Crystal Structure of Human RhoA in a Dominantly Active Form Complexed with a GTP Analogue*

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The 2.4 Å resolution crystal structure of a dominantly active form of the small guanosine triphosphatase (GTPase) RhoA, RhoA, complexed with the nonhydrolyzable GTP analogue, guanosine 5′-3-O-(thio)triphosphate (GTPyS), reveals a fold similar to RhoA-GDP, which has been recently reported (Wei, Y., Zhang, Y., Derewenda, U., Liu, X., Minor, W., Nakamoto, R. K., Sомlyo, A. V., Somlyo, A. P., and Derewenda, Z. S. (1997) Nat. Struct. Biol. 4, 699–703), but shows large conformational differences localized in switch I and switch II. These changes produce hydrophobic patches on the molecular surface of switch I, which has been suggested to be involved in its effector binding. Compared with H-Ras and other GTPases bound to GTP or GTP analogues, the significant conformational differences are located in regions involving switches I and II and part of the antiparallel β-sheet between switches I and II. Key residues that produce these conformational differences were identified. In addition to these differences, RhoA contains four insertion or deletion sites with an extra helical subdomain that seems to be characteristic of members of the Rho family, including Rac1, but with several variations in details. These sites also display large displacements from those of H-Ras. The ADP-ribosylation residue, Asn, by C3-like exoenzymes stacks on the indole ring of Trp with a hydrogen bond to the main chain of Glu. The recognition of the guanosine moiety of GTPyS by the GTPase contains water-mediated hydrogen bonds, which seem to be common in the Rho family. These structural differences provide an insight into specific interaction sites with the effectors, as well as with modulators such as guanine nucleotide exchange factor (GEF) and guanine nucleotide dissociation inhibitor (GDI).

Rho is a small GTPase that was first purified from mammalian tissue membrane (1) and cytosol (2) fractions and was identified as the gene product of the ras homologue gene, rho (3). Rho has three mammalian isoforms, RhoA, RhoB, and RhoC, that exhibit high sequence homology with 83% identities (4). Rho cycles between GTP-bound and GDP-bound forms in a similar manner as Ras and other small GTPases. The level of the active GTP-bound form is regulated by its own GDI, GEF, and GAP. The interconversion between the GTP-bound and GDP-bound forms allows Rho to act as a molecular switch that regulates intercellular signaling pathways. Rho is implicated in the cytoskeletal responses to extracellular signals including lysophosphatidic acid and certain growth factors, which result in the formation of stress fibers and focal adhesion (5–7). Recent isolation and characterization of putative target proteins for Rho from the bovine brain (8, 9) have led to a possible mechanism by which Rho regulates cytokinesis, cell motility, or smooth muscle contraction (10, 11). These proteins contain the MBS made up of myosin phosphatase and a novel serine/threonine kinase, Rho-kinase, that has been shown to phosphorylate MBS to inactivate myosin phosphatase and also to phosphorylate the MLC. Accumulation of phosphorylated MLC induces a conformational change in myosin II that increases its interaction with actin and enables the formation of myosin filaments (12). Rho-kinase is identical to ROCK from the rat brain (13) and p16ROCK from human megakaryocytic leukemia cells (14), which are members of a growing family of serine/threonine protein kinases that include myotonic dystrophy kinase. Other target proteins for Rho contain PKN (15, 16), RhoGDS (16), RhoA due (17), and Citron (18). Further signaling pathways for actin polymerization have appeared to involve PtdIns 4-phosphate 5-kinase (19) and p140mDia (20), as downstream effectors.

Rho has two related small GTPases, Rac and Cdc42, that are also involved in regulating the organization of the actin cytoskeleton, whereas the cell morphological effects induced by these GTPases are clearly different in appearance. Rac regulates lamellipodium formation and membrane ruffling, and Cdc42 regulates filopodium formation. Rac is also known to be involved in the activation of NADPH oxidase in phagocytes. Rac has two mammalian isoforms, Rac1 and Rac2, that exhibit a high sequence homology with 90% identities. Rac and Cdc42 binding subunit; MLC, myosin light-chain; PKN, protein kinase N; PtdIns 4-phosphate 5-kinase, phosphatidylinositol 4-phosphate 5-kinase; PAK, p21(Cdc42/Rac1)-activated protein kinase; CNF, cytotoxic necrotizing factor; GTPyS, guanosine 5′-3′-O-(thio)-triphosphate; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PEG8000, polyethylene glycol 8000; r.m.s., root-mean-square; GMP-PCP, guanosine 5′-β,γ-imino)triphosphate; GMP-CP, guanosine 5′-β-(methylene)triphosphate; Adr, ADP-ribosylation factor; smgDGS, small GTP-binding protein guanine nucleotide dissociation stimulator.

* This work was supported in part by Grants in Aid for Scientific Research on Priority Areas (06276104) and Biometallics (09235220) (to T. H.) and for Cancer Research (to K. K.) from the Ministry of Education, Science and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by a research fellowship from the Japan Society for the Promotion of Science.

