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Crystal structures of the small G protein Rap2A in complex with its substrate GTP, with GDP and with GTPγS

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The small G protein Rap2A has been crystallized in complex with GDP, GTP and GTPγS. The Rap2A–GTP complex is the first structure of a small G protein with its natural ligand GTP. It shows that the hydroxyl group of Tyr32 forms a hydrogen bond with the γ-phosphate of GTP and with Gly13. This interaction does not exist in the Rap2A–GTPγS complex. Tyr32 is conserved in many small G proteins, which probably also form this hydrogen bond with GTP. In addition, Tyr32 is structurally equivalent to a conserved arginine that binds GTP in trimeric G proteins. The actual participation of Tyr32 in GTP hydrolysis is not yet clear, but several possible roles are discussed. The conformational changes between the GDP and GTP complexes are located essentially in the switch I and II regions as described for the related oncoprotein H-Ras. However, the mobile segments vary in length and in the amplitude of movement. This suggests that even though similar regions might be involved in the GDP–GTP cycle of small G proteins, the details of the changes will be different for each G protein and will ensure the specificity of its interaction with a given set of cellular proteins.

Keywords: crystal structure/G proteins/GTP hydrolysis/Rap/Ras

Introduction

Small G proteins form a large family of structurally related proteins which have the essential property of cycling between a GDP- and a GTP-bound form, each recognizing separate sets of cellular partners (reviewed in Boguski and McCormick, 1993). The cycle is used to filter, amplify or time upstream input signals, depending on the spatiotemporal properties of the different subfamilies. The molecular features of the family likeness are well established from crystallographic studies of various small G proteins (reviewed in Hilgenfeld, 1995; Kjeldgaard *et al*., 1996), but the structural differences between the GDP and GTP conformations have been described only for the H-Ras oncoprotein (reviewed in Bourne *et al*., 1991; Valencia *et al*., 1991). The high-resolution structures of H-Ras bound to GDP (Milburn *et al*., 1990) and to the non-hydrolysable GTP analogues GPPNP (Pai *et al*., 1990) and GPPCP (Milburn *et al*., 1990) revealed two polypeptide stretches, termed switch I and II, that take on different conformations according to which nucleotide is bound. The switch I region (residues 30–40) had been mapped genetically as a site for interaction with effectors (Willumsen *et al*., 1986), and the recent crystal structure of the H-Ras homologue Rap1A complexed with the Ras effector Raf has identified the molecular nature of these interactions (Nassar *et al*., 1995, 1996). This region probably constitutes a general docking site for the effectors of small G proteins. The switch II region, on the other hand, is not essential for proper effector recognition, but may interact with guanine nucleotide exchange factors (reviewed in Polakis and McCormick, 1993). As most of these data were obtained for H-Ras, it remains essentially unsettled whether the numerous small G proteins of the Ras family undergo the same GDP/GTP structural transition as H-Ras (Valencia *et al*., 1991).

The H-Ras structures, by locating essential protein–nucleotide interactions, have also been a reference in the longstanding debate on how the GTPase activity of small G proteins works (reviewed in Maegley *et al*., 1996). The rate of hydrolysis of GTP is important for the duration of the association of the G protein with its GTP-specific partners, and it is usually very low (compiled in Zerial and Huber, 1995). The crystal structures of H-Ras define candidate residues for activation of the water molecule that attacks the γ-phosphate of GTP, and for the stabilization of the transition state of the GTPase reaction. It is not known, however, how the GTPase reaction is designed to act as a timer and/or wait for interactions triggered by GTPase-activating proteins (GAPs).

Rap proteins, which include Rap1A, Rap1B, Rap2A and Rap2B, have ~50% sequence identity with Ras proteins (reviewed in Bokoch, 1993). Rap1 was independently cloned by sequence homology (Pizon *et al*., 1988) and by its ability to revert the transformed phenotype of v-Ki-Ras-transformed fibroblasts (Kitayama *et al*., 1989), from which it was hypothesized that Rap1 may compete for binding to downstream targets of Ras. Although the physiological function of the Rap proteins is still unknown, it is now established that Rap1 binds to several Ras targets including p120–GAP (Frech *et al*., 1990), and the Ras effectors Raf and RalGDS (Herrmann *et al*., 1996). Indeed, the docking site for the Ras-binding domain of Raf (RafRBD) at the surface of Rap1A differs from that of H-Ras by only two amino acids (Nassar *et al*., 1995). However, the intrinsic GTPase of Rap1A is not activated by p120–GAP (Frech *et al*., 1990) and Rap1A does not activate c-Raf (Nassar *et al*., 1996). Although the physiological relevance of this competition is not estab-

lished, it accounts well for the 'anti-Ras' effect of Rap1 under overexpression set-ups. Such effects are not observed with the other member of the family, Rap2 (Jimenez *et al*., 1991): despite its identity of ~60% with Rap1, its overexpression is unable to antagonize the growth-promoting effects of Ras. Why Rap1 and Rap2 have differential pattern of cross-interactions with Ras targets is presently unexplained.

