J. Biochem. **134**, 327–331 (2003)
DOI: 10.1093/jb/mvg149

Structural Basis of the Rho GTPase Signaling

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Received May 21, 2003; accepted May 28, 2003

Small GTPases of the Rho family serve as conformational switches in a wide variety of signal transduction pathways that regulate diverse cellular functions. The GTP-bound forms of Rho GTPases are capable of interacting with downstream effectors that control cytoskeletal rearrangements. Regulators that stimulate nucleotide exchange, the hydrolytic cycle and distribution between the membrane and cytosol control the switch. Detailed pictures of Rho GTPase switching, effector recognition and regulation by regulators have emerged from recent structural investigations. These include the most extensively studied Rho GTPases, RhoA, Rac1, 2 and Cdc42, and their complexes with effectors and regulators. These studies have revealed the general diversity of effector and regulator structures, and in particular the structural features concerning the specific interactions involved in Rho effector recognition and regulator interactions with Rho GTPase. These findings provide a critical insight into the nature of Rho GTPase activity and consequently allow for a detailed manipulation of signaling pathways mediated by these proteins.

Key words: Cdc42, GAP, GDI, GEF, Rac, RhoA.

Abbreviations: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; GDI, guanine-nucleotide dissociation inhibitor; PKN, protein kinase N; ACC finger, antiparallel coiled-coil finger; CRIB, Cdc42/Rac-interactive binding; ACK, the activated Cdc42-associated kinases; WASP, Wiskott-Aldrich syndrome protein; Par6, partitioning-defective 6; PAK, p21 activated kinase; TPR, tetratrico-peptide repeat; dbl, diffuse B-cell lymphoma oncogene product; DH, Dbl homology; PH, pleckstrin homology; Dbs, Dbl's Big Sister; Tiam1, the T-lymphoma invasion and metastasis factor 1; NTF, nuclear transport factor; GMPPNP, guanosine 5′-(β,γ-imido) triphosphate; GTPγS, guanosine 5′-*O*-(γ-thiotriphosphate).

Rho GTPases belong to the Ras superfamily of small GTPases and control a wide variety of cellular processes such as actin cytoskeleton rearrangement, microtubule dynamics, cell adhesion and polarity (for review, *1*). Like all members of the Ras superfamily, Rho GTPases function as conformational switches by cycling active GTP- and inactive GDP-bound forms. The cycle is regulated by two classes of protein, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs catalyze nucleotide exchange. This results in the activation of Rho GTPases due to the higher concentration of GTP than GDP in cells. GAPs stimulate GTP hydrolysis, which results in the inactivation of Rho GTPases. Furthermore, Rho GTPases are regulated by guanine nucleotide dissociation inhibitors (GDIs). These extract GDP-bound forms from the membrane to the cytosol and inhibit the release of GDP from the GTPases. Rho GTPases in the GTP-bound form are able to bind a variety of downstream target proteins, called effectors, which initiate a variety of cellular responses. This review briefly summarizes our current structural understanding of the conformational changes, effector recognition and activation/inactivation mechanisms of Rho GTPases. The discussion will be based on three recent and extensive structural determinations of Rho GTPases, RhoA, Rac1, 2 and Cdc42, and their complexes with effectors and regulators (Table 1).

Overall fold and conformational switch of Rho GTPases

Rho GTPases share a common G-domain fold, which consists of a six-stranded β-sheet surrounded by α-helices (*2*). The differences between the GDP- and GTP-bound structural forms of RhoA are confined primarily to two segments, referred to as switch I (residues 28–44 in human RhoA) and switch II (residues 62–69) (Fig. 1a) (*3*). These regions basically correspond to Ras switches I (residues 32–38) and II (59–97), respectively, although RhoA switch I is longer than Ras switch I and contains residues in the N-terminal half of β-strand B2. The conformations of switches I and II in the GTP-bound form of RhoA are stabilized by hydrogen bonds between the γ-phosphate group and the main-chain amide groups of the invariant Thr 37 (switch I) and Gly 62 (switch II). The GDP-bound forms of Rho GTPases lack these interactions and show a large variation in switch conformations (*4*, *5*). The 13-residue insertion, which is characteristic of Rho GTPases, is folded into a compact α-helical structure (Fig. 1a). This insertion has no significant conformational impact as determined by a comparison of the GDP- and GTP-bound forms. The recognition of GTP by RhoA is similar to that by Ras, but water-mediated interactions are observed