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also share a significant homology with 68% identities and, actually, bind to some common target proteins for activation. Rho, however, exhibits a relatively low similarity to those GTPases, an approximately 45% identity with both Rac and Cdc42. These differences in similarity are thought to be essential for the activation of several downstream target proteins of each small GTPase, although we do not yet understand the molecular basis of the specificities. Based on these differences, RhoA, RhoB, and RhoC are hereafter referred to as the RhoA subfamily, and Rac1, Rac2, and Cdc42 as the Rac1 subfamily. The Rho-binding domains of the target proteins consist of less than 100 residues and have been classified into at least two motifs (9, 21). The class 1 of the Rho-binding motif is characterized as a polybasic region followed by a leucine-zipper-like motif and is found in PKN, Rhophillin, Rhotekin, and MBS. Rho-kinase and Citron make up another class of the Rho-binding motif, the class 2, that has a putative coiled-coil motif located at the C terminus of the segment that is similar to myosin rod. It is of considerable interest that these sequences of the Rho-binding domains have no similarity to the binding domain of an activated Cdc42Hs-associated kinase (ACK) (22), a p21(Cdc42/Rac1)-activated protein kinase (PAK) (23), or the Ras-binding domain of Raf-1 (24).

Rho and the related small GTPases are the most common targets for bacterial toxins and are of major importance for the entry of bacteria into mammalian host cells. It is well known that various bacterial toxins can modify Rho by ADP-ribosylation, -glycosylation, and -deamidation. These toxins are classified into three families, C3-like exoenzymes such as Clostridium botulinum C3 ADP-ribosyltransferase, large clostridial cytotoxins such as Clostridium difficile toxins A and B, and Rho-activating toxins such as Escherichia coli CNFs (25). The C3-like exoenzymes act on members of the RhoA subfamily, but most of the large clostridial cytotoxins inactivate all members of the Rho family. The Rho-activating toxins activate members of the RhoA subfamily and Cdc42. No activity for Ras, Rap, and Ran has been reported for the bacterial toxins of these three families, but Clostridium sordelli HT, one of the large clostridial cytotoxins, is known to inactivate Ras and Rap. There is no interpretation for these emerging differences in the specificity of the small GTPases. Hence, it becomes essential to examine the three-dimensional structures of Rho to understand how their interactions with the target proteins control the various signaling processes and how the modifications by bacterial toxins change the activities of their target GTPases for bacterial invasion. We report here the crystal structure of recombinant human RhoA, which is dominantly activated with substitution of Gly14 by valine (RhoA V14), complexed with GTP analogue, GTPγS, and we compare it with the structures of H-Ras and other related GTPases.

**EXPERIMENTAL PROCEDURES**

**Preparation and Crystallization of RhoA V14**—The cloning, expression, and purification of the dominantly active form of recombinant human RhoAV14 was carried out according to the methods described previously (8, 9, 11, 15). Details procedures will be described elsewhere. The resulting active sample, used in this study, is verified with MALDI-TOF MS (JMS-ELITE, PerSeptive Inc.) and N-terminal analysis (M492, Applied Biosystems). The protein is truncated at Ala181 and has one additional serine residue at the N terminus. Crystals were obtained at 4 °C by the hanging-drop vapor diffusion method from solutions containing 10 mg/ml GTP-S-RhoAV14, 50 mM Tris-HCl buffer, pH 8.5, 10% PEG8000, 7.5% 1,4-dioxiane equilibrated against 100 mM of the same buffer containing 20% 2-propanol. Crystals had hexagonal or trigonal lattice parameters with a rather long c axis (a = b = 60.80 Å, c = 214.56 Å) and diffracted at 3.0 Å.

**Data Collection and Structure Determination**—The structural analysis was performed using Form A. Intensity data were collected at 10 °C using an R-AXIS IIC imaging plate detector with CuKα x-rays generated by a rotating anode RU-300H (RIGAKU, Japan). The diffraction data were processed with PROCESS (RIGAKU). A summary of the data processing statistics are given in Table I. The initial phases were calculated by molecular replacement with the program AMoRe (26) using a search model based on the structure of human H-Ras (Protein Data Bank code 5P21, Brookhaven National Laboratory), with which RhoA shares a 27.5% identity. Several searches with a polyalanine model using different ranges of intensity data and integration radii resulted in a unique solution. Rigid body refinements of the searched model were performed with X-PLOR (27). The model obtained was divided into the secondary structure elements, and again, rigid body refinements were performed, followed by solvent flattening/histogram matching with the program DM (28). Four regions of insertions and deletions were inspected on the resulting 2Fo-Fc maps that was generated with the program O (29). The structure was built and refined through alternating cycles using the programs O and X-PLOR, respectively.

