Crystal Structure of the Core Domain of RhoE/Rnd3: A Constitutively Activated Small G Protein[†,‡]

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ABSTRACT: We report the 2.1 Å crystal structure of the core G protein domain of the unusual Rho family member RhoE/Rnd3 in complex with endogenous GTP and magnesium. Unlike other small G proteins, RhoE, along with two other proteins Rnd1/Rho6 and Rnd2/RhoN, does not hydrolyze GTP. The main reason for this is the presence of serines in the positions equivalent to Ala59 and Gln61 in Ras. The structure shows that there are still water molecules in similar positions to the waters thought to be involved in the hydrolysis reaction in other G proteins. The structure suggests three not necessarily exclusive explanations for the lack of hydrolysis. The lack of the conserved glutamine raises the energy of the transition state inhibiting hydrolysis. The serines may restrain the waters from moving closer to the GTP, a step that is required to attain the transition state. They also stabilize the GTP-bound conformation of switch II and could prevent conformational changes required during hydrolysis. By superposition of the RhoE structure on structures of Rho family proteins in complex with binding partners, we make predictions on RhoE interactions with these partners.

The Rho family of GTPases forms a subgroup of the Ras superfamily of regulatory GTPases. Members of this superfamily characteristically act as molecular switches, cycling between a GTP-bound "active" state and a GDP-bound "inactive" state (*1*). The transition occurs when the γ phosphate group of the bound GTP nucleotide is hydrolyzed. Conformational changes of the GTPase on hydrolysis, in particular in segments called switch regions reduce binding to downstream effectors and turn the protein "off".

The activity of the Rho GTPases, and of the Ras superfamily in general, is regulated by three different types of proteins. These are (i) guanine nucleotide exchange factors (GEFs),[1] which promote the exchange of bound GDP for bound GTP and the formation of the active form of the GTPase; (ii) GTPase-activating proteins (GAPs), which have the opposite effect and increase the intrinsic rate of hydrolysis of bound GTP; and (iii) guanine nucleotide dissociation inhibitors (GDIs), which can inhibit both the exchange of nucleotides and the hydrolysis of bound GTP (*2*). The regulators exhibit specificity for particular GTPases: p190RhoGAP, for example, acts preferentially on Rho (*3*).

The Rho subfamily contains at least 20 proteins in humans (*4*) several of which are known to regulate cytoskeletal organization and cell migration (*4*). Three members of the family known to regulate the organization of the cytoskeleton are Rho, Rac, and Cdc42. For example, in Swiss 3T3 fibroblasts Rho controls the formation of contractile actin-myosin filaments which make up stress fibers (*5*). Rac regulates the formation of lamellipodia (*6*) and Cdc42 the formation of filopodia (*7*).

The human form of RhoE was first identified by Foster and co-workers (*8*), who showed by sequence alignments that RhoE exhibits sequence similarity to several members of the Rho GTPase family, and has the strongest homology to RhoA, B, and C (54% sequence identity). Despite this finding, RhoE has several unusual properties which distinguish it from the other Rho proteins. In a comparison with RhoA and B, Ras, Rac1, and Cdc42 under similar conditions, it was found that RhoE binds GTP but does not exhibit any appreciable ability to hydrolyze GTP (*8*). Furthermore, it is resistant to RhoGAP activity, and both p190RhoGAP and Cdc42GAP failed to promote any GTP hydrolysis by RhoE. Only the GTP-bound form of RhoE can be isolated, with no evidence of a GDP-bound form. Thus, RhoE appears to be constitutively associated with GTP in vivo, a feature that distinguishes it from the majority of GTPases in the Ras superfamily. However, by mutation of three residues to the amino acids found in active GTPases (S32G, S79A, S81Q), GTP hydrolysis activity was conferred on RhoE (*8*).

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[1] Abbreviations: ESRF, European Synchrotron Research Facility; GAP, guanosine triphosphate hydrolase activating protein; GDI, guanine nucleotide dissociation inhibitor; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; GTP, guanosine triphosphate; GTPase, guanosine triphophate hydrolase; GTPγS, guanosine 5′-(3-*O*-thio)-triphosphate; GppNHp, guanosine-5′-(α,γ-imido)triphosphate; MDCK, Madin-Darby canine kidney cells; PDB, Protein Data Bank; PEG, polyethlene glycol; RMSD, root-mean-square deviation.

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RhoE has also been found to induce different effects on the cytoskeleton to those of RhoA. Rho proteins generally stimulate stress fiber formation, but experiments with MDCK epithelial cells showed that, while RhoA promoted stress fiber formation as expected, RhoE caused rapid disappearance of these fibers as well as the loss of focal adhesions (*9*). This led to the proposal that RhoE may in fact compete with RhoA for binding to downstream effectors, but not switch on their activity.