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Table 1. **Rho GTPase structures and complexes with regulators/effectors.**

	Structures	PDB id	Reference
RhoA	GTP-form	1A2B	*3*
	GDP-form	1FTN	*4*
GEF	Dbs- RhoA	1LB1	*22*
GAP	p50rhoGAP-RhoA(GDP-AlF_4)	1TX4	*24*
Effector	PKN-RhoA(GTPγS)	1CXZ	*8*
Rac1	GTP-form	1MH1	*31*
	GDP-form	not reported	
GEF	Tiam1-Rac1	1FOE	*20*
GDI	LyGDI-Rac2(GDP)	1DS6	*27*
	RhoGDI-Rac1	1HH4	*28*
Effector	p67phox-Rac1(GTP)	1E96	*13*
Cdc42	GTP-form	not reported	
	GDP-form	1A4R	*5*
GEF	Dbs-Cdc42	1KZ7	*21*
	Intersectin-Cdc42	1KI1	*22*
GAP	p50rhoGAP-Cdc42(GMPPNP)	1AM4	*23*
	Cdc42GAP-Cdc42(GDP-AlF_3)	1GRN	*25*
GDI	RhoGDI- Cdc42(GDP)	1DOA	*26*
Effector	ACK-Cdc42(GMPPNP)	1CF4	*9*
	WASP-Cdc42(GMPPNP)	1CEE	*10*
	PAK-Cdc42(GMPPNP)	1E0A	*12*
	Arfaptin-Cdc42(GDP/GMPPNP)	1I4D,1I4L,1I4T	*17*
	Par6- Cdc42(GMPPNP)	1NF3	*11*

around the guanine base and the ribose ring. In the crystal structure, a putative nucleophilic water molecule was found to coordinate the γ-phosphate group and Gln 63, which is important for GTPase activity (Fig. 1b). Mg^{2+} ions are essential for guanine nucleotide binding as well as GTPase activity. Interestingly, the elimination of Mg^{2+} induces large conformational changes in the switch I region of RhoA, which results in opening of the nucleotide-binding site (*6*).

Rho effector recognition

A number of RhoA effectors have been identified which specifically bind Rho GTPases in a GTP-dependent manner. These include protein kinase N (PKN), Rho-kinase and its homologs, rhophilin, rhotekin, citron, citron kinase, mDia and kinectin (for review, *7*). All of these effectors are assumed to have a coiled-coil motif that binds RhoA. Our crystal structure of the Rho-PKN complex reveals the antiparallel coiled-coil (ACC) finger fold of the effector domain that directly contacts switch I, the inter-switch region forming β-strands B2 and B3, and the C-terminal α-helix A5 (Fig. 2a, left) (*8*). These regions on the RhoA molecular surface contain RhoA-specific residues that directly participate in the interactions with PKN. This assists in discriminating RhoA from other members of small GTPase families and Rho GTPases such as Rac and Cdc42. RhoA residues important in discriminating between RhoA and Rac/Cdc42 are likely to be Lys27 in switch I, Glu47 in β-strand B2, Gln52 in β-strand B3 and Glu169 in α-helix A5.

