Structural basis of activation and GTP hydrolysis in Rab proteins

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Background: Rab proteins comprise a large family of GTPases that regulate vesicle trafficking. Despite conservation of critical residues involved in nucleotide binding and hydrolysis, Rab proteins exhibit low sequence identity with other GTPases, and the structural basis for Rab function remains poorly characterized.

Results: The 2.0 Å crystal structure of GppNHp-bound Rab3A reveals the structural determinants that stabilize the active conformation and regulate GTPase activity. The active conformation is stabilized by extensive hydrophobic contacts between the switch I and switch II regions. Serine residues in the phosphate-binding loop (P loop) and switch I region mediate unexpected interactions with the γ phosphate of GTP that have not been observed in previous GTPase structures. Residues implicated in the interaction with effectors and regulatory factors map to a common face of the protein. The electrostatic potential at the surface of Rab3A indicates a non-uniform distribution of charged and nonpolar residues.

Conclusions: The major structural determinants of the active conformation involve residues that are conserved throughout the Rab family, indicating a common mode of activation. Novel interactions with the γ phosphate impose stereochemical constraints on the mechanism of GTP hydrolysis and provide a structural explanation for the large variation of GTPase activity within the Rab family. An asymmetric distribution of charged and nonpolar residues suggests a plausible orientation with respect to vesicle membranes, positioning predominantly hydrophobic surfaces for interaction with membrane-associated effectors and regulatory factors. Thus, the structure of Rab3A establishes a framework for understanding the molecular mechanisms underlying the function of Rab GTPases.

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Introduction

Guanine nucleotide binding proteins (G proteins) cycle between active (GTP-bound) and inactive (GDP-bound) states by a highly conserved molecular mechanism (reviewed in [1,2]). Activation is tightly regulated by guanine nucleotide exchange factors (GEFs), which promote exchange of GTP for GDP in response to extracellular or intracellular signals [3,4]. Subsequent inactivation is regulated by GTPase-activating proteins (GAPs) that stimulate a weak intrinsic GTPase activity [5,6]. The net rate of GTP hydrolysis serves as a timing mechanism to limit the duration of interaction with effectors that preferentially bind to the GTP-bound conformation.

Rab GTPases comprise a large family with more than forty functionally distinct members regulating discrete steps in exocytic and endocytic vesicle trafficking pathways [7–9]. The first members of the Rab family were identified in secretion-deficient yeast mutants [10,11] and, on the basis of similarities with other GTPases, a role as molecular switches mediating vesicular transport between subcellular compartments was proposed for these proteins [12]. Rab3 isoforms associate with presynaptic vesicles to regulate Ca^{2+}-dependent exocytosis and neurotransmitter release [13,14]. Recent studies of Rab3A-deficient mice demonstrate that Rab3A acts during a late step in the fusion of presynaptic vesicles with active-zone membranes to control the quantity of neurotransmitter released [15]. Rab3A is also required for long-term potentiation in hippocampal neurons and thus may play a role in learning and memory [16].

Despite conservation of the catalytic Gln residue common to most GTPases, intrinsic rates of GTP hydrolysis in the Rab family vary by more than an order of magnitude [17]. These differences are thought to reflect the requirements of particular trafficking steps with faster processes, such as neurotransmitter release from presynaptic vesicles or the fusion of endocytic vesicles to form early endosomes, regulated by Rab proteins with higher intrinsic GTPase activities [18]. However, the structural determinants responsible for the large variation in the intrinsic rates of GTP hydrolysis have not been identified [17].

Rab GTPases interact with a variety of regulatory and effector proteins including GEFs, GAPs, GDIs (GDP dissociation inhibitors), effectors, and proteins required for

prenylation and targeting to specific intracellular compartments [9]. Studies of mutant and chimeric Rab proteins have implicated both switch regions, the α3–β5 loop and the hypervariable N- and C-terminal regions as important functional determinants [19–21]. The amino acid sequences of Rab effectors, GEFs, and GAPs indicate that they are not related to regulators or effectors for other GTPases. Thus, the extent to which Rab interactions with effector and regulatory proteins will resemble those of other GTPases remains to be determined.

Although GTPases have a similar overall fold, numerous variations in structure have been observed [2,22–30]. Rab GTPases are distantly related (<30% amino acid identity) to Ras and other monomeric GTPases. Although the residues essential for nucleotide binding and GTP hydrolysis are retained in the amino acid sequences of Rab proteins, many non-conservative substitutions (e.g. hydrophobic for charged residues) within functionally important regions suggest that significant structural variations are likely to underlie differences in functional properties and contribute to the specificity of interactions with effectors and regulatory factors.

Here we describe the 2.0 Å crystal structure of the active form of Rab3A bound to the nonhydrolyzable GTP analog GppNHp. The active conformation of Rab3A exhibits significant but localized structural differences compared to Ras. In addition, Ser residues from the phosphate-binding loop (P loop) and putative switch I regions are observed to mediate novel and unexpected contacts with the γ phosphate of the nucleotide. These Ser residues, along with other residues that are important for stabilizing the active conformation of the switch regions, are conserved in Rab GTPases involved in the regulation of exocytic vesicle trafficking pathways. Thus, the Rab3A structure provides a structural basis for understanding the functional properties of Rab GTPases, and identifies new interactions that are predicted to be important in the regulation of GTPase activity.

Results and discussion

Structure determination

Compared to the GTPase domain of Ras, the amino acid sequences of Rab proteins exhibit N- and C-terminal extensions that are hypervariable in length and sequence. These extensions are required for prenylation and targeting to specific intracellular membranes but are not essential for nucleotide binding or hydrolysis and are not thought to participate directly in protein–protein contacts with effector or regulatory proteins [31,32]. The N terminus of Rab3A contains a 19-residue extension, one of the longest in the Rab family. The N terminus is sensitive to proteolysis by both Lys-C and Glu-C (DGL, unpublished results) and attempts to crystallize Rab3A with an intact N terminus were unsuccessful. However, diffraction-quality crystals of the active (GppNHp-bound) form were obtained from a deletion construct (residues 15–186) that lacked both hypervariable regions (hereafter referred to as Rab3A for simplicity). These results are consistent with the observation that the hypervariable regions of Rab7 do not have a well-defined secondary structure in solution [33].

Crystals of Rab3A bound to a nonhydrolyzable GTP analog (GppNHp) were grown by vapor diffusion in microseeded hanging drops. A native data set complete to 2.0 Å was collected on a laboratory source and the structure was solved by molecular replacement using the coordinates of Ras [22] as a search model (see Table 1 and Figure 1). The final refined model includes residues 18–186, one molecule of GppNHp, a Mg^{2+} ion, 123 ordered water molecules and has a crystallographic R value of 0.191 and an R_{free} value of 0.237 based on 5% of the reflections randomly omitted prior to refinement. The stereochemistry is excellent and there are no mainchain φ–ψ values outside allowed regions of the Ramachandran plot.