Recently, three new proteins have been identified which form a distinct subgroup of the Rho family: Rnd1, Rnd2, and Rnd3 (*10*). Rnd3 is identical to RhoE, with an extra 15 N-terminal amino acids that were lacking in the sequence published by Foster et al. (*8*). Like RhoE, Rnd1 has no detectable GTPase activity, does not appear to have a specific RndGAP, and does not respond to p50RhoGAP (also known as Cdc42GAP), and has very low affinity for GDP. It is therefore thought to be constitutively in the GTP-bound form. Rnd1, like RhoE, promotes the loss of actin stress fibers, and in fact Rnd means "round", and refers to the cell rounding observed when cell-substrate adhesion is lost. Thus, Rnd1, Rnd2, and RhoE/Rnd3 share unusual properties that place them in a distinct subgroup of the Rho family.

We report here the crystal structure of RhoE at 2.1 Å resolution. The structure shows a fold very similar to that of RhoA with bound GTP, containing the characteristic switch regions that bind the nucleotide, and a coordinated magnesium ion. Variations in individual residues in the active site suggest possible reasons why RhoE does not hydrolyze GTP and may not have a GDP-bound form.

MATERIALS AND METHODS

Protein Purification, Crystallization, and Data Collection. The coding region for residues 16 to 200 of mouse RhoE was cloned into pET28a Expression Vector with an N-terminal polyhistidine tag. The construct was transformed into *Escherichia coli* BL21(DE3) competent cells, which were grown at 37 °C in LB containing 30 mg/L kanamycin. When the cell culture reached an optical density of $A_{600} = 0.8$, protein expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactoside and cells were harvested after 4 h.

RhoE was purified using Ni-NTA resin (Qiagen). The protein was bound to the resin in a lysis buffer of 50 mM Tris, 300 mM NaCl, 10 mM imidazole, and 5 mM $MgCl_2$ at pH 8. Washes of the resin were performed with the same buffer containing 50 mM imidazole, and elution was performed with 500 mM imidazole. The eluted protein was concentrated and loaded onto a Superdex 75 gel filtration column for further purification in a buffer containing 20 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$ at pH 8.

RhoE was crystallized at 16 °C under oil. A drop of 1 μL of protein at a concentration of 30 mg/mL was mixed with 1 μL of precipitation solution consisting of 0.1 M Hepes pH 7.5, 10% propan-2-ol, and 20% (w/v) PEG 4000. Crystals appeared within 7 days with dimensions 0.2 × 0.05 × 0.05 mm. Flash freezing in liquid nitrogen was performed in the same solution with 30% propan-2-ol as cryoprotectant. Data were collected on Station ID14-EH2 at ESRF Grenoble at wavelength of 0.933 Å. Data were processed using MOSFLM (*11*), and intensities were scaled using SCALA (*12*, *13*). The results are shown in Table 1. The cell dimensions were $a = 59.5$, $b = 59.5$, $c = 233.0$ Å in point group $P422$.

Table 1: Data Collection and Refinement Statistics

data collection	all data	highest resolution shell
resolution (Å)	18.8−2.1	2.2−2.1
observations	115 552	15240
reflections	25 276	3592
completeness (%)	99.8	100
redundancy	4.5	4.2
I/σ	16.3	3.5
R-merge	0.079	0.343

refinement	all data	highest resolution shell
resolution (Å)	223−2.1	2.15−2.10
refined observations	23005	1758
free observations (5%)	1260	81
R-factor (%)	18.3	21.8
R-free (%)	21.2	25.4

model	
no. of atoms	3062
RMS bond length (Å)	0.021
RMS bond angle (°)	1.92

chain	all	A	C
average B (Å^2)	22.3	20.5	20.8
main chain B (Å^2)	19.1	19.1	19.1
side chain B (Å^2)		22.1	22.6
water B (Å^2)	48.0		
average B GTP (Å^2)	16.5	16.1	16.9
Wilson B (Å^2) from TRUNCATE	31.2		

Structure Solution and Refinement. Molecular replacement was carried out with the protein coordinates of the GTPγS bound form of RhoA from the structure in complex with the effector domain of the protein kinase PKN (PDB 1CXZ) (*14*), using the program MOLREP (*15*). In space group $P4_32_12$, a clear solution with two molecules in the asymmetric unit was found (R factor 0.514, correlation 0.430, compared with R factor 0.567 and correlation 0.275 in $P4_12_12$). Clear electron density for the GTP and Mg were seen. Refinement against 2.1 Å data was performed using CNS (*16*) with rounds of manual rebuilding in O (*17*). Final refinement was performed using Refmac5 (*18*) with TLS parameters for each RhoE/GTP/Mg group as a single body.

Structure Analysis. Structural superpositions and calculations were carried out using Swiss PDB viewer (*19*); http://www.expasy.ch/spdbv/.

