motif, thereby anchoring the GTPase to the membrane. This feature is essential for Rho protein function. Of the Ras-superfamily, only two subgroups, the Rho-family and Rab-family proteins, are known to be regulated by GDIs that conceal the C-terminal prenyl modification to promote cytosolic sequestration of these GTPases (Olofsson 1999; Seabra and Wasmeier 2004). In the cases of RhoB, TC10, and TCL, however, the cysteine residue of the CAAX motif can be palmitoylated, thus preventing recognition by RhoGDI and promoting membrane localization (Michaelson et al. 2001).

In support of the structural importance of the Switch domains and the CAAX motif to Rho protein function, Dvorsky and Ahmadian calculated the relative number of interactions of all residues of Rho GTPases with GDIs, GEFs, GAPs, and effectors using available structures for the most well-studied members of the Rho-subfamily, RhoA, Rac1, and Cdc42, and concluded that most interactions occur at Switch domain 1, 2 or at the extreme C-terminus (Dvorsky and Ahmadian 2004). The relevance of the CAAX motif brings into question the inclusion of the “atypical” Rho GTPases in the Rho family, as most of the atypical Rho GTPases (except for Wrch-1) lack a CAAX motif and (with the exception of Wrch-2) are not membrane associated (Ridley 2006).

The translocation of Rho proteins from the membrane fraction into the cytosolic fraction is a key step in the inhibition of Rho GTPase proteins. Analysis of inactive forms of Rho proteins have also revealed that some classes of Rho proteins may become inactivated via phosphorylation. RhoA, for example, is phosphorylated by PKA at serine 188, and this phosphorylation has been shown to increase the apparent strength of RhoA interactions with GDIs by inhibiting binding of RhoA to other downstream targets independent of RhoA’s GDP/GTP state (Dong et al. 1998; Forget et al. 2002).

Rho Regulatory Proteins

Guanine Nucleotide Exchange Factors (GEFs)

The first identified Rho GEF was cloned from a screen for transforming genes in diffuse B-cell lymphoma cells, hence the name Dbl, and has been demonstrated to function as a GEF for the Rho-family member Cdc42 (Eva et al. 1988; Hart et al. 1991). Dbl represented the first member of a newly recognized family of GEFs that specifically regulate Rho GTPases (Schmidt and Hall 2002). Dbl contains two regions of homology with the yeast GEF Cdc24; the Dbl-homology (DH) domain, which catalyzes the release of GDP from Rho family proteins by stabilizing GTPase intermediates that are devoid of nucleotide and Mg^{2+} , and an adjacent C-terminal pleckstrin homology (PH) domain, which is thought to promote Dbl

Table 2.1

	Rho subfamily																				
	Rho	Rac			Cdc42			RhoBTB			Miro	Rnd	RhoH	RhoD							
Cancer type	Rho A	Rho B	Rho C	Rac 1	Rac 2	Rac 3	RhoG	Cdc 42	TC10	TCL	Wrch-2*	Wrch-1*	RhoBTB-1*	RhoBTB-2*	Miro-1*	Rnd 1	Rnd 2	Rnd 3	Rho/TTF	RhoD	Rif
Bladder	X	S	X	X			X	X					X								
Breast	X	X	X	X		X	i	X			X		X								
Cervical	X	S		S							i										
Colon	X		X	X			X	X			X						X				
Gastric	X	X	X	X			X	X			i										
Head and Neck	X	S	X	X	X		X	X					S								
Hepatocellular	X		X	i			i														
Leukemia	X			X	X		X	X			i								X		
Lung	X	S	X	X			X	X			i		X				X				
Lymphoma	X	X	X	i			X	X									X		X		
Melanoma	X	S	X	X			X	X									S				
Osteosarcoma	X	S		i																	
Ovarian	X		X				X	X													
Pancreatic	X	S	X	i				X			i										
Prostate	X		X	X		X	X	X										S			

X = oncogene

S = tumor suppressor

i = indirect/observational cancer involvement

* = atypical

localization to the plasma membrane via binding to phosphoinositides, leading to subsequent interaction with Rho GTPases (Rossman et al. 2005). A misnomer in the field is that GEFs recruit GTP to the GDP/GTP-binding motif of GTPases. In actuality, the concentration of GTP is significantly higher in vivo than that of GDP; thus, a concentration gradient drives the “preferential” loading of GTP into the GTPase.

Through analysis of several structures, interactions between the DH domain and Rho GTPases have been well characterized. DH domains have three conserved regions (CR1-CR3), which form the core of this domain’s three-dimensional structure (Rossman et al. 2005). The most significant difference in the structure of DH domains occurs at the helix, which is C-terminal to CR1-CR3 and contains the $\alpha 6$ -helix. Several groups have demonstrated that the DH domain interacts extensively with the Switch 1 region of Rho GTPases, and that CR3 and the $\alpha 6$ -helix contact Switch 2, anchoring the two proteins through a hydrophobic cleft. Likewise, a conserved basic residue and semi-conserved Asn in CR3 make important contacts with Switch 2 that contributes to the DH domain’s exchange potential (Rossman et al. 2005).