We report here a comprehensive crystallographic study of the GDP/GTP cycle of the small G protein Rap2A, which was crystallized with its natural ligand GTP, with GDP and with the GTP analogue GTPγS. The GTP complex is the first report of the high-resolution structure of a G protein with its true substrate. GTP interacts with the G protein in a different way than previously reported for GTP analogues, including the Rap2A–GTPγS complex. On the other hand, the comparison of the GDP/GTP structures of Rap2A with their Ras counterparts shows that Rap2A, in the context of the usual overall fold of G domains, undergoes conformational changes of different amplitude and location from those of H-Ras. Altogether, the Rap2A structures provide a new framework for discussing the GTPase mechanism and the structural switches that allow the segregation of GDP- from GTP-specific partners by small G proteins.

Results

Crystallization and structure determination of the Rap2A–GTP complex

Recombinant Rap2A was purified from *Escherichia coli* essentially in the GDP-bound form, which yielded monoclinic crystals of Rap2A–GDP with freshly purified protein. Under these conditions, protein stocks that had been stored for several months at –80°C yielded hexagonal crystals of Rap2A–GTP without any attempt to exchange GTP for GDP. The nucleotide content of the protein stock was tested by ion exchange chromatography, and revealed the presence of GMP, GDP and GTP in equal amounts. Reproducible crystals were indeed obtained by loading GTP onto Rap2A with the method used for GTPγS. Rap2A–GTP crystals are stable for at least 1 week at 4°C, after which they begin to melt and are progressively replaced by monoclinic Rap2A–GDP crystals. The presence of nucleotides other than GDP may be attributed to the 2 GDP ↔ GMP + GTP spontaneous equilibrium, or to the presence of trace amounts of an *E.coli* contaminating enzyme capable of catalysing this reaction, thereby regenerating GTP and stabilizing the crystals. The same causes were suspected for the presence of ADP and fructose-1,6-bisphosphate in crystals of phosphofructokinase grown with ADP and fructose-6-phosphate (P.Evans, personal communication).

At a wavelength of 1 Å on the synchrotron beam lines, Rap2A–GTP crystals were stable enough for the collection of a complete data set. However, we found that the quality of the electron density map improved with diffraction amplitudes merged from two crystals that were exposed to X-rays for no more than 3 h, keeping GTP hydrolysis to a minimum. The stability of the GTP conformation during data collection is probably due to the low intrinsic GTPase rate of Rap2A (2×10^{-3}/min at 37°C; Lerosey *et al*., 1991), and possibly to an inhibitory effect arising from crystal packing forces.

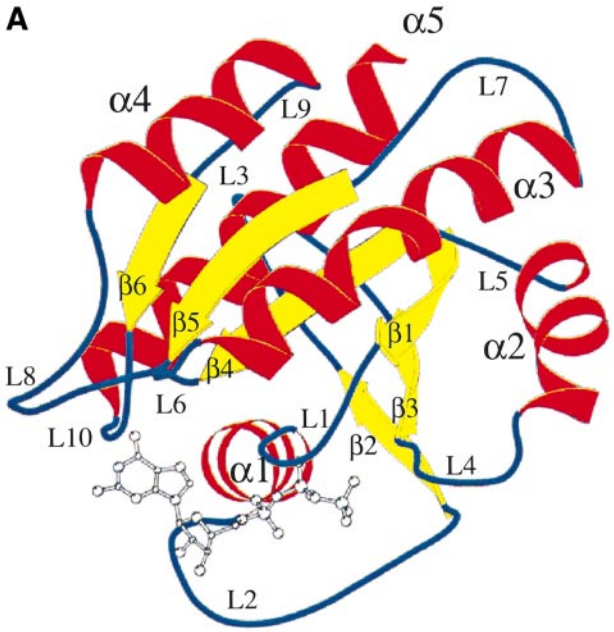
Structure of Rap2A–GTP and comparison with Rap2A–GTPγS