Although large single crystals of the GDP-bound form were also obtained, these exhibited weak diffraction ($d_{min} < 4$ Å), preventing an experimental determination of the specific

Table 1

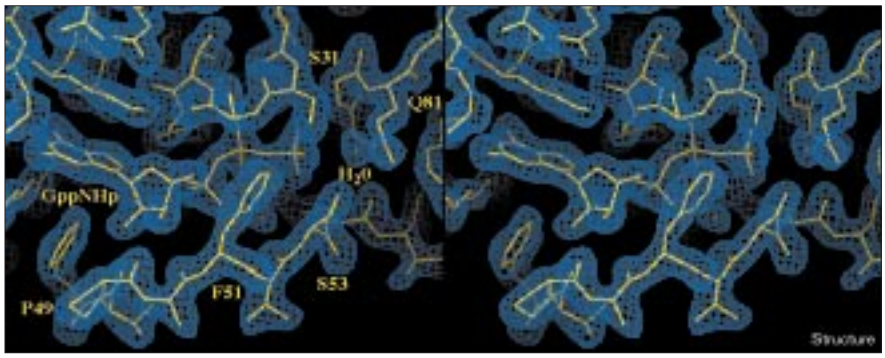
Structure determination and refinement.

Data collection	
Resolution limit* (Å)	20.0–2.0 (2.1–2.0)
R_{sym}† (%)	6.3 (19.7)
Completeness (%)	99.5 (99.5)
$< I/\sigma >$	17.1 (6.3)
Rotational search	
Search model	Ras mainchain
Euler angles (Q_1, Q_2 , Q_3)	262.4, 120.8, 94.0
Correlation coefficient (%)	
highest peak	15.0
highest false peak	13.5
Translation search	
Space group	C2
Fractional coordinates (x, y, z)	0.198, 0.000, 0.220
Correlation coefficient (%)	
highest peak	23.7
highest false peak	12.0
Refinement	
Resolution (Å)	8–2.0
R factor‡	19.1
R_{free}§	23.7
Number of reflections	10,595
Rmsd from ideal geometry	
bond lengths (Å)	0.007
bond angles (°)	1.3

*Values in parentheses correspond to those for the highest resolution shell. †$R_{sym} = \Sigma |I_{hkl} - <I_{hkl}>| / \Sigma I_{hkl}$, where $<I_{hkl}>$ includes Friedel mates and symmetry-related reflections.
‡R factor = $\Sigma_{hkl} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$ for all data above 2σ.
§$R_{free} = \Sigma_{hkl} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$ for 5% of the reflected data.

Figure 1

Stereoview of the nucleotide-binding region in the SigmaA-weighted $2F_{obs}–F_{calc}$ electron-density map calculated with phases derived from the final refined model and contoured at 1.2σ.



conformational changes accompanying activation of Rab3A. To facilitate discussion, we will designate putative conformational switch regions in Rab3A that correspond with the experimentally established switch regions in Ras [34], recognizing that the actual conformational changes will probably differ in both extent and amplitude [35]. The putative switch I and switch II regions span residues 49–57 and 80–95, respectively.

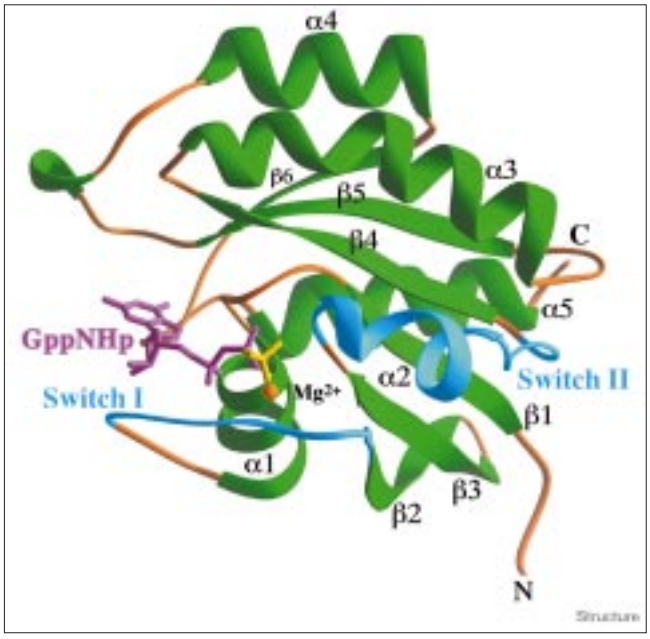
Rab3A structure

As depicted in Figure 2, Rab3A has a typical GTPase fold consisting of a six-stranded β sheet, comprised of five parallel strands and one antiparallel strand, surrounded by five α helices. Although the overall structure is similar to GppNHp-bound Ras and other GTPases [2,22–28], localized but significant differences are observed in the vicinity of the putative conformational switch regions and the α3–β5 loop (Figure 3). Each of these regions has been previously implicated in the function of Rab proteins [20,21,36]. In contrast to Ras, all of the mainchain atoms in the putative switch I and switch II regions adopt well-ordered conformations in the structure of Rab3A. There are relatively few contacts between the switch regions and symmetry-related molecules, indicating that the structure within the switch regions reflects intrinsic molecular determinants of the active conformation and is minimally perturbed by crystal packing. The α3–β5 loop in Rab3A is one residue shorter than its counterpart in Ras, accounting for at least part of the difference in the conformation of this loop. However, residues 125–127 in the α3–β5 loop are not well ordered, and crystal contacts involving adjacent residues raise the possibility that the conformation and/or flexibility of this loop might differ in solution. Consequently, the following discussion will focus primarily on the structural differences within the switch regions and their implications concerning the function of Rab GTPases in vesicle trafficking.

Switch I region

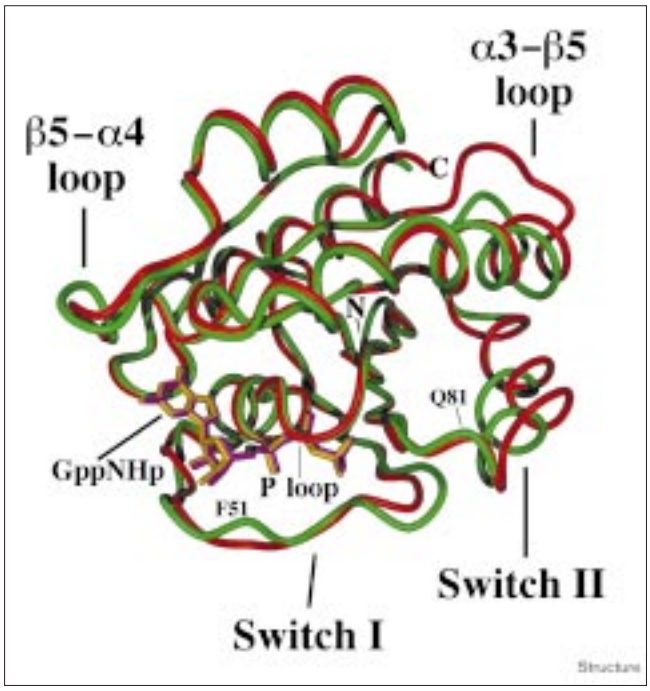
The switch I–β2 region of Rab3A contains a sequence, ^{51}FVS<u>T</u>VGIDFKV^{61} (single-letter amino acid code; invariant Thr is underlined), that is highly conserved in exocytic Rab GTPases. Despite two nonconservative substitutions, residues ^{52}VS<u>T</u>^{54} adopt a mainchain conformation nearly identical to that of the analogous residues in Ras (^{33}DP<u>T</u>^{35}), allowing the invariant Thr54 to mediate characteristic interactions with the γ phosphate and the Mg^{2+} ion typical of the GTPase superfamily. The most significant structural difference in the switch I region occurs within a short stretch of residues immediately C-terminal to Thr54. This region corresponds to the 'effector-binding loop' of Ras and is encoded by a