The functions and mechanisms of the PH domain appear to be more diverse than those of the DH domain, and at this point are less well understood. Structural data from numerous Rho GEFs indicate that positioning of the PH domain relative to the DH domain is highly variable, likely contributing to the observation that PH domain interaction with Rho GTPases also varies greatly between Rho GEFs (Rossman et al. 2005).

Although PH domains are characterized by their ability to bind phosphoinositides, the importance of this binding to Rho GEF activity remains hotly contested. Early studies demonstrated that loss of function due to deletion of the PH domain in Dbl-family members could be restored by replacing the PH domain with another membrane localization signal (Rossman et al. 2005). Subsequent studies, however, have shown that Dbl-family PH domain binding to phospholipids occurs with low affinity and specificity and is dispensable, in some cases, for proper GEF localization (Rossman et al. 2005).

GTPase-Activating Proteins (GAPs)

GAPs exhibit a wide array of specificity and activity. Recently, experiments introducing the RhoGAP domains of either p122RhoGAP or GRAF into fibroblasts blocked the formation of RhoA-mediated actin stress fiber formation, despite the indiscriminate in vitro activity of these proteins toward Rac1 or Cdc42 (Sekimata et al. 1999; Taylor et al. 1999). In addition to the fact that many identified RhoGAPs contain several domains other than just a RhoGAP region, the aforementioned data add support to the underappreciated hypothesis that various signaling pathways converge on RhoGAPs by interacting with their regulatory motifs to control RhoGAP specificity.

Protein kinases can regulate RhoGAP activity, the best example being the regulation of p190 RhoGAP by Src family tyrosine kinases. Observationally, Src activation leads cells to exhibit characteristics commonly associated with inactivated Rho proteins, namely disruption of actin stress fibers and reduction of focal contacts. As such, it was not surprising that Src activation was shown to correlate with the phosphorylation of two Tyr residues on p190 RhoGAP proximal to the RhoGAP domain, and that this double phosphorylation is critical for RhoGAP activity (Roof et al. 1998). Phosphorylation control of the p190 RhoGAP is not limited to tyrosine phosphorylation. In fact, protein kinase C activity, which leads to phosphorylation of Ser/Thr residues, can modulate the spatial distribution of p190 RhoGAP in the cell (Brouns et al. 2000).

In addition to p190 RhoGAP, several other GTPase-activating proteins have been identified, the most famous of which is BCR, the 5' partner of an oncogenic and reciprocal fusion cloned from the breakpoint region in Philadelphia-positive chronic myeloid leukemia, BCR-ABL (Diekmann et al. 1991; Rowley 1973). The 3' end of BCR actually contains the RhoGAP domain, suggesting that the RhoGAP activity of BCR may be maintained because the translocation and fusion are reciprocal (producing both BCR-ABL and ABL-BCR chimeras). Despite this notion, however, ABL-BCR actually loses its ability to function as a RhoGAP and has negative consequences on cell adhesion, in particular adherence to endothelial cells (Zheng et al. 2006). The regulation of Rho activity as mediated by RhoGAPs is crucial to an integrated understanding of Rho function in cancer.

Rho Effector Interactions: General Considerations

Rho proteins regulate cellular structure by controlling microtubules and actin cytoskeleton structure. To accomplish this, Rho proteins regulate the function of the Diaphanous-related formins, such as mDial1. mDial1 contains a Rho-binding domain, a polyproline FH1 domain that regulates F-actin, and an FH2 domain, which regulates microtubule structure. Interestingly, the introduction of mDial1 mutants lacking the Rho-binding domain into HeLa cells resulted in the bipolar elongation as microtubules aligned parallel to F-actin bundles (Ishizaki et al. 2001). These data demonstrated the essential role of Rho signaling through mDial1 in the maintenance of normal cell morphology.

In an effort to determine whether RhoA's abilities to induce nuclear signaling and oncogenic effects were due to the effect on the cytoskeleton or through other mechanisms, transfection of several different RhoA mutant expression vectors into NIH3T3 cells followed by a colony formation assay revealed that substitutions of Leu-39, Glu-39, or Cys-42 failed to stimulate serum response factor-mediated gene expression or induce neoplastic transformation. In transient assays, however, the introduction of these three different mutants did induce significant changes in actin stress fiber formation, providing some of the first evidence that in certain cases the Rho-effector proteins regulating the structure of

actin filaments are distinct from those signaling to the nucleus and hijacking growth control (Zohar et al. 1998).