Rac and Cdc42 effectors contain a common sequence motif referred to as a CRIB (Cdc42/Rac-interactive binding) motif, which consists of about 15 residues. The existence of a common binding motif for Rac and Cdc42 corresponds to the fact that Rac and Cdc42 exhibit relatively high (~70%) sequence identity and bind certain common

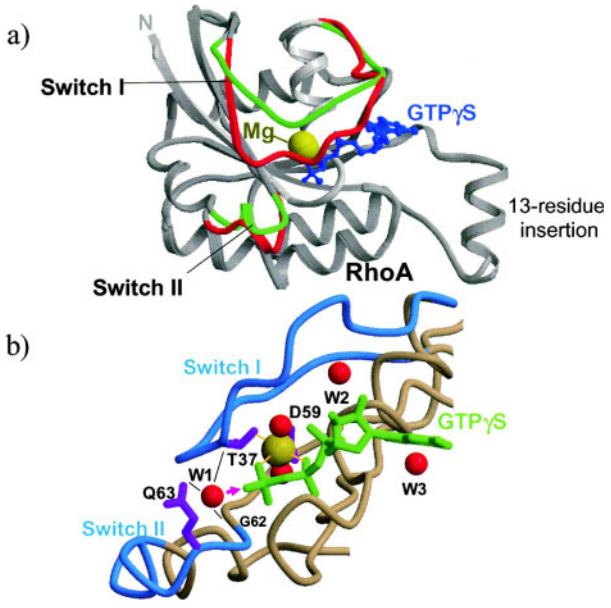

Fig. 1. **Conformational changes in the switch regions and water molecules around the guanine nucleotide-binding site.** (a) Superposition of RhoA Cα-carbon atom tracing of GTP- and GDP-bound forms. Switch regions in GTP- (PDB id: 1A2B) and GDP-bound forms (1FTN) are highlighted in red and green, respectively. GTPγS and Mg^{2+} are displayed as ball-and-stick and space-filled models. (b) Close-up view of the guanine nucleotide-binding site in the GTP-bound form. Water molecules and Mg^{2+} are displayed as red and yellow balls, respectively. All figures are made by using Molscript (*32*) and Radter3d (*33*). Three water molecules, which are frequently observed in crystal structures of Rho GTPases, are also shown as red balls (W1-W3). W1 is the putative nucleophilic water molecule close to the γ-phosphate group. W1 is stabilized by hydrogen bonding interactions with the side chain of Gln63 and the main chains of Thr37 and Gly62. W2 and W3 mediate RhoA interactions with the ribose and the guanine base of GTP, respectively.