RESULTS

Structure Determination and Description. The protein crystallized in space group $P4_32_12$ (Table 1). The search model was the dominantly active form of recombinant human RhoA^{V14} consisting of residues 1 to 181 of RhoA described by Maesaki et al. (*14*), in which RhoA is bound to the non-hydrolyzable GTP analogue GTPγS (guanosine 5′-3-*O*-thiotriphosphate) and to an Mg^{2+} ion, and is also complexed to the effector domain of the protein kinase PKN/PRK1. The structure of RhoA in this complex was itself obtained by molecular replacement using the uncomplexed form of RhoA bound to GTPγS and Mg^{2+} (*20*). The molecular replacement process included only the protein chain, not the GTP ligand, but the electron density clearly indicated the presence of GTP in the active site (Figure 1). The nucleotide had remained

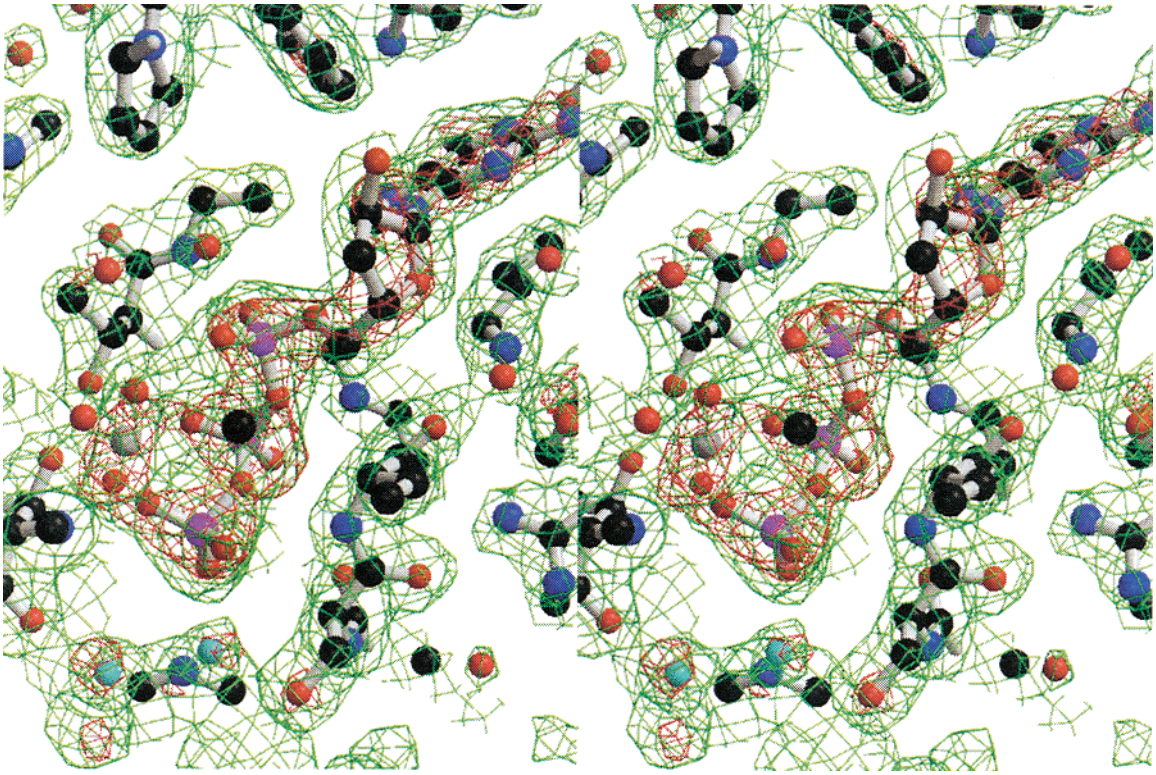

FIGURE 1: Final $2F_o-F_c$ map (green) in the region of the GTP contoured at 1σ and the initial F_o-F_c map (red) after rigid body and B factor refinement of the molecular replacement model but before the addition of any ligand atoms, showing clear evidence that it is GTP that is bound in the active site. Drawn with BOBSCRIPT (*43*).

bound throughout the protein purification procedure and had not undergone any hydrolysis. The B factors for the γ phosphate atoms are only slightly larger than for the α or β phosphates. The refinement statistics to a resolution of 2.1 Å are presented in Table 1.

The structure contains two copies of RhoE, each binding GTP and a Mg^{2+} ion, and 193 water molecules. Although the crystallized protein comprised residues 16 to 200 of RhoE and an uncleaved HisTag, there is only visible electron density for residues 23 to 200 in chain C, and residues 22 to 200 in chain A. The loop from 66 to 69 in both copies has poor electron density. In chain C, residue 66 is modeled as an alanine instead of an isoleucine because of poor electron density for that residue. In chain A, residue 65 is modeled as an alanine instead of a glutamate, 68 as an alanine instead of a threonine, and residue 200 is an alanine in place of a lysine. Residues 67, 68, and 69 are poorly ordered in both chains, more particularly in chain A. These residues form part of a flexible loop, which is distorted due to different crystal packing in the two copies of the molecule. The side chain of Asp67 makes hydrogen bonds in crystal contacts in both copies and is clearly visible in the electron density, but the main chain and adjacent side chains have poor density, presumably because they are distorted from an energetically favored conformation and take multiple conformations which are not resolved. The torsion angles of A67 fall in a disallowed region of the Ramachandran plot. The side chains of Tyr A60 and Asn C164 are modeled as two conformations. There is an intramolecular disulfide bridge between residues 24 and 197 in both copies. There is no reason to suggest that this is physiological, the cysteine at the equivalent of 197 is only found in Rnd1 and not Rnd2 and only the Rnd/RhoE proteins have cysteines at the equivalent of position 24. In the reducing conditions of the cytosol these surface accessible residues would probably remain reduced. The buried surface area in the contact between the monomers is 813 Å^2 per chain, which is high enough to suggest a dimer. However, analytical gel filtration indicates a monomer in solution.