**Overall Structure**—The major features of the fold, consisting of a six-stranded β-sheet surrounded by helices connected with loops, are basically conserved as found in H-Ras (31, 32) and other related small GTPases (33–36) (Fig. 1). The β-sheet is formed by the anti-parallel association of two extended β-strands (B2 and B3) and the parallel association of five extended β-strands (B5, B1, B4–B6). RhoAV14 contains five α-helices (A1, A3, A3’ A4, and A5) and three 310-helices (H1–H3). There are three insertion and one deletion sites, which are common in the members of the Rho family, as can be seen from the sequence and secondary structure element alignment of RhoA and H-Ras (Fig. 2). The 13-residue insertion (Asp-Glu-Asp-Asp-Glu-Ala-Asp-Ile-Val-Arg-Ile) is located at the loop between strand B5 and helix A4. Excluding the deletion and insertion residues, the Cα-carbon atoms of RhoAV14 and the corresponding dominantly activated H-Ras 12, which is complexed with GTP (37), superimpose with a root mean square (r.m.s.) deviation of 1.68 Å for 163 common residues and part of the antiparallel β-sheet, consisting of the C-terminal half of strand B2 and the N-terminal half of strand B3, in addition to the insertion and deletion sites described above (Fig. 3A). Recently, Wei *et al.* have reported the crystal
Structure of RhoAV14-GTPγS complex

| Intensity data processing of RhoAV14 crystals | Refinement statistics |
|-----------------------------------------------|-----------------------|
| Resolution                                     | 2.4 Å                 |
| Bravais                                        | 8.75% (26.7%)         |
| Number of measurements                        | 61,579                |
| Number of independent reflections             | 8,683                 |
| Completeness                                   | 89.3% (74.8%)         |
| Mean (I/σ(I))                                 | 7.91 (2.04)           |

a $R_{merge} = 100 \times \sum |F(h)| - \langle |F(h)| \rangle / \sum |F(h)|$, where $|F(h)|$ is the mean intensity for reflection $h$.

b Brackets are quantities calculated in the highest resolution bin at 2.5–2.4 Å.

c $R_{merge} = 100 \times \sum |F(h)| - \langle |F(h)| \rangle / \sum |F(h)|$, where $F(h)$ and $F(h)$ are observed and calculated reflections.

d $R_{free}$ is $R_{cryst}$ which was calculated using 10% of the data, chosen randomly and omitted from the subsequent molecular replacement and structure refinement.

e $\Delta\phi$ is the deviation of the peptide torsion angle from 180°.

Fig. 1. Structure of RhoAV14, GTPγS. Shown is a ribbon representation of RhoAV14 complexed with GTPγS (yellow) and Mg2+ (a gray ball) with β-strands (red), α-helices (green), and βαα* helices (blue). Three water molecules (pink balls) are also illustrated. One water molecule (Wat1) participates in the guanine-base recognition of GTPγS, the second (Wat2) participates in the binding of the ribose, and the last (Wat3) is a putative nucleolytic water molecule. The secondary structure elements, Mg2+ ion, water, and GTP molecules are labeled as well as the N and C termini.

The structure of RhoA bound to GDP (38). Compared with this RhoA-GDP structure, the significant conformational changes were found to be localized in the switch I and II regions (Fig. 3B), as described for H-Ras (31, 39). Excluding these regions, the Cα-carbon atoms of RhoAV14-GTPγS and RhoA-GDP superimpose with a r.m.s. deviation of 0.48 Å. The nonhydrolyzable nucleotide GTPγS binds to the protein with a Mg2+ ion that has a typical octahedral coordination sphere (Fig. 4).

Insertion Regions—The N-terminal segment (Glu125-Lys133) of the 13-residue insertion forms an α-helix designated as A3*, which is followed by an extended loop. A short β3α3′-helix, designated as H3 (Fig. 1), is induced at the segment flanking the N terminus of this insertion, with large displacements of Arg122 and Asn123 from those of H-RasV12 (3.0 Å and 6.1 Å, respectively). Compared with RhoA-GDP, however, no significant conformational change exists in the 13-residue insertion and its N-terminal flanking regions. This folding seems to be basically similar to that of Rac1 complexed with GMP-PNP (36) but shows many differences in details. It is notable that the sequences of this region of members in the RhoA subfamily is rather different from those of the Rac1 subfamily. Among the key residues in stabilization of helices H3 and A3* (Fig. 5A), Arg122 and Asp124 are conserved in the Rho family but Arg128, Glu137, and Lys140 are variant in the Rac1 subfamily. No water molecule is found to be involved in the structural stabilization of the RhoA insertion region, though Rac1 forms a water-mediated hydrogen bond between the main chains. The conserved residues Leu131 and Pro138 of helix A3* form a hydrophobic patch with Thr127 and Pro120, which are also conserved or conservatively replaced in the Rho family. Rac1 adds Ile126 (Arg128 of RhoA) to the hydrophobic patch.

The outer surface of helix A3* is covered with charged residues whose side chains form hydrogen bonds and/or ion pairs, Glu125, Arg128 and Glu139, Lys132 pairs. These residues are conserved or conservatively substituted in the RhoA subfamily, but are replaced by other amino acid residues in the Rac1 subfamily. It is interesting that, in the Rac1 subfamily, Glu130 and Arg129 are substituted by lysine and glutamic acid, respectively, and Glu130 and Lys133 are substituted by lysine/arginine and glutamic acid, respectively. Therefore, these pairs of acidic and basic residues of Rac1 could form hydrogen bonds or ion pairs as observed in the current structure, though most of the...
exposed side chains of the residues of Rac1 corresponding to the residues 125–135 of RhoA are highly mobile.