Figure 2



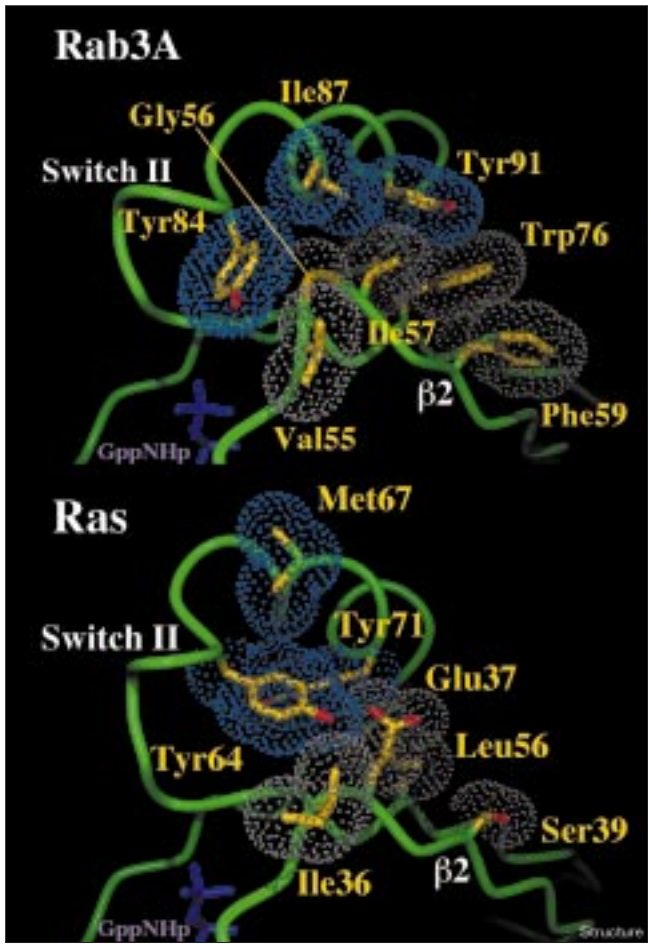
Ribbon drawing of the overall Rab3A–GppNHp structure with the nucleotide in purple, the γ phosphate in yellow and the Mg^{2+} ion represented by an orange sphere. The segments corresponding to the switch I and switch II regions of Ras are highlighted in light blue.

Figure 3



Comparison of the structures of Rab3a–GppNHp (green) and Ras–GppNHp (red; PDB code 5P21) following least-squares superposition. The overall structures are similar, with a root mean square deviation (rmsd) of 1.33 Å after superposition of 163 C_α atoms. Significant differences are observed in the switch I and switch II regions, as well as the α3–β5 and β5–α4 loops. GppNHp is purple in the Rab3A structure and yellow in the Ras structure.

Figure 4



Structural differences in the switch II region. The sidechains of selected residues are highlighted in yellow with the corresponding van der Waals surfaces represented by blue dots (switch II residues) or gray dots (other residues). The Ras–GppNHp structure corresponds to the PDB entry 5P21.

sequence motif, T(I/V)**G**(I/V/A)(D/E)**F**, that is conserved throughout the Rab family from yeast to mammals (bold residues are invariant within the Rab family but are not strictly conserved throughout the GTPase superfamily) [9]. In the Rab3A structure, the hydrophobic residues of this motif form an extensive hydrophobic interface with residues from the β3–switch II region (Figure 4). The strictly conserved Gly56 represents a sequence insertion relative to Ras and plays a pivotal role by forcing the mainchain of residues Val55–Ile57 to bulge outward in the direction the α2 helix, thereby allowing the Cα of Gly56 and the hydrophobic sidechains of the flanking residues (Val55 and Ile57) to pack against conserved hydrophobic sidechains from α2. Gly56 adopts mainchain ϕ–ψ angles that are incompatible with a β substituent, explaining the conservation of a Gly residue at this position. The hydrophobic interface between the switch I and switch II regions is reinforced by the indole ring of Trp76 from β3, which wedges between the sidechains of Ile57 and Phe59 and simultaneously packs against the phenyl ring of the Tyr91 in switch II. In addition to the 'Gly bulge' described above, the formation of this extensive hydrophobic interface is also facilitated by nonconservative substitutions whereby strictly hydrophobic residues in Rab3A (Ile57, Phe59, Trp76 and Ile87) replace charged, polar and dissimilar hydrophobic residues in Ras (Glu37, Ser39, Leu56 and Met67, respectively). Finally, heterotrimeric Gα subunits and Arf proteins also contain similar Gly insertions [23–25]; however, the resulting bulges differ in conformation and do not appear to increase interactions between the switch regions.

Structural differences are also observed in the loop segment spanning the C-terminal end of α1 and the first few residues of the switch I region, resulting in additional stabilizing interactions with the nucleotide in comparison with Ras. An interaction between the 3′ OH of the ribose and the backbone carbonyl oxygen of Pro49 is analogous to a similar contact in α subunits of heterotrimeric G proteins, whereas a hydrogen bond between the sidechain OH group of Ser37 and an α-phosphate oxygen resembles

similar contacts in heterotrimeric G proteins Ran and Arf [23,25,26]. The aromatic ring of Phe51 lies in van der Waals contact with the 5′ CH_2 group of the ribose moiety, presumably increasing the barrier for nucleotide dissociation. In contrast, the corresponding Tyr32 in GppNHp-bound Ras is flipped outward, allowing solvent access to the nucleotide [22]. It should be noted, however, that in the solution structure Tyr32 is highly flexible [37], and in the crystal structure crystal contacts involving Tyr32 are observed [22]. The backbone conformation and sidechain orientation of Phe51 are reminiscent of similar structural arrangements in the active conformations of other monomeric GTPases including Rap2A [35], RhoA [30] and the Rap1A complex with the Ras-binding domain of Raf-1 [38]. In these structures, a conserved Tyr shields the nucleotide and is thought to impede dissociation. Thus, the closed conformation of Phe51 observed in the Rab3A structure appears to be a general feature of the active form of monomeric GTPases.