The overall structure of RhoE is shown in Figure 2a. The main features of the fold, which consists of a six-stranded β sheet surrounded by helices connected with loops, are the same as those found in RhoA (*20*), in H-Ras (*21*), and in other small GTPases (*22*−*24*). This core region of six β strands and five or six α helices carries out the basic functions of nucleotide binding and hydrolysis, and has been called the G domain (*1*). In H-Ras, there are six β strands and five α helices (*21*); the Rho family of proteins generally has six α helices, containing an additional and unique α helical sequence that replaces a loop in other Ras family members (*25*). RhoE also contains this insert region, which extends from residues 137 to 150.

Comparison of RhoE Structures to Themselves and RhoA. Figure 2b shows the superpositions of RhoA-GTPγS (PDB 1A2B) bound and RhoA-GDP (PDB 1FTN) bound forms on the two copies of RhoE in the asymmetric unit. The RMSD for the two copies of RhoE over 176 Cα atoms is 0.78 Å and the two chains superpose closely overall. The region with the largest discrepancy is the loop of residues 64−68, which is involved in different crystal contacts in the two copies.

RhoE superposes closely with RhoA-GTPγS with an RMSD of 1.02 Å for 176 Cα atoms with copy C and 1.32 Å with copy A (the loop 64−68 is closer to the RhoA structure in copy C). There is a single insert of residue 185 in the RhoE backbone compared to RhoA (Figure 2b). The

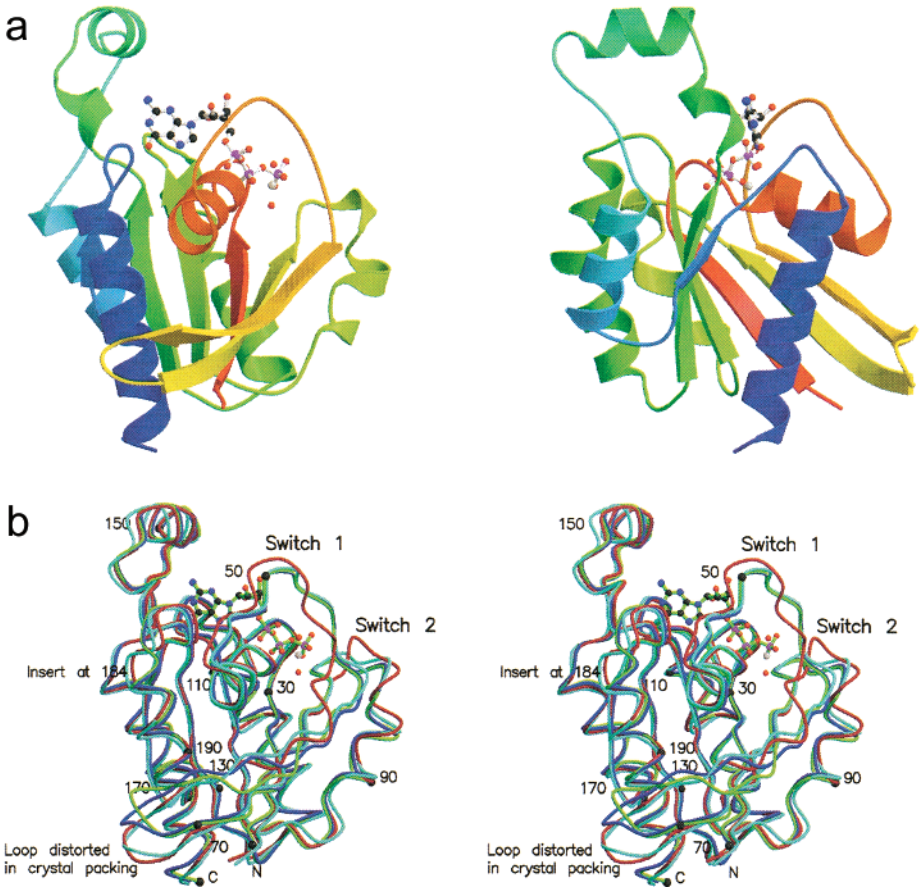

FIGURE 2: Overall Structure and superposition of RhoE. (a) Secondary structure cartoon of RhoE and GTP colored from red at the N terminus to blue at the C terminus. The second view is a 90 degree rotation round the vertical of the first. (b) Stereopair of the superposition of the Cα atoms of the two copies of RhoE with GTP in green and blue, RhoA with GTPγS (PDB entry 1A2B (*20*)) in cyan and RhoA with GDP (PDB entry 1FTN (*26*)) in brown. Superposition performed using Swiss PDB viewer (*19*). Figure drawn with MOLSCRIPT (*44*) and Raster3D (*45*).

main difference is in the loop corresponding to 64−68 of RhoE probably due to the crystal packing.