Compared with H-Ras, helix A3 has a one-residue insertion at the center and two Pro residues (Pro 96 and Pro 101), which cause a disruption of the normal hydrogen-bonding pattern of an α-helix, whereas H-Ras has no Pro residue on this helix. These differences induce a relatively large discrepancy (2.05 Å at Pro 96) of helix A3 from that of H-RasV12 (Fig. 3A). Both Pro 96 and Pro 101 face the solvent region so as to induce pronounced kinks that serve to maximize the contacts with strands B1 and B4.

Phosphate-binding Loop—The G14V mutation of RhoA and the G12V mutation of H-Ras exhibit less than one-tenth the GTPase activity of the wild-type GTPases. Crystal structures of H-RasV12 complexed with GDP (31, 39) and GTP (37) show that the mutation causes no significant conformational change at the phosphate-binding region, though there are large differences in mobility and conformation predominantly localized in the switch II region (see below). Similar results were obtained in RhoA. The r.m.s. deviation of the 12GXG(V)XXGKT/S motif between RhoAV14-GTPγS and H-RasV12-GTP complexes is indicated at the top and below the aligned sequences, respectively. The α-helices (A1–A5) are in green, the extended β (B1–B6) are in green, and the 3_10-helices (H1–H3) are in blue. Three functional regions of RhoA are also indicated with marks for the residues that participate in interactions with GTPγS (circles) and the Mg^2+ ion (rectangles). The dominantly active mutation of glycine to valine is indicated by a star. The sequences are taken from SwissProt. The accession numbers are P06749 (RhoA), P01121 (RhoB), P08134 (RhoC), P15154 (Rac1), P15153 (Rac2), P25763 (Cdc42), P01116 (K-Ras), P01111 (N-Ras), and P01112 (H-Ras).

FIG. 2 Sequence alignment of human RhoA with the related human GTPases. Conserved residues are highlighted in yellow for the RhoA subfamily (RhoA, RhoB, and RhoC), in red for the Rac1 subfamily (Rac1, Rac2, and Cdc42), in blue for the Ras family (K-Ras, N-Ras, and H-Ras) and in gray for all members. The secondary structure elements of RhoAV14-GTPγS and H-RasV12-GTP complexes are indicated at the top and below the aligned sequences, respectively. The α-helices (A1–A5) are in green, the extended β (B1–B6) are in green, and the 3_10-helices (H1–H3) are in blue. Three functional regions of RhoA are also indicated with marks for the residues that participate in interactions with GTPγS (circles) and the Mg^2+ ion (rectangles). The dominantly active mutation of glycine to valine is indicated by a star. The sequences are taken from SwissProt. The accession numbers are P06749 (RhoA), P01121 (RhoB), P08134 (RhoC), P15154 (Rac1), P15153 (Rac2), P25763 (Cdc42), P01116 (K-Ras), P01111 (N-Ras), and P01112 (H-Ras).
cantly different conformation from that of H-Ras V12-GTP. Large displacements of the residues of switch I begin from Asp28 and end at Pro36 with the largest displacement (4.1 Å) at Glu32 (Figs. 5C). These displacements, which result in differences in recognition of the ribose of the guanine nucleotide, seem to be caused by Pro31, which restricts the main chain torsion angles. Since Pro31 is well conserved in the Rho family but is replaced by other residues in Ras (Val), Rab (Val), and Ran (Asp), the displacements could be a common structural feature of members of the Rho family. While large displacements were observed in switch I as described, no significant difference in the position and orientation of Thr37, which coordinates to the Mg$^{2+}$ ion, is seen between RhoA V14 and H-Ras V12.

**Strands B2 and B3**—The switch I loop is connected to the anti-parallel β-sheet of strands B2 and B3, which is followed by switch II. This two-stranded sheet is located at the edge of the six-stranded β-sheet and is sitting on helices A1 and A5 to form a hydrophobic core. Compared with H-Ras V12-GTP, a large displacement of these strands expands between a stretch from Ala44 to Val53, which moves toward helices A1 and A5, with the largest shift being 2.9 Å at Asp45. It is notable that the sequence of this region is highly conserved in the Rho family. While large displacements were observed in switch I as described, no significant difference in the position and orientation of Thr37, which coordinates to the Mg$^{2+}$ ion, is seen between RhoA V14 and H-Ras V12.

**Switch II**—The segment between strands B3 and B4 contains the switch II region that has a key residue Gln63 (Gln61 of H-Ras) of GTPase activities. Mutation of Gln61 of H-Ras to almost any other amino acid blocks intrinsic and GAP-stimulated GTPase activity (43). In the crystal structures of cellular H-Ras complexed with either GDP (31), GMP-PCP (39) or GMP-PNP (32), the highly conserved 57DTAGQ58 motif of oncogenic H-Ras V12 complexed with GTP has only one major conformation (37). In RhoA-GDP, residues 63–65 are also disordered. The electron density of this