Following the realization that Rho signaled through a variety of effectors to induce different effects, crystal structure data helped to clarify classes of Rho-interacting proteins. In fact, the crystal structure of RhoA bound to the effector domain of protein kinase PKN/PRK1 revealed that an antiparallel coiled-coil finger (ACC finger) fold of the effector domain bound to the Switch 1, 2, and adjacent regions by specific hydrogen bonds in RhoA. Subsequent in silico sequence analysis demonstrated that the structure of the ACC finger domain is highly conserved in Rho effector proteins (Maesaki et al. 1999).

Rho GTPases Drive Cell Migration

The migration of cells in multicellular organisms is an essential part of development, wound repair, and immune surveillance that is controlled by extracellular signals which elicit intracellular changes in the organization of actin and microtubule cytoskeletons. As a cell begins its migratory journey to a new destination, leading edge protrusions or lamellipodium extend from the surface of the cell toward the attractive extracellular signal (or away from the repulsive signal) and establish new adhesion sites along this leading edge. The cell body then begins to contract and adhesions along the lagging edge are broken in a highly coordinated process leading to efficient, directional movement.

While the Rho family members Cdc42, Rac, and Rho form integrin-based matrix adhesion complexes at the cell surface, Cdc42 and Rac also regulate the polymerization of actin to form filopodial and lamellipodial protrusions, respectively, and Rho proteins regulate the assembly of contractile actin-myosin filaments (Nobes and Hall 1995; Ridley and Hall 1992; Ridley et al. 1992).

Although Rac and Cdc42 are known to catalyze a protrusive force by inducing localized actin polymerization at the leading edge of a cell during migration, studies in *Drosophila melanogaster* demonstrated that Cdc42 loss of function does not affect the rate of migration of peripheral glial cells, but does alter the direction of migration (Sepp and Auld 2003). Experiments using high-speed supernatants from *Xenopus* egg extracts demonstrated that constitutively active Cdc42 (generated by loading Cdc42 with GTP γ S - a non-hydrolysable GTP analogue) can induce F-actin polymerization in wild-type extracts, but not N-WASP (a downstream effector of Cdc42) immunodepleted extracts (Rohatgi et al. 1999). N-WASP belongs to the WASP/SCAR/WAVE family of scaffold proteins. Ensuing experiments in the Rohatgi et al. paper and several others demonstrated that members of this family stimulate actin polymerization either de novo or at the barbed edge of preexisting filaments through stimulation of the Arp2/3 complex (Rohatgi et al. 1999; Weaver et al. 2003). Interestingly, WASP and WAVE have also been shown to interact with profilin, a protein that increases the rate of actin polymerization by acting synergistically with the Arp2/3 complex (Blanchoin et al. 2000; Yang et al. 2000).

Rac activation occurs primarily at the leading edge of a migrating cell, thereby preferentially driving the formation of lamellipodium along this edge. In 2005, the mechanism regulating Rac activation to the leading edge was finally elucidated (Nishiya et al. 2005). Normally, the α_4 integrin–paxillin complex inhibits stable lamellipodia by recruiting Arf-GAP, which in turn inhibits Arf, thus blocking Rac activation. However, phosphorylation of α_4 integrin, which only occurs at the leading edge (Lim et al. 2007), prevents its interaction with paxillin and subsequently Arf-GAP, thereby releasing repression of Arf and allowing it to activate Rac.

Rho activity facilitates cell migration by orchestrating focal adhesion assembly, cell body contraction, and lagging edge retraction. The protein p160ROCK is known to work specifically downstream of Rho, not Rac, to mediate focal adhesions and stress fibers (Ishizaju et al. 1997). After becoming activated, p160ROCK stabilizes actin filaments in actin-myosin filaments by phosphorylating and activating LIMK, which then inactivates cofilin, an actin depolymerizer, through a subsequent phosphorylation (Maekawa et al. 1999; Sumi et al. 2001). As the enemy of my enemy is my friend, by inhibiting a protein that prevents actin reassembly, this p160ROCK phosphorylation cascade stabilizes actin filaments along the lagging edge of a migratory cell. Interestingly, p160ROCK has also been shown to phosphorylate and inactivate myosin light chain phosphatase, leading to increased myosin phosphorylation and the generation of a contractile force, which produces the desired movement (Mitchison and Cramer 1996).

Conclusions

It is clear that many of the Rho family members act in concert and share multiple signaling pathways to affect their functions under different stimuli. Future efforts to understand their biology should be, at least in part, focused on understanding the dynamics of the on–off switch that is at the heart of Rho protein function. We believe the era of using quantitative, sophisticated methods of modeling and imaging live cells will carry this field to the next level, where we may enhance targeted drug design by a deeper insight into how these proteins transmit information in the cell.

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