Comparison with the RhoA-GDP form (*26*) gives a RMSD of 1.45 Å for 176 Cα residues, with the greatest deviation as expected being in the switch regions, particularly switch I. The RhoA-GDP structure gives an indication of the extent to which the RhoE conformation would need to change to bind GDP. A structure of RhoA-GDP in a Mg^{2+}-free form (*27*) shows a much greater RMSD of 3.92 Å (superposition not shown) over 176 Cα atoms with RhoE. The region which moves most is the switch I region, and the elimination of Mg^{2+} opens up the nucleotide binding site. The structure of RhoE, then, is typical of a small G protein in the GTP bound form with one inserted residue compared to its closest sequence homologues. Uniquely there is endogenous GTP in the active site that is stable for weeks during crystallization.

DISCUSSION

Reasons for Lack of GTP Hydrolysis. There has been considerable dispute over the precise mechanism of GTP hydrolysis in GTP-binding proteins (*28*−*30*). The more favored view is for an associative hydrolysis mechanism with a trigonal-bipyramidal transition state of phosphoryl transfer. In this explanation, the reaction begins by proton transfer from the attacking water molecule to the γ-phosphate oxygen (*29*). The generated OH^- nucleophile then moves toward the γ phosphate, and the reaction proceeds via a pentacoordinate transition state, with GDP as the leaving group.

A crucial residue in the hydrolysis reaction is a conserved glutamine, which is located in the switch II region of RhoA and Ras. In Ras, this is residue Gln61, and it was found that mutations affecting this glutamine led to a significant decrease in GTPase activity (*31*). Rac also has an equivalent Gln61, and the corresponding residue in RhoA is Gln63 (Figure 3). Structural studies of complexes with GAP and the transition state analogue GDP·AlF_4^- (*32*) or GDP·AlF_3 (*33*) indicate that the function of this glutamine is to contribute to an electrostatic "envelope" around the nucleophile and its substrate that preferentially stabilizes the transition state. In the ground state, the carbonyl group of Gln61 (in Ras) interacts with the attacking water molecule (*29*); in the transition state the Gln61-H_2O structure moves about 2 Å toward the γ phosphorus, and that position is stabilized by Gln61 interacting with an arginine in the RasGAP, Arg789, as well as with oxygen of the γ phosphate.

In RhoE, the equivalent residue to Gln61 in Ras is Ser81. The side chain of the serine is pointing away from the GTP and forming an H bond with the main chain N of residue 83 stabilizing the main chain conformation promoted by the intervening Pro82. The primary cause of the loss of GTPase activity is the replacement of the conserved glutamine by serine in the Rnd proteins. The glutamine lowers the energy of the transition state enhancing the rate of hydrolysis. As

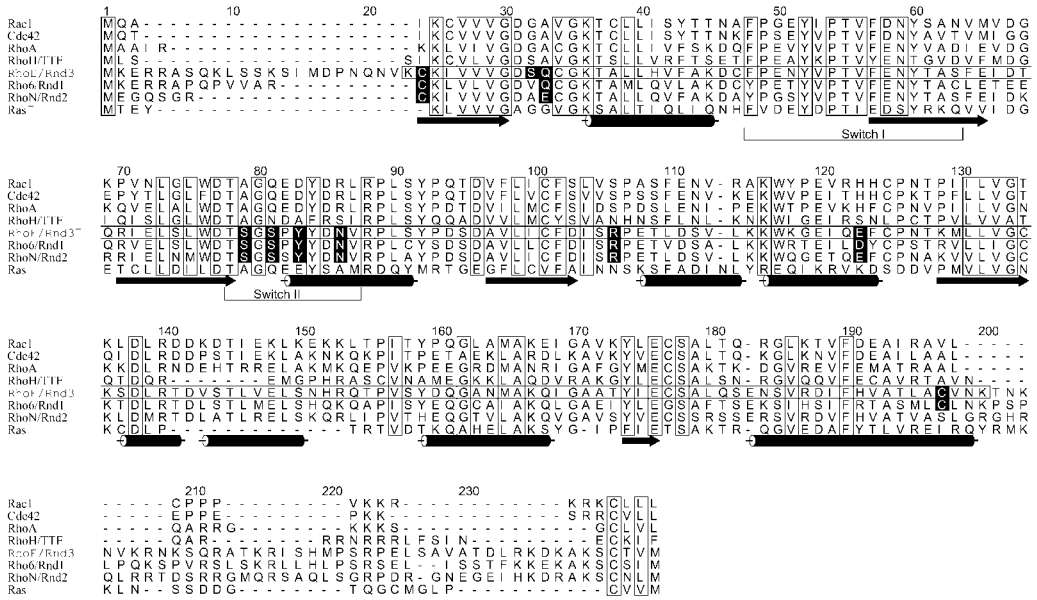

FIGURE 3: Alignment of RhoE/Rnd3 with the other Rnd proteins and with typical members of the Rho family and Ras. The numbering at the top is of RhoE. The boxed area is that seen in the crystal structure. The secondary structure of RhoE is shown below the alignment. Some of the residues discussed in the text are highlighted. Drawn with ALSCRIPT (*46*).

shown by Foster et al. (*8*), mutation of S81Q is necessary (but not sufficient) for GTP hydrolysis by RhoE. Replacement of serine 79 by alanine is also required for hydrolysis, and this structure offers possible explanations of this.