**Fig. 3. Structural comparison of RhoA V14-GTPγS with H-Ras V12-GTP and RhoA-GDP.** A, superposition of Cα-carbon atom tracings of RhoA V14 bound to GTPγS (magenta) and H-Ras V12 bound to GTP (green) (Protein Data Bank code 521P). Segments displaying large displacements are highlighted in red for RhoA and yellow for H-Ras. These include switch I (residues 27–36 for RhoA), the anti-parallel β-sheet formed by strands B2 and B3 (residues 43–53), and switch II (residues 59–78). The 13-residue insertion (residues 122–137), forming helix A3′, and the segments helix A3 (residues 90–106), L7 (residues 107–110), and L9 (residues 151–154), which have insertion or deletion to produce large displacements, are in blue for RhoA V14. B, superposition of Cα-carbon atom tracings of RhoA V14-GTPγS (magenta) and RhoA N25 bound to GDP (green) (38) with segments displaying large displacements in red and yellow, respectively. These include switch I and the C-terminal flanking region (residues 28–44) and the N-terminal region of switch II (residues 62–69). Val14 of RhoA V14 is highlighted in blue.
The stereospecificity of Mg\(^{2+}\) ions is well defined and has a single conformation at the present resolution. RhoA \(^{V14}\) has two \(\alpha\)-helices, H1 (\(^{64}EDY^{66}\)) and H2 (\(^{70}KPL^{72}\)), which are separated by a short loop of three residues (\(^{67}DRL^{69}\)). The sequence of this region is well conserved in the Rho family but is different from those regions in the Ras family (Fig. 2). A similar conformation is also seen in the crystal structure of Rac1-GMP-PNP. In contrast, H-Ras\(^{V12}\) has a \(3_{10}\)-helix at the position corresponding to the short loop of RhoA\(^{V14}\) with an \(\alpha\)-helix of five residues corresponding to residues 71–75 of RhoA\(^{V14}\). These differences induce a large displacement of Glu64 (3.8 \(\AA\)), together with reorientations of the side chains of Gln63, Glu64 and Asp65 from the corresponding residues of H-Ras\(^{V12}\) (Fig. 3A). Lys98 and Glu102, both of which are located at helix A3, play crucial roles in the conformation of the segment by forming multiple hydrogen bonds to switch II (Fig. 5D). These two residues are conserved in members of the Rho family but are replaced in H-Ras. Moreover, the segment from helices H1 to H2 makes hydrophobic contacts strands B2 and B3. In this hydrophobic core, H-Ras\(^{V12}\) has an additional residue Tyr71, which is replaced by a small residue (Ser73) in RhoA. This difference causes a movement of the helix H2 toward strand B2. These differences seem to be one of the main reasons why the conformations of the segment are so different between RhoA\(^{V14}\) and H-Ras\(^{V12}\).

**Magnesium Ion Binding**—The strong GTP/GDP-binding and the GTPase activity of small GTPases have been shown to be absolutely dependent on the presence of divalent ions. The Mg\(^{2+}\) ion of the present structure is located at a position similar to those in H-Ras\(^{V12}\)-GTP and in H-Ras-GMP-PNP, as well as that in RhoA-GDP. The displacement of the ion from the corresponding position in the GDP-bound form is 1.04 \(\AA\). The Mg\(^{2+}\) ion plays a key role in bringing together the functional regions of the phosphate-binding, switches I and II, as observed in H-Ras. Actually, the stereochemistry of Mg\(^{2+}\) coordination in RhoA-GDP is different from the current form but also is different from that in H-Ras-GDP.

**Guanosine Nucleotide Binding**—The glycosyl conformation of GTP\(\gamma\)S is anti with the C2-endo sugar pucker. The guanine base is trapped in a hydrophobic pocket, in a manner similar to H-Ras\(^{V12}\)-GTP, to be recognized by several interactions with the conserved residues of the \(^{116}GXKKDL^{121}\) and \(^{162}SAK^{162}\) motifs (Figs. 5C). A major difference in base recognition is the water-mediated hydrogen bonds to the N7 and O6 atoms of the guanine base. The water molecule (Wat-1) is completely buried inside the hydrophobic binding pocket with a hydrogen bond to Gly77. The space for the accommodation of this water molecule is mainly produced by a rearrangement of the side-chain packing of the pocket, involving Leu\(^{21}\), Asn117, and Cys\(^{150}\) (Fig. 5E).

In H-Ras, Cys\(^{150}\) of RhoA is replaced by a Thr residue. In addition to the base recognition, the 2'-hydroxyl group of the ribose also has a water-mediated hydrogen bond to switch I, although in H-Ras\(^{V12}\)-GTP, the hydroxyl group of the ribose forms direct hydrogen bonds with the main chains corresponding to Pro\(^{31}\) and Glu\(^{32}\) of RhoA. As mentioned above, these differences in the recognition of the ribose are caused by the large displacements of switch I. Similar water-mediated hydrogen bonds in base and sugar recognition have also been found in RhoA-GDP and in Rac1-GMP-PNP.