Switch II region

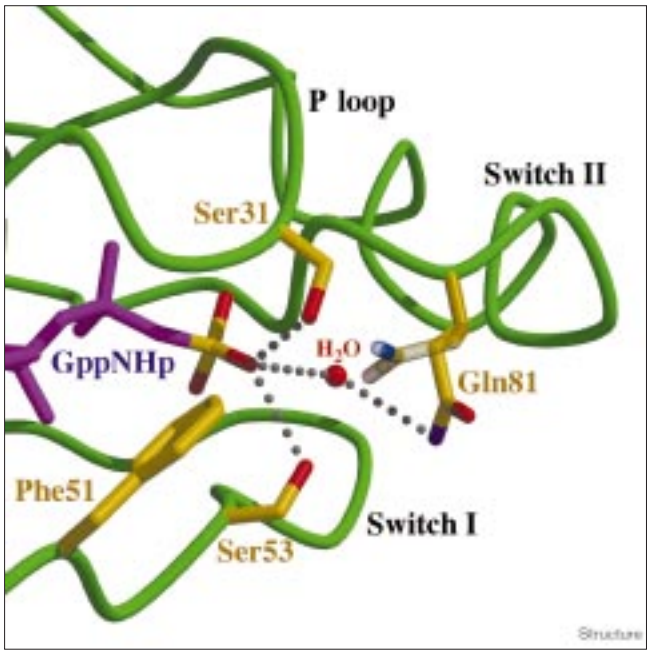
The β3–switch II region of exocytic Rab GTPases (e.g. Rabs 1, 3, 8 and 10, Ypt1 and Sec4) exhibits a highly conserved consensus sequence, ^{76}**W**DTAGQER(**Y/F**)RT**I**T(S/T)(A/S)**YY**RGA^{95}, that contains the invariant Asp and Gly residues (underlined) of the DxxG motif common to all GTP-binding proteins. The hydrophobic residues in this sequence (bold) are identical or similar in endocytic Rabs and most other monomeric GTPase families including Ras. The conformation of the ^{76}WDTAGQ^{81} segment in Rab3A is virtually identical to that of other GTPases, thus allowing Asp77 and Gly80 to form critical contacts with a water liganded to the Mg^{2+} ion and the γ phosphate, respectively. The remainder of the switch II region (from Glu82 to Ala95) adopts a well-ordered but discontinuous secondary structure consisting of a 3_{10} helical turn followed by a five-residue α-helical segment and two sequential β turns (Figure 2). This peculiar secondary structure appears to be a consequence of the extensive hydrophobic interface with the switch I region. The α-helical segment encoded by the ^{85}RTITT^{89} sequence is rotated by ~60° about an axis tilted towards the β2 strand with respect to the α2 helical axis in Ras. These mainchain rearrangements have several important consequences. First, Ile87 in α2 is drawn towards the Gly bulge in the switch I region, thereby positioning its hydrophobic sidechain to pack against the Cα of Gly56 and the sidechain of Ile57. Second, the aromatic sidechains of two conserved Tyr residues (Tyr84 and Tyr91) flip outward in comparison with the location of their counterparts in Ras (Tyr64 and Tyr71) such that one face of each aromatic ring is solvent-exposed while the other packs against hydrophobic sidechains from the switch I region (Ile57 and Phe59) and the β3 strand (Trp76). The sidechain OH groups of Thr88 and Thr89 are buried in the interface with the β2–β3 hairpin where they stabilize the secondary structure of the foreshortened α-helical segment by donating direct and water-mediated hydrogen bonds to the mainchain carbonyls of Tyr84 and Arg85. The sidechains of Arg85 and Thr86 occupy solvent-exposed positions and do not participate in intramolecular interactions, but they may be conserved for interaction with regulatory or effector proteins. Finally, the residues in the β3–α2 loop adopt an ordered mainchain conformation. In contrast, the analogous residues in Ras are poorly ordered [22]. The decreased mobility of the β3–α2 loop in Rab3A reflects the differences in secondary structure of the switch II region as well as the more extensive interaction with residues in the switch I region. The interactions described above appear to be the major factors contributing to the stability of the active conformation of the switch II region. Disruption of the hydrophobic interface between the switch I and II regions would account for the large changes in intrinsic tryptophan fluorescence associated with Trp74 and Trp114, which accompany predicted nucleotide-dependent conformational changes in Rab5 [39].

Interactions with the nucleotide, γ phosphate and Mg^{2+} ion

All of the characteristic interactions with the nucleotide and Mg^{2+} ion, as observed in other GTPases, are preserved with nearly identical stereochemistry in Rab3A. The active conformation of Rab3A is stabilized by interactions between the γ phosphate of GTP and two critically conserved residues, Thr54 in switch I and Gly80 in switch II (Thr35 and Gly60 in Ras, respectively) [1]. Specifically, the O3 oxygen of the γ phosphate accepts a hydrogen bond from the backbone NH of Gly80 while the O2γ oxygen is coordinated by the Mg^{2+} ion and accepts hydrogen bonds from the backbone NH and sidechain hydroxyl groups of the invariant Thr54. The Mg^{2+} ion coordinates six ligands with octahedral geometry: two phosphate oxygens (O2β and O2γ), the hydroxyl groups of two threonine residues (Thr36 of the GX_4GKS/T motif and Thr54) and two water molecules.

In addition to the canonical nucleotide contacts, the sidechain hydroxyl groups of two Ser residues (Ser31 in the P loop and Ser53 in switch I) donate hydrogen bonds to the O1 oxygen of the γ phosphate (Figure 5). Both serine residues are conserved in exocytic Rab GTPases but are variable in endocytic Rabs and are not present in the P-loop sequences of most other GTPase families, which instead have a conserved glycine at this position. Although the sidechain hydroxyl of Ser31 is optimally oriented for interaction with the γ phosphate, the hydrogen bond with Ser53 is markedly longer (3.0 versus 2.5 Å), indicative of a weaker interaction. The stereochemistry of the canonical interactions with the γ phosphate is not perturbed by the additional contacts, which instead appear to increase the stability of the active conformation and reduce the likelihood of spontaneous GTP dissociation. As the additional γ phosphate contacts occur only in the GTP-bound form, they may play a role in triggering release from nucleotide exchange factors during activation.

Figure 5



Novel interactions with the γ phosphate. Selected residues within the P loop, switch I and switch II regions are shown in yellow. The OH groups of Ser31 and Ser53 provide additional contacts to the γ phosphate that have not been observed in previous GTPase structures. The amino group of the conserved Gln81 donates a hydrogen bond to the nucleophilic water molecule. The orientation of Gln61 in the Ras–RasGAP complex [42] is shown as a ghosted residue. Although the sidechain of Gln81 is ordered, the orientation of the carboxamide group can not be unambiguously deduced and it is possible that the sidechain carbonyl accepts a hydrogen bond from the nucleophilic water. Ser31, Phe51 and Ser53 are conserved among exocytic Rab GTPases.