The effect of removal of the conserved glutamine is not to exclude water as there are still two water molecules in the active site close to the γ phosphate in both copies of RhoE, although neither of them are in line for attack on the bridging oxygen bond. The first water molecule (Wat1) forms hydrogen bonds to the side chain of Ser79 and main chain carbonyl of residue 55 and to the other water molecule (Wat2), which also hydrogen bonds to the side chain of Ser32 and a γ phosphate oxygen (Figure 4a).

In most structures (17 found in PDB Jan 2002) of small G proteins with GTP or a GTP analogue that have any waters reported in the region of the phosphate, there is only one water molecule present. This water is close to the position of Wat1 in the RhoE structure and is usually hydrogen bonded to the Gln61 equivalent as well as nearby main chain atoms, although this water is still present in essentially the same place when the glutamine side chain has been removed by mutation (eg PDB 1E96 which has a Q61L mutation). In the six reported structures of a GAP protein complexed to a G protein with a transition state analogue, there is still a single water molecule, but it is closer to the phosphorus atom of the γ phosphate. The exceptions are structures of Ras PDB 1QRA (temperature 100 K, ligand GTP), 1CTQ (100 K, GppNHp), 5P21 (277 K, GppNHp), 621P (277 K, GppNHp, Q61H). These have two waters in similar positions to RhoE. In three of the structures (not 621P), there is a third water molecule close to the position of the oxygen of the serine 32 of RhoE. This residue is a glycine in Ras. The superposition of the three waters with the two waters and serine O in RhoE is almost perfect in 1CTQ. In particular, Wat2 in all the structures at 100 K (1QRA, 1CTQ, the RhoE structures) is also within hydrogen bonding distance of an oxygen of the γ phosphate leading to a proposed two water model (*29*), where the primary hydroxyl generation is by transfer of a proton from Wat2 to the phosphate. The presence of this water in the non-hydrolyzing RhoE argues against this model, as does its absence in many of the structures of active G proteins.

Foster et al. (*8*) have shown that the serine residues at positions 79 and 81 of RhoE are critical for GTP hydrolysis. They mutated these residues to their equivalents in activated Ras, Ala59 and Gln61. The S79AS81Q double mutant of RhoE was able to hydrolyze GTP at levels comparable to those for RhoA. Mutating a third residue, Ser32, to its Ras equivalent Gly12 produced a triple S32GS79AS81Q mutant of RhoE that could hydrolyze GTP to levels greater than those seen for RhoA. Thus, the serines at positions 32, 79, and 81 in RhoE are critical in affecting its ability to hydrolyze GTP. It is noteworthy that Cdc42, Rac1, and RhoA all have glycine, alanine, and glutamine, like Ras, in the equivalent positions, and RhoE alone is different (Figure 3). The equivalent to Ser32 in Rnd1/Rho6 is Val, Rnd2/RhoN is Ala but is a Ser in RhoH/TTF (*34*). All three Rnd proteins have Ser at 79 and 81 but RhoH has an Ala and Asn. Like the Rnd proteins, RhoH is unable to hydrolyze GTP (*35*), suggesting that a Ser at 32 together with Asn at 81 is sufficient to prevent hydrolysis.

We conclude that RhoE does not hydrolyze GTP as there is no active site glutamine, but this mutation does not exclude water molecules from the active site. The water distribution in the active site is similar to active G proteins, in particular ras with GppNHp at 100 K. However, the hydrolytic water has to move closer to the γ phosphate for activity. Ser79 cannot alter its torsion angle enough to allow this and retains the water in the ground state. In agreement, the single S81Q mutation of RhoE does not hydrolyze GTP and additional removal of the serine OH at S79 is required to confer GTPase activity in the S79AS81Q double mutant (*8*).

GDP Binding. A further unusual feature of the RhoE/Rnd group of proteins is that they do not bind GDP with any measurable affinity. Methods used to exchange GDP for GTP in vitro do not work for RhoE (*9*; K. Riento, unpublished data) or Rnd1 (*10*). This suggests that RhoE may not be able to adopt a GDP-like conformation of its switch regions.