**Triphosphate Binding**—GTP and GDP bind to small GTPases with dissociation constants on the order of nanomolar. This strong binding affinity is well demonstrated in the current structure. The triphosphate moiety of GTP\(\gamma\)S has 21 direct and 9 water-mediated hydrogen bonds to the protein, together with 2 magnesium coordinations. These involve six residues of the phosphate-binding loop, four residues of switch I, three residues of switch II, and four residues of base-recognition motifs. It is notable that most of these residues of the phosphate-binding loop interact with the triphosphate through their main-chains, especially the amino groups. This is the reason...
why the amino acid sequence of the \(12\text{GXGXXGKT/S19}\) motif contains many variant residues. The residues whose side chains participate in the interactions with the triphosphate are invariant Lys18, Tyr34, and Asp59. The conformation of the triphosphate exhibits similarity to that of GDP bound to RhoA, with relatively small displacements of the \(\alpha\)- and \(\beta\)-phosphates from those of GDP, 0.75 \(\text{Å}\) and 0.67 \(\text{Å}\), respectively. Two oxygen atoms of the \(\gamma\)-phosphate make contact with the protein by several hydrogen bonds, together with the coordination to the Mg\(^{2+}\) ion buried inside the pocket formed by switches I and II and the phosphate-bonding loops. These heavy interactions allow the \(\gamma\)-phosphate to orient the \(\gamma\)-sulfur atom toward Val14, Tyr34, and Pro36. Similar configurations of the \(\gamma\)-thiophosphate were also observed in the crystal structures of transducin-\(\alpha\) (44) and \(G_{\text{n1}}\) (45) complexed with GTP\(\gamma\)S. In all these crystals complexed with GTP\(\gamma\)S, the \(\gamma\)-sulfur atom is the closest atom to the side-chain amide group of Gln63 (Gln200 of transducin-\(\alpha\) and Gln204 of \(G_{\text{n1}}\)) among the \(\gamma\)-thiophosphate atoms.

**Putative Nucleophilic Water Molecule**—We identified one water molecule (Wat-1) that is close enough to the \(\gamma\)-phosphate to perform an in-line nucleophilic attack. The water molecule is located at a position 10° off from this line at a distance of 3.6 \(\text{Å}\) from the phosphorus atom and forms a hydrogen bond (3.3 \(\text{Å}\)) to the \(\gamma\)-sulfur atom, although the distance to the \(\gamma\)-oxygen atoms of the phosphate group is too long to form a hydrogen bond (3.6 \(\text{Å}\) for both). Similar water molecules have been located in analogous positions close to the \(\gamma\)-phosphate in the crystal structures of transducin-\(\alpha\) and \(G_{\text{n1}}\) complexed with GTP\(\gamma\)S, as...
well as of H-Ras, Rac1, and EF-Tu (46) complexed with GMP-PNP, although no water molecule corresponding to Wat-3 has been found in RhoA-GDP. Gln63 positions the side-chain oxygen atom at a distance of 3.8 Å from the hydrolytic water and the side-chain nitrogen atom at a distance of 3.8 Å from the side-chain carboxyl group of Asp65.

**DISCUSSION**

*Modification Sites by Bacterial Toxins—C. botulinum C3 ADP-ribosyltransferase transfers an ADP-ribose moiety of NAD to Asn41 of Rho (47). The side-chain of Asn 41, which is located at strand B2, forms a hydrogen bond (3.1 Å) to the main-chain carbonyl group of Glu40. This hydrogen bond allows Asn41 to interact with the indole ring of Trp58 of strand B3 (Fig. 5F). The distances between the nearest atoms of the indole ring and the carbonyl oxygen atom of the side chain of Asn41 range from 3.3 to 3.5 Å, which indicates the existence of a stacking interaction between them. Because the indole ring is a strong electron-donor, this interaction may help to enhance the nucleophilic properties of the side-chain nitrogen atom of Asn41. It should be noted that the hydrophobic side chains of Val38, Phe39, and Val43 are exposed to the solvent region around Asn41, together with Trp58. This unusual feature of the molecular surface may be related to the interaction with C3-like exoenzymes. Asn41 orients the side chain away from the switch I loop. This is consistent with the fact that the ADP-ribosylation on Rho affected neither the GTPγS binding nor its intrinsic GTPase activity. Furthermore, the ADP-ribosylation on Rho did not affect its interaction with rhoGAP (48). Recent data using Swiss 3T3 cells indicates that the ADP-ribosylation of Rho enhances its binding to PtdIns 4-phosphate 5-kinase and acts as a dominantly negative inhibitor (19, 49). This also suggests that the ADP-ribosylation does not impair the intrinsic properties of the switch I conformation though PtdIns 4-phosphate 5-kinase could bind to the GDP-bound form, and therefore, the binding may be different from that of other effectors that do not bind to the GDP-bound form. Rac and Cdc42 are not subjected to ADP-ribosylation (50). This may be related to the global conformation of the anti-parallel β-sheet formed by strands B2 and B3 since the sequence of this region of RhoA subfamily is conserved but is different from that in the Rac1 subfamily. On the molecular surface around Asn41, Val43 is replaced by Ser/Ala and Glu40 is replaced by Asp in Rac and Cdc42.

Recent biochemical data have shown that Thr37 is glucosylated by the major virulence factors of *C. difficile*, toxin A and B (51). The glucosylated RhoA induces the disaggregation of actin filaments. It also appears that GDP-bound RhoA is a superior substrate for Toxin B to GTP-bound RhoA. This is
switch II of Arf forms a long (E30D/K31E), which mimics Ras.