Structural determinants of GTPase activity

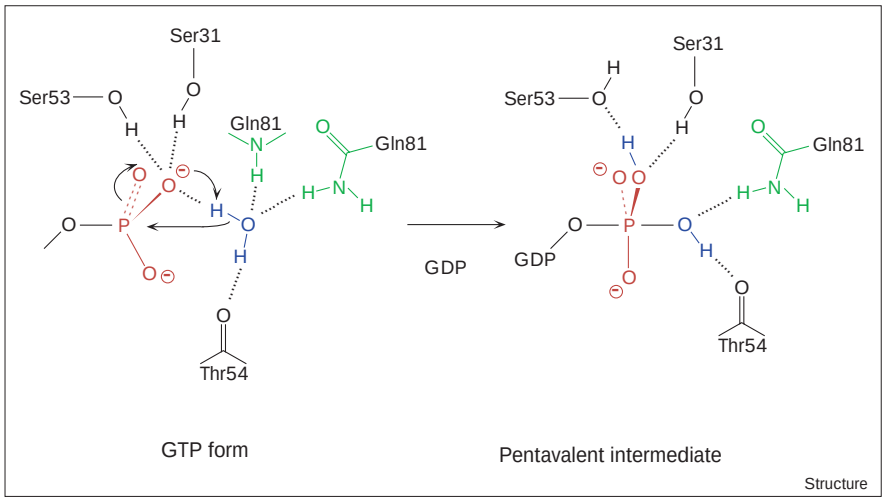
Biochemical studies have implicated a highly conserved glutamine residue (Gln61 in Ras and Gln81 in Rab3A) in the mechanism of GTP hydrolysis [40]. Crystal structures of several GTPase complexes with GDP–AlF_4^- (a 'transition state' mimic) alone [24,41] or with GAPs [42,43] have shown that the sidechain carboxamide of the conserved Gln stabilizes the transition state through hydrogen-bonding interactions with an equatorial (O2γ) oxygen and the axial oxygen derived from the nucleophilic water. In Rab3A, the sidechain carboxamide of Gln81 contacts the putative nucleophilic water; however, interaction with the γ phosphate is blocked by the sidechain of Ser31 (Figure 5). Although the geometry of the hydrogen bond between Ser31 and the γ phosphate is almost ideal, the sidechain orientation of Ser31 combined with the rigid P-loop conformation should result in a considerably weaker interaction in the transition state. Mutation of Ser31 to Gly increases intrinsic GTPase activity several-fold while retaining GAP sensitivity [44], whereas the corresponding Gly12→Ser substitution in Ras impairs basal GTPase activity, abolishes GAP sensitivity and is oncogenic [45]. These observations imply that Ser31 acts as a negative determinant of GTPase activity by preferentially stabilizing the active conformation and sterically preventing Gln81 from contacting the equatorial oxygen. In $G_{z\alpha}$ an analogous Ser replaces the otherwise invariant second Gly of the GAGES motif in the P loop of G_α subunits. Mutation of this Gly to either Val or Ser in $G_{i\alpha1}$ impairs catalytic activity, and in the structure of the Gly→Val mutant, the Val sidechain blocks access to the catalytic Gln [46]. A stereochemistry similar to that observed for the interaction of Ser31 with the γ phosphate in Rab3A may also account for the tenfold lower intrinsic GTPase activity of $G_{z\alpha}$ compared to other G_α subunits [47].

Unlike the stereochemically constrained interaction with Ser31, a simple rotation about the C_α–C_β bond would allow the sidechain hydroxyl of Ser53 to maintain or even improve the hydrogen-bonding geometry with the γ phosphate in the pentavalent transition state. Minor backbone readjustments within the inherently flexible switch I region could further optimize the stereochemistry of this interaction. This structural hypothesis is consistent with observed hydrolysis rates in Rab GTPases. Rab3A and Rab5 have similar GTPase activities [17,44] whereas the rate of GTP hydrolysis in Rab7 is 20-fold slower [17]. Both Rab5 and Rab7 have a Ser corresponding to Ser31 in Rab3A. Rab5 also has a Ser corresponding to Ser53; however, Rab7 has an Ala at the equivalent position. The intrinsic GTPase rate for Ras, which lacks both Ser residues, is intermediate between Rab5 and Rab7 [17]. Moreover, mutation of Ser53 to Ala in Rab3a results in a tenfold decrease in GTPase activity [48]. These observations suggest that Ser53 may play a catalytic role by preferentially stabilizing the transition state. Figure 6 illustrates a potential mechanism for GTP hydrolysis in which the γ phosphate serves as a proton acceptor, as has been proposed for G_α subunits [41] and Ras [49]. In this mechanism, Ser31 and Ser53 may also influence activation of the attacking water by modulating the basicity of the γ phosphate.

A partially purified Rab3A-specific GAP activity has been reported [50], and recently several RabGAPs have been cloned. These include a Rab3-specific GAP [51] and two yeast GAPs [52,53] that stimulate the GTPase activity of the yeast Rab homologs Ypt6 and Sec4. However, none of the RabGAPs identified to date share homology with the catalytic domains of RasGAPs or RhoGAPs and the determinants of GAP activity in RabGAPs have yet to be characterized. In G_α subunits, the guanidinium group of a conserved Arg residue stabilizes the transition state by donating hydrogen bonds to both the β–γ bridging oxygen and the equatorial O1γ oxygen [24,41]. Regulators of G-protein signaling (RGS) accelerate GTP hydrolysis by stabilizing the switch

Figure 6

Schematic diagram illustrating a possible mechanism for conversion of the GTP-bound form of Rab3A to the putative transition state for GTP hydrolysis.



regions and inducing minor conformational adjustments that reorient the sidechains of the conserved Gln and Arg residues for optimal interaction with the transition state [54]. Biochemical and structural studies of a Ras–RasGAP–GDP–AlF_3 complex have shown that RasGAP supplies a catalytic Arg residue that stabilizes the transition state in a manner similar to that observed for the intrinsic catalytic Arg in the α subunits of heterotrimeric G proteins [42,55]. An analogous catalytic Arg is observed in the RhoA–RhoGAP–GDP–AlF_4- complex [43].

As shown in Figure 7, access to the γ phosphate in Rab3A is blocked by the sidechains of Phe51, Ser31 and Ser53. Phe51 represents an obvious impediment to the insertion of a catalytic arginine residue. In principle, RabGAPs might easily overcome such a barrier by inducing an open conformation of Phe51 similar to that observed for the analogous Tyr residues in the Ras–RasGAP and RhoA–RhoGAP complexes [42,43]. However, the backbone carbonyl oxygen of the catalytic Arg residue in both RasGAP and RhoGAP approaches quite closely to the conserved P-loop Gly such that any β substituent at this position would sterically disrupt the interaction with the respective GAPs. By analogy, the β carbon of Ser31 in Rab3A would be expected to pose a significant steric barrier to the insertion of potential catalytic residues by RabGAPs. It is possible that RabGAPs employ a different mode of interaction in order to avoid unfavorable interactions with Ser31. Alternatively, RabGAPs might accelerate GTP hydrolysis by reorienting intrinsic residues (Ser31, Ser53 and Gln81) to stabilize the transition state in a manner similar to that of the RGS proteins [54]. Thus, whether RabGAPs share a common mode of action with RasGAPs and RhoGAPs remains an open question.

Figure 7

Space-filling models highlighting the differences between Rab3A–GppNHp and Ras–GppNHp in the nucleotide-binding region. The nucleotide is shown in purple, except for the γ phosphate (white). Residues contacting the γ phosphate in Rab3A are highlighted in yellow, as are the corresponding residues in Ras. Selected oxygen and nitrogen atoms are shown in red and blue, respectively. The γ phosphate is solvent accessible in Ras but completely buried in Rab3A.