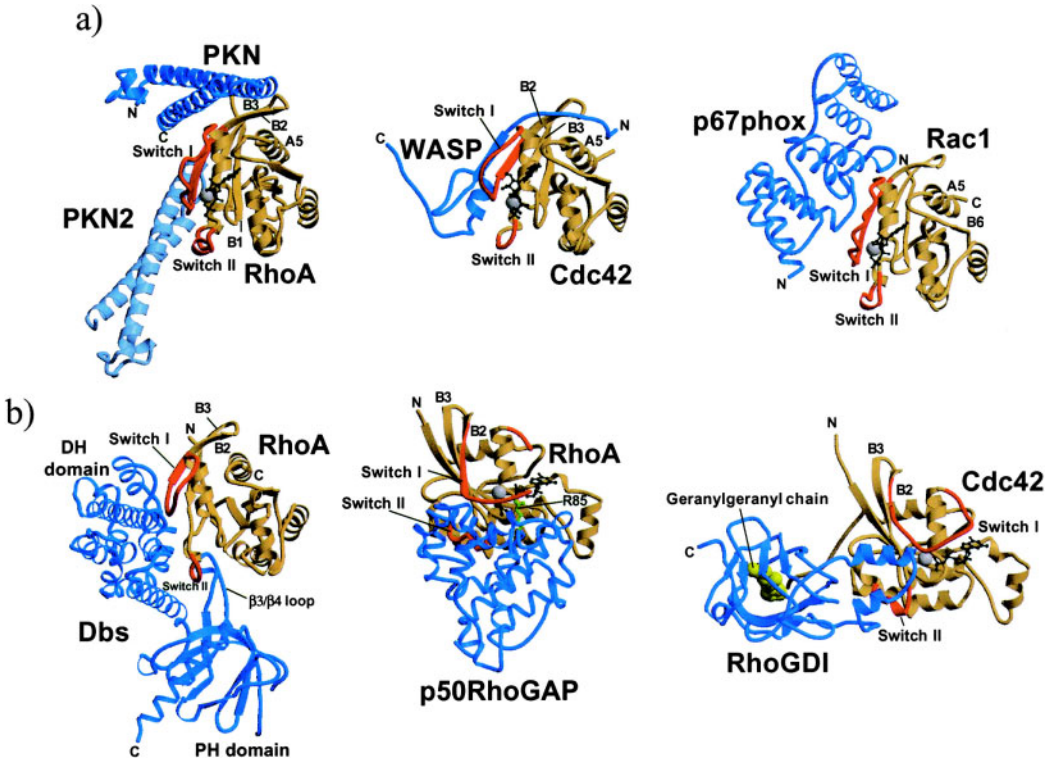

Fig. 2. **Effector and regulator interactions with Rho GTPases.** (a) Rho GTPases, RhoA (left), Cdc42 (middle) and Rac1 (right), complexed with the effector domains; RhoA-PKN (PDB id: 1CXZ), Cdc42-WASP (1CEE) and Rac1-p67phox (1E96) complexes. Switch regions are colored in red. In the RhoA-PKN complex (left), PKN bound to the second binding site is shown in right blue (PKN2). (b) Structures of the Rho GTPases complexed with the regulators GEF (left), GAP (middle) and GDI (right): Dbs-RhoA (PDB id: 1LB1), p50RhoGAP-RhoA (1TX4), RhoGDI-Cdc42 (1DOA) complexes. Switch regions are colored in red.

effectors. The sequence identity of RhoA with Rac and Cdc42 is ~45%. Recent crystal structures of Cdc42 effectors such as activated Cdc42-associated kinase (ACK) (*9*) and Wiskott-Aldrich syndrome protein (WASP) (*10*), and the partition-defective protein Par6 (*11*) and a Rac/Cdc42 effector, p21-activated kinase (PAK) (*12*) have shown that the CRIB motif forms an intermolecular β-sheet with β-strand B2 of Rac/Cdc42 (Fig. 2a, middle). Additionally, these effectors make contact with switches I and II and α-helices A1 and A5. The C-terminus of both PAK and WASP forms a β-hairpin and α-helix that makes contact with Cdc42, while that of ACK wraps around Cdc42 in an extended conformation. Discrimination between Cdc42 and Rac is likely to be governed by contacts with switch I, β-strand B2 and α-helix A5. In particular, contacts involving Val42, Gly47 and Leu174 of Cdc42 (Ala, Asp, and Arg in Rac, respectively) have been pointed out in the complex structures. Interestingly, $p67^{phox}$, which is one of the subunits of phagocytic NADPH oxidase, is an effector for Rac lacking the CRIB motif. A tetratrico-peptide repeat (TPR) domain has been identified as a binding domain for Rac. A recent crystal structure of the TPR domain complexed with Rac has revealed that the N-terminal end of switch I and the loop connecting β-strand B6 to α-helix A5 of Rac interact with the TPR domain comprising repeated α-helical motifs and β-hairpin insertion (*13*) (Fig. 2a, right).

In the RhoA-PKN crystal, the RhoA switch II region makes hydrophobic contacts with the symmetry-related PKN ACC finger domain. This observation of two contact sites on RhoA is consistent with experimental results (*14*, *15*) that have detailed the multiple interactions of PKN with RhoA through two homologous effector domains, ACC-1 and ACC-2. Interestingly, similar interactions between switch II and an effector helical domain have been observed in the Rab3A-rabphilin-3A complex (*16*) and in the Rac-Arfaptin complex (*17*). Arfaptin binds specifically to GTP-bound Arf1 and Arf6, and binds to Rac-GTP and Rac-GDP with similar affinities. Notably, the interaction between Arfaptin and Rac is quite unlike that of Rac-CRIB complexes and most closely resembles those interactions found in the RhoA-PKN complex, in which the antiparallel coiled-coil region of PKN packs against the helical segment of switch II. The Rac-Arfaptin complex is also interesting as it is the first example showing cross-talk between small GTPase families, Rho and Arf families.