Switches I and II are shown in red and blue, respectively. This surface also contains most of the residues corresponding to the effector-binding residues as seen in the complex between the Ras-binding domain of Raf1 and a double mutant Rap1A (E30D/K31E), which mimics Ras.

consistent with the crystal structures: in RhoA-GDP, the side chain of Thr^{37} does not participate in either Mg^{2+} ion or phosphate binding, whereas it participates in both in the current structure. Since Thr^{37} orients the side chain inside the loop, its glucosylation must accompany a structural deformation of the loop. This structural change could extend to strand B2. Actually, it has been shown that the glucosylation of Thr^{37} inhibits ADP-ribosylation by C3-like exoenzymes (52).

CNFs from E. coli and dermonecrotic toxins (DNTs) from Bordetella species induce the massive reorganization of the actin cytoskeleton and inhibit cell division, leading multinucleated cells. Recently, CNF1 has been shown to cause the deamidation of Gln^{63} of RhoA, resulting in a dominantly active form, RhoA^{Gln63} (53–54). CNF1 acts preferentially with RhoA but also inhibits the GAP-stimulated GTPase activity of Cdc42 and of Rac at high concentrations. These actions of CNF1 may be related with the unique conformation and/or conformational properties of switch II. The differences in the CNF1 activity on RhoA, Cdc42 and Rac probably indicate that this toxin may interact with these small GTPases through segments other than switch I, though it remains unclear.

GTP/GDP Switching and Effector Binding—The fundamental mechanism of the molecular switch, which involves the significant conformational changes in switch I and II regions, in signal transduction seems to be common in small GTPases and G_{i} subunits of trimeric GTPases, as described for H-Ras (31), transducin-α (41), and G_{i/15} (55) and Rap2A (56). However, the present RhoA^{V14} structure reveals large conformational deviations from H-Ras^{V12} in the regions containing switches I and II. The structures of two other small GTPases, ADP-ribosylation factor (Arf) (33) and Ran (34), also showed significant conformational variations in the switch regions, whose structures are also different from those of the present RhoA^{V14}; switch II of Arf forms a long β-strand, and Ran has a completely different orientation of switch II that contains a short β-strand. All these results indicate that small GTPases from different families may have a similar fold but with significant variations in the switch regions.

There are several biochemical data indicating that the switch I region of RhoA is involved in its effector binding. It has been suggested from analyses of the chimeric proteins of Rho and Ras that the switch I region (residues 32–42) is essential for the induction of actin stress fiber formation (57). Either Cdc42 or Rac shows no significant binding to the target proteins of RhoA, such as Rho-kinase and the others described above. At switch I and its fracting regions of RhoA^{V14}, residues that are exposed to the solvent region are well conserved in the RhoA subfamily but are replaced in the Rac1 subfamily. It is of interest that most of the side chains of these residues protrude onto the same molecular surface (Fig. 6). The double mutant Rap1A (58), which mimics Ras, binds to the Ras-binding domain of c-Raf1 through several residues that are located at the same side of the corresponding molecular surface of RhoA^{V14}. Among them, residues whose side chains form the specific hydrogen bonds to the Ras-binding domain are located at the N-terminal half of strand B2. It is notable that most of these residues are replaced by non-conservative, mainly hydrophobic, residues (Val^{33}, Val^{35}, Phe^{39}-Asn^{41}, and Val^{43}) in RhoA^{V14}-GTP^{γ}S to form hydrophobic patches on the molecular surface, as described.

In addition to switch I, the second effector site is suggested in the C-terminal two-thirds of the molecule (57). However, little is currently known about the possible second effector site of RhoA. Recent mutagenesis experiments have indicated that the 13-residue insertion region of Rac1 participates in the interaction with p67phox but not in the interaction with PAK, and a combinational use of the multiple effector-binding sites has therefore been proposed (59). Since there are several structural differences in the 13-residue insertion regions between RhoA^{V14}-GTP^{γ}S and Rac1-GMP-PNP, it may be possible that RhoA also utilizes the insertion region in the specific binding with its own effector proteins, but this remains to be seen in future experiments. It should be noted that the 13-residue insertion region has no significant displacement from that in RhoA-GDP and, therefore, has no switching function between GTP-bound and GDP-bound forms. It is well known that the G_{i} subunits of trimeric GTPases contain four insertion regions if compared with small GTPases. The 13-residue insertion region
of the members of the Rho family corresponds to the third insertion region that forms an additional helix at the N-terminal portion of the segment (44), although no homology has been detected between RhoA and each of G$_S$ subunits and no possible function has been assigned to this insertion region.