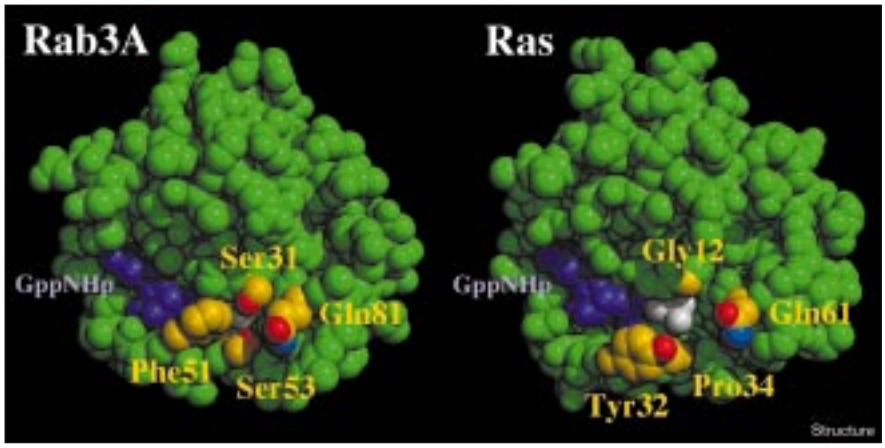
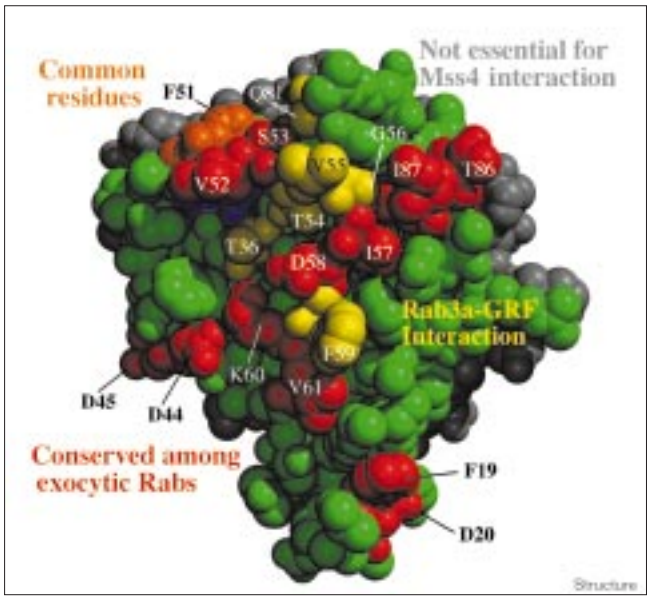


Figure 8

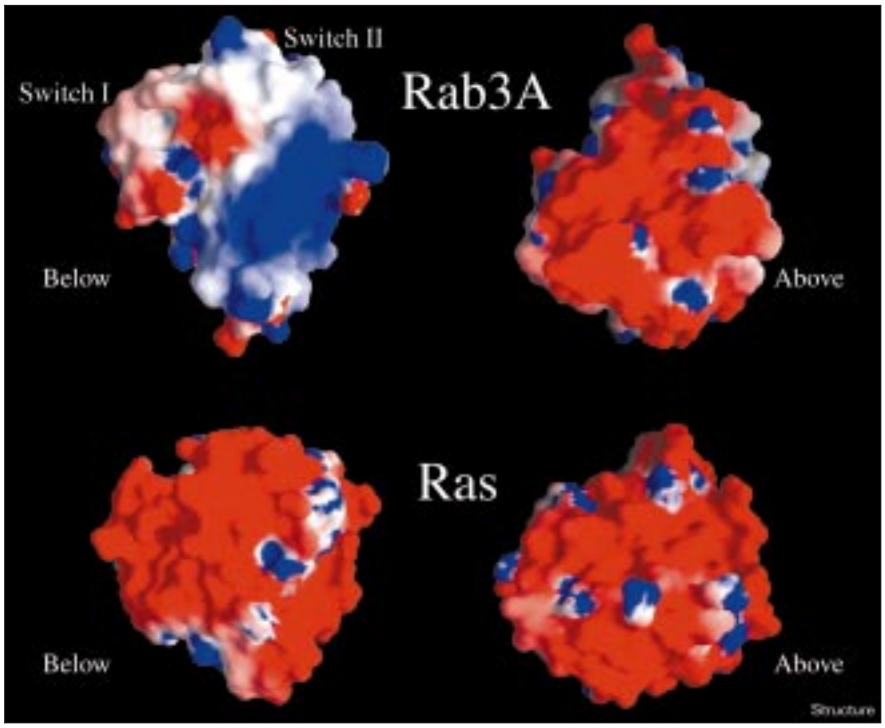


Interaction determinants at the surface of Rab3A. Space-filling model highlighting functionally important and highly conserved residues in Rab3A. Regions not required for binding to the exchange factor Mss4 are shown in gray [56], residues highly conserved among exocytic Rabs are in red and residues involved in the interaction with Rab3A GRF are in yellow. Residues that are both highly conserved and Rab3A-GRF-sensitive are shown in orange. The view is from below with respect to the orientation in Figure 7.

Interactions with membranes, regulators and effectors

Studies of Rab3A–Rab5A chimeras have demonstrated that the N-terminal region of Rab3A is required for interaction with Mss4, a GEF with broad specificity for exocytic Rab proteins [56]. Mutations in the switch I region of Rab3A disrupt interaction with Rab3A-GRF, a partially purified GEF of unknown sequence [57]. Residues implicated in the interaction with Mss4 and Rab3A-GRF are located on a common surface of Rab3A (Figure 8). Many of the residues that are conserved in exocytic Rab GTPases but are variable in endocytic Rabs occupy exposed positions on this surface, suggesting that they are not required for structural integrity but may be important for interaction with other proteins. Two Rab3A-specific effectors (Rabphilin [58] and Rim [59]) have been identified, each interacting with Rab3A via a Zn^{2+}-finger domain. The regions of Rab3A required for effector interaction have not be extensively mapped. Synthetic peptides corresponding to residues 52–67 of Rab3A, roughly analogous to the effector-binding loop in Ras, block vesicular transport [60] and stimulate membrane fusion [61] in different systems. In other monomeric GTPases, the switch I and/or switch II regions are critical for effector binding [621]. Additional regions in G_{α} subunits, such as the α3–β5 loop in $G_{s\alpha}$ [63,64], participate in effector binding and are important determinants of specificity. The high degree of sequence conservation within the switch regions of Rab GTPases suggests that interactions with adjacent variable regions (e.g. the α3/β5

Figure 9



Comparison of the solvent-accessible surfaces of Rab3A–GppNHp and Ras–GppNHp, colored according to electrostatic potential using the program GRASP [72]. The electrostatic potential is contoured in the range from $-3\ k_bT$ (red) to $+3\ k_bT$ (blue) where k_b is Boltzmann's constant and T is the absolute temperature (K). Both molecules are viewed from above and below as defined by the orientation in Figure 7.

loop) will contribute to the specificity of effector binding. The recent crystal structure of Rab3A bound to the effector domain of Rabphilin-3A reveals that both switch regions and the α3–β5 loop of Rab3A mediate the interaction with Rabphilin-3A [65].