Regulator RhoGEF

RhoGEFs are typical multifunctional proteins containing multiple domains. More than 10 such domains have been identified in Trio. These domains are believed to be essential in enabling RhoGEFs to receive signals by interacting with upstream proteins, resulting in the activation of Rho GTPases. Generally, RhoGEFs possess a dbl homology (DH) domain and pleckstrin homology (PH) domain linked in tandem. In most cases, the DH-PH module is the minimal structural unit required for full

GEF activity (*18*, *19*). The DH domain is structurally distinct from the Cdc25 domain in RasGEF and the Sec7 domain of ArfGEF, although all of these domains consist of α-helices. Recent structural studies have revealed a conserved mechanism of nucleotide exchange in Rho GTPases catalyzed by RhoGEFs (*20*–*22*). There are three highly conserved regions in all DH domains (CR1-3). CR1 and CR3, together with certain other helices, constitute the major binding surface for GTPases and contact switches I and II, and the inter-switch region (β-stands B2 and B3) of the GTPase (Fig. 2b, left). CR2 sits on the opposite side of the helical bundle relative to CR1 and CR3, and presumably functions mainly to stabilize the helical bundle. The DH domain helices engage and reshape the switch regions of the cognate GTPase with disruption of Mg^{2+} ion and nucleotide binding. The PH domain, which in general has been implicated in membrane localization via phospholipid binding, directly regulates exchange independent of phospholipid binding. In fact, a β3/β4 loop of the PH domain makes direct contact with the GTPase in the Dbs-Cdc42 (*21*) and Dbs-RhoA (*22*) complexes.

RhoGEFs possess a wide assortment of specificities toward Rho GTPases. Tiam1 acts specifically on Rac1, whereas intersectin is specific for Cdc42. In contrast, Dbl and Dbs catalyze exchange within both Cdc42 and RhoA, whereas Vav and Vav2 activate Rac1, Cdc42 and RhoA. A structural comparison of Tiam1-Rac1, Dbs-RhoA, Dbs-Cdc42 and intersection-Cdc42 complexes and mutation experiments have suggested that residues determining GTPase specificity are likely to be located on the inter-switch region (β-strands B2 and B3; Val43, Asp45, Glu54, Ala56, Trp58 for RhoA) and the N-terminus (Arg5) (*22*). These residues make contact with RhoGEF helices formed by CR3 and its N-terminal flanking residues (752–785 for Dbs).

Regulator RhoGAP

GAP enhances the rate of GTP hydrolysis by up to 10^5 times. Recent mechanistic studies from a structural viewpoint have provided insights into the RhoGAP-mediated GTPase activation reaction (*23*–*25*). As with GEFs, the structures of GAP domains for various small GTPase subfamilies including heterotrimeric GTPases are different, while all GAP domains are folded into all-α helical structures. The GAP domain of p50RhoGAP consists of nine α-helices and binds Rho GTPases to stabilize both switches I and II (Fig. 2*b*, *middle*). The structure of RhoGAP complexed with RhoA bound to GDP and AlF_4^-, a transition state mimic, revealed a 20° rotation between RhoA and GAP in comparison with the RhoGAP-RhoA structure complex bound to GMPPNP, a non-hydrolysable GTP analog. Consequently, in the transition state but not in the ground state, GAP acts by inserting a highly conserved Arg, referred to as an "arginine finger", into the GTPase active site. This interacts directly with Gln61 of the GTPase, which is responsible for the correct positioning of the hydrolytic water molecule for phosphoryl transfer. Stabilization of this glutamine residue restricts the freedom of the water molecule and may reduce the energy barrier for GTP hydrolysis. The guanidium group of the Arg finger also interacts with GTP and a water molecule of the hydrated Mg^{2+} ion to stabilize the configuration of the triphosphate group and the ion, as well as to neutralize the charges of the phosphate group. Asn 194 in p50RhoGAP, which is conserved in all RhoGAP domains, interacts with the main-chain and is thought to stabilize the effector loop.