**GEF and GDS Binding**—GEFs for small GTPases of the Rho family have been identified as Dbl-containing proteins that contain a region with a sequence homology to the dbl oncogene product (60). While most of these Dbl-containing proteins can act on multiple members of the Rho family in vitro, some have a limited specificity for one type of the GTPases in vitro. Among them, Lbc shows selectivity for Rho (61) but Tiam-1 for Rac (62) and Cdc24 for Cdc42 (63). Analysis of RhoA/Cdc42Hs chimeric proteins has suggested that residues of switch I and switch II are involved in the specific interaction with Lbc (64). Based on mutation analyses, Lys27, Tyr34, Thr37, and Phe39 in switch I and Asp76 in switch II have been identified as Lbc-sensitive residues. These residues are located at nearly the same side of the molecule, which may form a surface of interaction for Lbc. It is of interest that this surface is almost the same as that for a tentative effector binding (Fig. 6). The side chains of all these residues are highly projected toward the solvent region but a tentative effector binding (Fig. 6). The side chains of all these residues. These residues are located at nearly the same side of the membrane containing Asn41 since ADP-ribosylation of the Dbl-containing proteins but has some homology to Cdc25 of the members of the Rho family corresponds to the third insertion region that forms an additional helix at the N-terminal portion of the segment (44), although no homology has been detected between RhoA and each of G$_S$ subunits and no possible function has been assigned to this insertion region.

**GDI Binding**—In addition to inhibiting nucleotide dissociation, GDIIs mediate partitioning their cognate small GTPases between the membrane and the cytosol (68). RhoGDI inhibits the guanine nucleotide exchange of all members of the Rho family. Recent structural studies have suggested that rhoGDI binds to the cognate GTPases via an immunoglobulin-like domain that has a hydrophobic pocket for binding to the C-terminal isoprenyl group (69, 70). Although this immunoglobulin-like domain has little effect on the rate of nucleotide dissociation from the GTPases, it has been suggested that this binding directs the flexible N-terminal arm of rhoGDI to GTPases, resulting in the inhibition of nucleotide exchange. It is of interest to question how the N-terminal arm interacts with GTPases because the C terminus having the isoprenyl group is located on the molecular surface of the GTPases opposite to the nucleotide binding surface. It has been reported that GDI effectively prevents ADP-ribosylation by C3-like exoenzymes and the nucleotide-exchange activity of smgGDS (67). Furthermore, the nucleotide-exchange activity of Dbl also was remarkably reduced (66). Taken together, these results suggest that the N-terminal arm of rhoGDI may interact with GTPases on the molecular surface, which has residues interact with GEF and GDS as well as C3-like exoenzymes (Fig. 6). This hypothesis provides a framework for analyzing the interactions of rhoGDI, GEF, and GDS with RhoA.

**Effects of the γ-Sulfur Atom and the G14V Mutation on the GTPase Activity**—The GTP·γS molecule exhibits its resistance to hydrolysis, which is conferred by the γ-thiophosphoryhothioate. In the present structure, the γ-thiophosphoryl turns the sulfur-phosphorus bond toward Gln63 and positions the γ-sulfur atom to come into contact with the putative nucleophilic water molecule. Therefore, the bulky sulfur atom, which has a van der Waals radius (1.8 Å) much larger than that of the hydroxyl group (1.4 Å), sterically shields the phosphorus atom from the close approach of the nucleophilic water molecule and could interfere with the stabilization of the transition state by Gln63. The γ-sulfur atom could also interfere with the stabilization of the transition state by the Arg residue from GAP (71). Similar mechanisms for the resistance of the GTP·γS molecule to hydrolysis, which is conferred by the γ-thiophosphoryhothioate, are possible for transducin-α and G$_S$. Based on the crystal structure of transducin-α complexed with GTP·γS, it has also been pointed out that Arg74, which is a key residue stabilizing the transition state, prevents the thiophosphate from reaching the transition state, due to a steric clash between the firmly anchored guanidino group and the sulfur atom (44).

It has been suggested that the role of the key Gln residue (Gln63 of RhoA) is to stabilize the transition state by direct hydrogen bonds doubly bonded to the γ-phosphate and the putative nucleophilic water molecule. The transition state should induce conformational changes around the active site of the current structure since the present conformation of Gln63 directs the side-chain carbonyl group toward the nucleolytic water molecules (3.8 Å) but also positions the side-chain amide group away from the phosphorus. The γ-sulfur atom of the phosphatase is closest to the side-chain amide group of Gln63, as described above, but the distance between them is more than 5 Å. The contacts of the branched side chain of Val14 with the N terminus of switch II seem to push Gln63 away from the γ-phosphate group and reduce the conformational flexibility of the side chain of Gln63. Actually, the Cγ carbon atoms of Val14 and Gln63 have a contact of 3.6 Å. Any rotation around the side-chain torsions of Gln63 could not bring the side-chain amide group to a position close enough to interact with the γ-sulfur atom because of the steric hindrance of the bulky side chain of Val14. We postulate that these steric effects are a possible means of inhibiting GTP hydrolysis by the dominantly active mutation V14 of RhoA. Thus, the mechanism of dominant activation by the G14V mutation of RhoA seems to be similar to that of G12V of H-Ras even though the conformations of switches I and II are quite different.

**Acknowledgments**—We thank Drs. S. Takayama, F.-S. Che, and T. Katsuragi for technical assistance and helpful discussions. We acknowledge Dr. Z. S. Derewenda for providing the coordinates of RhoA-GDP.

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*J. Biol. Chem.* 1998, 273:9656-9666.
doi: 10.1074/jbc.273.16.9656

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