The targeting of Rab GTPases to specific intracellular membrane compartments requires isoprenylation of one or two Cys residues at the C terminus [19]. The isoprenyl groups presumably insert into the lipid bilayer, anchoring Rab proteins to the membrane surface. Once associated with a particular membrane compartment, it is not clear whether the GTPase domain is oriented in a particular way with respect to the membrane surface, or simply anchored near the membrane surface via a flexible, hypervariable tether to the isoprenylated C terminus. In contrast to Ras, which exhibits a predominantly negative and uniform charge distribution, the electrostatic potential at the surface of Rab3A is asymmetric (Figure 9). It is plausible that the positively charged and relatively flat surface interacts with negatively charged vesicle membranes, thereby orienting the switch regions, the α3–β5 loop and the known determinants of GEF binding to facilitate interaction with membrane-associated regulators and effectors.

Biological implications

The cycle of activation and inactivation in Rab proteins plays a critical role in the trafficking of vesicles between various intracellular compartments. Basal rates of nucleotide release from Rab GTPases are very slow and their activation by nucleotide exchange is tightly regulated by the catalytic action of guanine nucleotide exchange factors (GEFs). Rab proteins exhibit a wide range of intrinsic GTPase activity, despite conservation of a catalytic Gln residue common to the GTPase superfamily, and can be stimulated by Rab-specific GTPase activating proteins (GAPs). The net rate of GTP hydrolysis serves as a timing mechanism to control the extent of vesicle docking and fusion. The structure of Rab3A reveals the stereochemical determinants that stabilize the active conformation and participate in the mechanism of GTP hydrolysis. Although the overall structure is very similar to that of the active form of Ras, key differences occur in the switch regions and the α3–β5 loop.

Residues conserved throughout the Rab family comprise the primary structural determinants of the active conformation. Like other GTPases, the conformation of the switch regions is coupled to the presence of the γ phosphate by contacts with invariant Thr and Gly residues in switch I and switch II, respectively. In Rab3A, however, the active conformation is further stabilized by an extensive hydrophobic interface involving conserved residues in the switch I and switch II regions. The formation of this interface is facilitated by insertion of a conserved Gly residue in the switch I region and by structural rearrangements involving conserved hydrophobic residues in the switch II region. The high conservation of residues in the switch regions indicates a common mode of activation and suggests that the specificity determinants for interaction with isoform-specific GEFs, GAPs and effectors will reside in other regions such as the α3–β5 loop.

In addition to universal interactions between the guanine nucleotide and invariant residues in the GTPase superfamily, novel contacts with the γ phosphate in Rab3A are mediated by Ser residues that are highly conserved in exocytic Rab GTPases but are variable in endocytic Rabs. The interaction with Ser31 has optimal stereochemistry for stabilization of the ground-state geometry and, consequently, exerts a negative effect on GTPase activity. The stereochemistry of the interaction with Ser53, on the other hand, is not optimal and thus might favor the transition-state geometry. We suggest that the Ser-mediated interactions observed in Rab3A are likely to comprise the primary structural determinants of the large variation in GTP hydrolysis rates within the Rab family. In concert with global structural perturbations, these interactions would modulate the kinetics of GTP hydrolysis to suit the functional requirements and time scale of particular membrane-trafficking steps. Additional mutational studies will be required to test these predictions and determine precisely how the configuration of residues interacting with the γ phosphate regulates intrinsic and GAP-stimulated GTPase activity within the Rab family.

Materials and methods

Expression and purification

A construct (residues 15–186) encompassing the GTPase domain of Rab3A was expressed in *Escherichia coli* using a modified pET15b vector (Novagen) containing an N-terminal 6 x His peptide followed by a thrombin cleavage site. This construct eliminates the hypervariable extensions at the N and C termini. BL21(DE3) cells harboring the modified pET15b plasmid containing Rab3A were grown at 37°C in 6 l of 2 × YT media, induced at an OD_{600} of ~0.6 by addition of 1 mM IPTG and harvested after 3 hours at 25°C. The cells were resuspended in 50 mM Tris, pH 8.0, 0.1% mercaptoethanol, lysed by sonication, centrifuged at 35,000 × g for 1 hour and the supernatant loaded onto a Ni-NTA-agarose column (Qiagen). After washing with ten column volumes of 50 mM Tris, pH 8.0, 500 mM NaCl, 10 mM Imidazole, 0.1% mercaptoethanol, the fusion protein was eluted with a gradient of 10–150 mM Imidazole. The His tag was removed by incubation with a 1:2000 (w/w) ratio of thrombin (Hematologic Technologies) at 4°C for 12 hours in 50 mM Tris, pH 8.0, 2 mM $CaCl_2$, and 0.1% mercaptoethanol. Subsequent incubation with Ni-NTA-agarose eliminated any uncleaved fusion protein. Anion-exchange chromatography on Resource Q (Pharmacia) yielded 90 mg of a preparation that was > 99% pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The GppNHp-bound form of Rab3A was prepared by a method similar to that described for Ras [66]. Rab3A was incubated for 24 hours at room temperature in the presence of 5 mM EDTA, a tenfold excess of GppNHp and 10 units of calf intestinal alkaline phosphatase (CIP) immobilized on agarose beads (Sigma). After supplementing with 10 mM $MgCl_2$, immobilized CIP was removed by filtration through a 0.22 μm filter and GppNHp-bound Rab3A separated from excess nucleotide by gel filtration over Superdex-75 (Pharmacia). Unless otherwise noted, all buffers contained 0.5 mM $MgCl_2$.

Crystallization and data collection

Crystals of GppNHp-bound Rab3A were grown by vapor diffusion in microseeded hanging drops containing equal volumes of protein solution (15 mg ml^{-1} in 5 mM Tris-HCl, pH 7.5, containing 0.5 mM $MgCl_2$) and reservoir solution (14% PEG-8000, 50 mM NaMES, pH 6.5, and 200 mM NaCl) at 4°C. Crystals appeared in 24 hours and grew to maximum dimensions of $0.05 \times 0.1 \times 0.6$ mm over two weeks. The largest crystals diffract beyond 2.0 Å resolution and are in the centered monoclinical space group C2 with cell constants a = 88.9 Å, b = 35.0 Å, c = 58.6 Å, and β = 107.2°. The volume of the unit cell is consistent with one molecule in the asymmetric unit and a solvent content of 44%. Crystals were soaked for five minutes at 4°C in a cryoprotectant-stabilizer solution containing 30% PEG-8000, 50 mM MES, pH 6.5, and 10% glycerol prior to flash freezing in a nitrogen stream. A complete data set was collected at 100K on a 30 cm MAR image plate system, processed with the program DENZO [67] and scaled with the program SCALEPACK [67].

Structure determination and refinement

The structure of GppNHp-bound Rab3A was solved by molecular replacement (MR) [68] using a polyalanine search model derived from the coordinates of GppNHp-bound Ras [22] (PDB code: 5P21; Table 1). A rotation search resulted in a unique solution. A translation search including the top 50 rotation-function solutions also yielded a unique solution with an R value of 49.8% after rigid-body refinement against data from 8 to 3 Å. Initial MR phases were improved by histogram matching using the program DPHASE (G van Duyne) and solvent flipping using the program Solomon (CCP4) [69]. Difference maps calculated with SigmaA weights to reduce model bias revealed poor density for the putative switch I and switch II regions as well as the β4–α3, α3–β5 and β5–α4 loops. After seven rounds of model building and positional refinement, in which poorly defined regions were omitted, clear density for the nucleotide was observed. Following addition of the nucleotide and clearly identifiable sidechains within the hydrophobic core, multiple rounds of simulated annealing and manual fitting were interleaved with gradual extension of the resolution to 2.0 Å. At this point, the phases were sufficiently good to allow the amino acid sequence within the omitted regions to be deduced directly from the maps. Manual fitting of the omitted regions and placement of the remaining sidechains was followed by additional rounds of simulated annealing and positional refinement. Inclusion of 123 water molecules followed by restrained B-factor refinement resulted in a final model with outstanding stereochemistry and low R values (see Table 1). All computations for molecular replacement were conducted with the program Amore (CCP4) [69] whereas subsequent refinement was carried out with the program X-PLOR 3.1 [70]. Interactive model building was performed with the program O [71].