Regulator RhoGDI

The C-termini of Rho GTPases contain a conserved -CAAX sequence, which is the site of a series of post-translational modifications, the most significant of which is the isoprenylation of the conserved cysteine residue. GDIs maintain the Rho GTPases by shielding this lipid-modified hydrophobic tail from the aqueous environment, where GDI-GTPase complexes constitute a cytoplasmic pool of prenylated proteins. Crystal structures of GDI complexed with Rho GTPase revealed that GDI folds into an immunoglobulin-like β sandwich that comprises nine β-strands and contains a short helix followed by a helix-loop-helix motif in the N-terminal region (*26*–*28*) (Fig. 2b, right). The geranylgeranyl chain plunges into a hydrophobic pocket of the immunoglobulin-like domain. The pocket is lined with conserved hydrophobic residues that form favorable van der Waals contacts. GDI makes contact primarily with switch II and the C-terminal part of switch I. The side chain of the conserved Asp from the helix-loop-helix region of GDI is hydrogen bonded to Thr in switch I of Rho GTPase. This interaction stabilizes the Mg^{2+} coordination through the main chain carbonyl of Thr and consequently enhances the affinity for the nucleotide. GDI makes extensive contacts with the switch II region and, concomitant with the stabilization of switch I, leads to the inhibition of both GDP dissociation and GTP hydrolysis by Rho GTPase.

It is well known that at least three members of RhoGDIs, 1–3, exist. GDI2, which is also referred to as LyGDI, D4-GDI or GDIβ, has been found to be 10- to 20-fold less potent toward Cdc42 than RhoGDI (*29*). This difference results from the weaker binding to Cdc42, which is caused by the Asn 174 (at strand β9) substitution in GDI2 to Ile in GDI1 located in the cavity for isoprenyl binding. Thus, the structural determination of complexes can provide clear answers to questions concerning even small functional differences.

A GDI has also been identified for Rab GTPases, but RhoGDI and RabGDI consist of completely unrelated structural folds. The crystal structure of RabGDI in an uncomplexed form reveals two main structural units, a large complex α/β domain I and a smaller α-helical domain II (*30*). Domain I is surprisingly closely related to functionally unrelated enzymes, FAD-containing monooxygenases and oxidases.

Conclusion

Six years have passed since the first crystal structure of a Rho GTPase, Rac1, was reported (*31*). Since then an explosion in three-dimensional structural studies of Rho GTPases, together with their regulators and effectors has occurred concomitant with an explosion in molecular cell biology. These investigations have revealed the existence of a canonical structure and a conserved switch mechanism involving the GTPases. Furthermore, some underlying common principles concerning GEF and GAP mechanisms have been revealed, despite differences in the

regulator structures distinct from those of other family GTPases such as Ras. RhoGDI, which is structurally unrelated to RabGDI, makes novel hydrophobic interactions with the lipid-modified C-terminus of Rho GTPases. A variety of effectors display different structural roles for the various subtypes of Rho GTPases and interact with their cognate GTPases in somewhat different manners, while Rac and Cdc42 share some interactions in terms of effector recognition. Interactions with effectors and how they generate the biological response in a particular system are not well understood in most cases. Structural studies can provide critical insights into the structural features necessary for the proper pairing of effectors and regulators with Rho GTPases. Furthermore, these studies allow us to manipulate Rho signaling pathways *in vivo*.

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