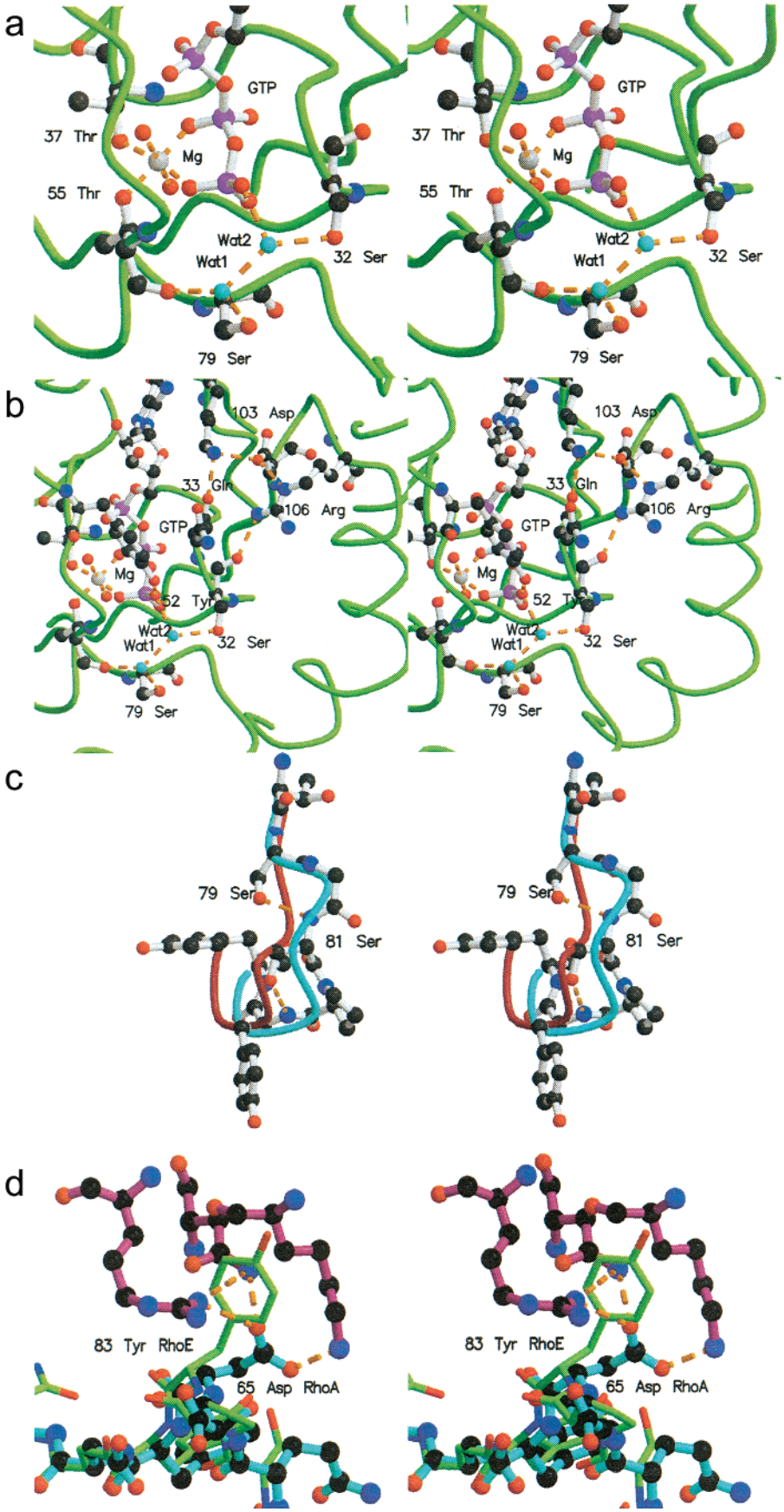

FIGURE 4: (a) Stereopair of the active site in the region of the γ phosphate showing the serines unique to the RhoE/Rnd proteins. (b) Stereopair of the structure of RhoE in the vicinity of Gln 33. (c) Stereopair of the region of Ser79 and 81 superimposed with the Cα trace of RhoA-GTPγS (cyan) and RhoA-GDP (brown). (d) Stereopair of the superposition of the Rho-GAP (purple) interaction with RhoA (cyan) in the region of switch II with the RhoE structure (green). All figures drawn with MOLSCRIPT (*44*) and Raster3D (*45*).

Gln33 in RhoE is equivalent to Ala15 in RhoA, and Rac1 and Cdc42 also have alanine at this position. Rnd1 also has a glutamine at this position and Rnd2 has a glutamate. It can be seen from Figure 4b that the long side-chain of Gln33 covers the GTP-binding site more than an Ala or Gly found in active G proteins would. It could potentially interact with nearby Tyr52 during its move of 11 Å to its position in the GDP state, inhibiting Tyr52 from taking up its GDP-bound conformation. Furthermore, the side-chain of Arg106, which is again unique to RhoE/Rnd1−3, replaces a serine in RhoA, and hydrogen bonds to the carbonyl of Ser32. Tyr52 lies in switch I (residues 48−63 of RhoE), which shows only three sequence changes from RhoA. Asn51 in RhoE is a valine in RhoA; Thr61 is also a valine in RhoA, and Ser63 is a glutamate.