Accession numbers

The atomic coordinates of Rab3A–GppNHp have been deposited in the Protein Data Bank with accession code 3RAB.

Supplementary Material

Figures describing interactions with the nucleotide and a sequence alignment of Rab GTPases are available with the internet version of this paper.

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Figure s1

Interactions between Rab3A and the nucleotide. Schematic illustration of hydrogen-bonding interactions between Rab3A and GppNHp. Residues providing contacts not observed in Ras are highlighted in yellow and the additional hydrogen bonds are in red.

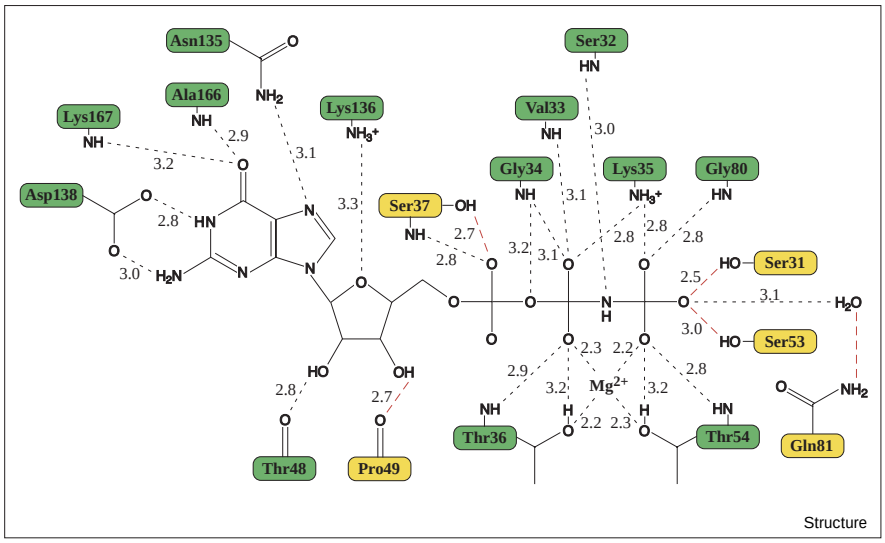
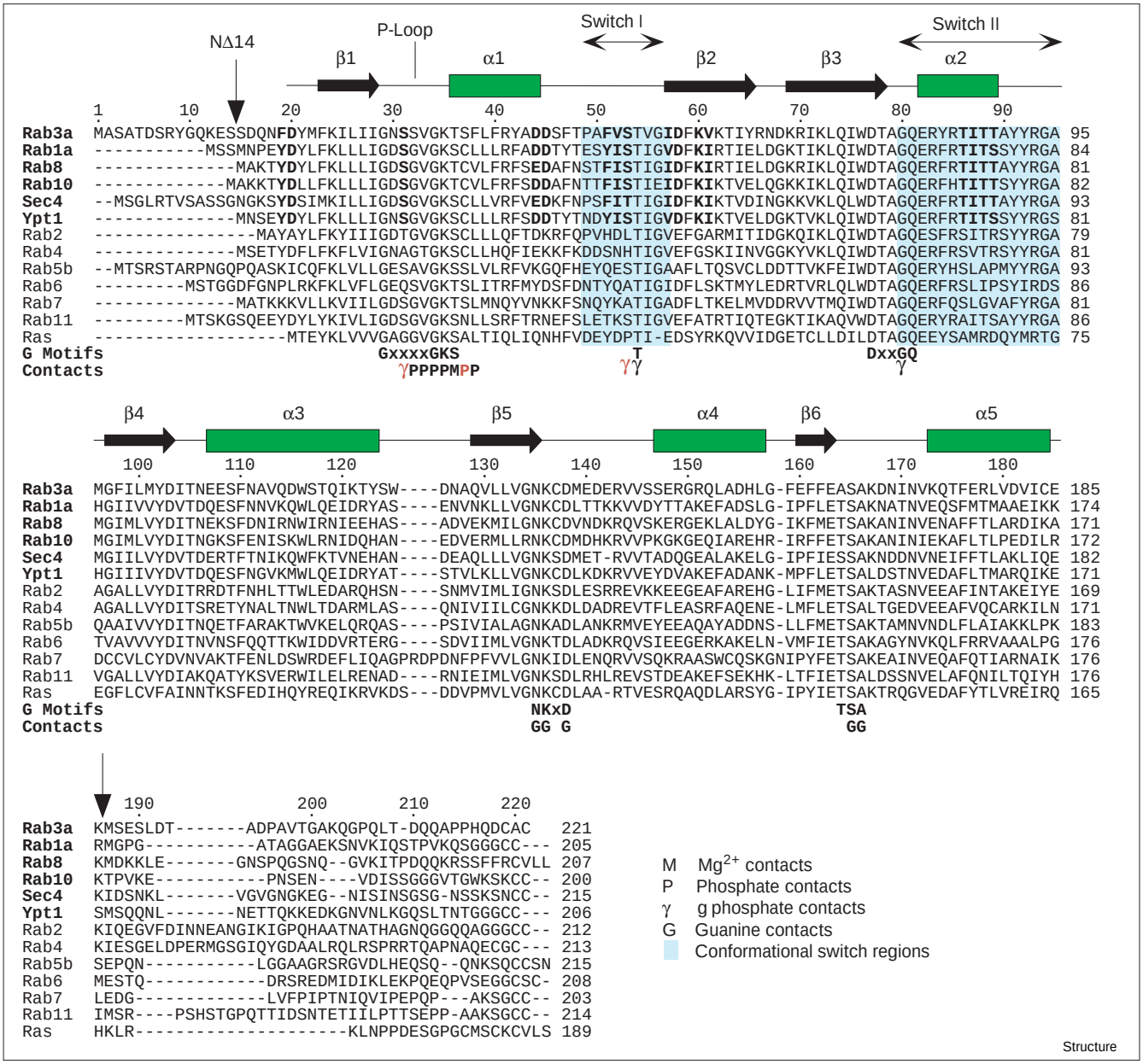


Figure s2



Sequence alignment of Rab isoforms and Ras, with the secondary structure corresponding to Rab3A shown above the aligned sequences. Residues in the putative switch regions are highlighted in light blue. Residues contacting the nucleotide are indicated below the aligned sequences. Additional contacts with the nucleotide (compared with Ras–GppNHp) are highlighted in red. Residues conserved in exocytic (but variable in endocytic) Rab GTPases are shown in bold.