The switch II region is less well conserved, with six differences in residues at positions 79, 81, 82, 83, 86, and 87 of RhoE. A second explanation for the crucial role of Ser79 and Ser81 in preventing hydrolysis is that they both form hydrogen bonds to the N+2 main chain nitrogens and the SGSP sequence is thereby stabilized in the GTP conformation, as these bonds would not be possible if RhoE adopted the backbone conformation of the GDP form of RhoA (PDB 1FTN) (Figure 4c). These differences may contribute toward the stability of the GTP-bound form of RhoE and its apparent inability to move to a GDP-bound conformation.

GAP Binding. In the RhoA-p50RhoGAP (PDB 1TX4) structure with the transition-state analogue GDP·AlF^{4-} two residues from RhoA are particularly involved in an extensive hydrogen-bonding network with the GAP, and these are Asp65 and Tyr66 (*31*). In the Cdc42 (GppNHp)−p50RhoGAP complex (PDB 1AM4) (*36*), the equivalent residue to Asp65 (Asp63) is important for making salt bridge and hydrogen bonding interactions with residues Lys122, Arg126, and Asn202 of p50RhoGAP. In RhoE, the equivalent of Tyr66 in RhoA is Tyr84, but the equivalent of Asp65 is Tyr83 (Figure 3). Superposition of the RhoE structure on the complexes (Figure 4c) shows that Tyr83 would probably interfere with both the ground and transition states formed with p50RhoGAP. However, RhoE was reported to bind to p190RhoGAP (*8*). Lys122 is extremely highly conserved in RhoGAPs. [In an alignment of about 70 nonredundant putative human RhoGAP domains from the SMART database (http://smart.embl-heidelberg.de/) it was totally conserved]. Arg126 is also found in most sequences (about 60) but strikingly not in p190RhoGAP-A and B, which have the sequence KSFFS and KAFFA compared to KTFLR in the equivalent of region 122−126 of p50RhoGAP. It may be that RhoE/Rnd proteins can compete better with RhoA for p190RhoGAP binding than for p50RhoGAP binding. This suggests that a function of RhoE may be to act as a competitive inhibitor for a subset of RhoGAPs. This could be the small set lacking the Arg126 or the larger set with a Phe at the equivalent position to Leu125, which would be predicted to be surface exposed and therefore potentially able to stack with Tyr83 of RhoE/Rnd proteins. Further experimental investigations of these suggestions are required.

Binding to other Rho Family Binding Partners. Members of the Rho family of GTPases bind different effectors, which initiate a cascade of protein−protein interactions that ultimately leads to rearrangements of the cytoskeleton. A recent review of GTP-binding proteins in the Ras superfamily (*37*) points out that 31 structures of GTPases complexed with various partners have been reported.

Structural superpositions with Rho family complexes suggest a number of conclusions about RhoE. The only structure of a Rho effector complex is that of RhoA with the effector domain of the protein serine/threonine kinase PKN, also known as PRK1 (*14*). The extra residue in RhoE, Ser185, clashes with PKN binding making it unlikely that PKN will bind to RhoE. There are no clear-cut mutations in RhoE relative to Cdc42 which would preclude interaction with CRIB domain effectors based on the structures of Cdc42 with WASP (Wiskott-Aldrich Syndrome protein) (PDB 1CEE) (*38*), ACK (activated Cdc42-associated kinase) (PDB 1CF4) (*39*), and PAK (p21-activated kinase) (PDB 1EES, 1E0A) (*40*−*41*), but no binding of these effectors to RhoE has been demonstrated so far.

Two residues have been shown by mutation studies (*42*) to be responsible for the specificity of the RhoGDI interaction with Rho family GTPases. They are Arg66 and His103. With regard to RhoE, the equivalent of Arg66 is Asn86, and the equivalent of His103 is Glu123. In the Rac1−RhoGDI complex, $Arg66_{Rac}$ forms salt bridges with $Glu121_{GDI}$ and $Asp185_{GDI}$, and $His103_{Rac}$ forms a salt bridge with $Asp184_{GDI}$. These salt bridges cannot form with the RhoE equivalent residues so it is unlikely that RhoE would be able to bind to RhoGDI. Furthermore, RhoE has been reported to be farnesylated (*8*) rather than geranylgeranylated. Binding of geranylgeranyl groups is strongly favored by RhoGDI. It is therefore very unlikely that there is a large cytosolic pool of RhoE associated with RhoGDI.

SUMMARY AND CONCLUSION

RhoE does not hydrolyze GTP as GTP is stable in crystals for weeks. This is caused by the substitution of serines in the RhoE sequence for residues known to be important in hydrolysis in other G proteins. These serines hydrogen bond to the waters in the active site and may restrain them in the ground state, preventing progression to a transition state. The lack of the conserved glutamine found in active G proteins does not change the ground-state water configuration. The serines also stabilize the GTP-bound form of switch II, which may prevent the conformational switch to the GDP state required for hydrolysis. Superposition of the structure on known Rho family complexes indicates that binding to PKN, RhoGDI, and p50RhoGAP are very unlikely due to loss of important interactions and steric clashes. The true binding partners of RhoE have not yet been definitively identified, which will be important in understanding its physiological role. Work is under way in our laboratories to find these proteins and characterize them biochemically and structurally.

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