An overall view of the structure is shown in Figure 1A. The Rap2A–GTP electron density map unambiguously shows the GTP nucleotide (Figure 2). The switch I and switch II regions, defined by analogy to H-Ras as residues 30–37 and 60–75, are well defined. The ligands of the γ-phosphate of GTP are the hydroxyl groups of Tyr32 and Thr35, the amide nitrogen of Gly60, the NH_3^+ of Lys16 and the Mg^{2+} ion (Figure 3). Whereas most of these ligands are seen in other triphosphate structures of small G proteins, the Tyr32–γ-phosphate distance has never been observed closer than 3.9 Å in H-Ras–GPPCP (Milburn *et al*., 1990) or 4.3–4.8 Å in the Rap1A–GPPNP–RafRBD complexes (Nassar *et al*., 1995, 1996; Geyer *et al*., 1996), this being too long for a direct hydrogen bond. In Rap2A–GTP, the hydroxyl group of Tyr32 is located at 3.0 Å from a γ-phosphate oxygen and protects it from contact with the solvent. Its configuration is stabilized by a hydrogen bond with the amide nitrogen of Gly13, and by the packing of its aromatic ring on the ring of Pro34 (Figure 4). The nitrogen of Gly13 also forms a hydrogen bond with the oxygen that bridges the β- and γ-phosphates. In the crystal structures of H-Ras and Rap1A with the GTP analogues GPPNP or GPPCP, the replacement of this oxygen by NH or CH_2 prevents the existence of the Gly13 hydrogen bond.

Crystals of Rap2A–GTPγS, whose structure we also report at 3 Å resolution, are isomorphous to the Rap2A–GTP crystals. Both structures are essentially similar, except that Tyr32 no longer forms a hydrogen bond with the corresponding atom of the γ-thiophosphate, which is probably the sulfur atom (Figure 5). The Tyr32–γ-thiophosphate distance is 7.2 Å. The conformation of switch I and II and their interaction with the nucleotide are otherwise identical. Thus, the Tyr32–γ-phosphate hydrogen bond forms only in the presence of the true ligand, but is probably not essential for the triphosphate conformation of Rap2A nor for proper binding of the nucleotide. In particular, the position of Thr61 compares well between the GTP and GTPγS complexes. The equivalent Gln61 in Ras is a site of oncogenic mutations, most of which impair catalysis (Der *et al*., 1986). Indeed, mutation of Thr61 into Gln in Rap1A restores a GTPase rate similar to that of Ras (Frech *et al*., 1990). The crystal structure of H-Ras–GPPNP suggests that Gln61 may either position or polarize the water molecule that attacks the γ-phosphate. In Rap2A–GTP, the Oγ of Thr61 is located 6 Å from the γ-phosphate, and there is no density for a water molecule that would be positioned for nucleophilic attack.

The GDP/GTP structural cycle of Rap2A

In order to describe the GDP/GTP cycle of Rap2A, we have also determined the structure of its GDP-bound form. The 1.7 Å resolution electron density of the Rap2A–GDP complex is clear for the protein, GDP, Mg^{2+} and 76 water molecules, plus a second Mg^{2+} site at a crystal interface (Figure 6). However, several residues in the switch regions I and II are disordered. The chain trace is unambiguous for residues 32–36 in the switch I, but their side chains



B

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                ß1          α1          ß2          ß3          α2          ß4      α3
rap2A  MREYKVVVLGSGGVG KSALTVQFVTGTFIE KYDPTIEDFYRKEIE VDSSPSVLEILDTAG TEQFASMRDLYIKNG QGFILVYSLVNQQSF  90
rap1A  MREYKLVVLGSGGVG KSALTVQFVQGIFVE KYDPTIEDSYRKQVE VDCQQCMLEILDTAG TEQFTAMRDLYMKNG QGFALVYSITAQSTF  90
cHras  MTEYKLVVVGAGGVG KSALTIQLIQNHFVD EYDPTIEDSYRKQVV IDGETCLLDILDTAG QEEYSAMRDQYMRTG EGFLCVFAINNTKSF  90
       1           15 16          30 31          45 46          60 61          75 76          90

                α3          ß5          α4          ß6          α5
rap2A  QDIKPMRDQIIRVKR YEKVPVILVGNKVDL ESEREVSSSEGRALA EEWG-CPFMETSAKS KTMVDELFAEIVRQM NYA     167
rap1A  NDLQDLREQILRVKD TEDVPMILVGNKCDL EDERVVGKEQGQNLA RQWCNCAFLESSAKS KINVNEIFYDLVRQI NR      167
cHras  EDIHQYREQIKRVKD SDDVPMVLVGNKCDL -AARTVESRQAQDLA RSYG-IPYIETSAKT RQGVEDAFYTLVREI RQH     166
       91          105 106         120 121         135 136         150 151         165
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Fig. 1. Sequence and secondary structure of Rap2A. (**A**) Secondary structure of Rap2A–GTP. Figures 2, 4, 5, 7, 8, 9 and 10 are close-up views in the same orientation. (**B**) Sequence alignment of Rap2A, Rap1A and H-Ras. Amino acids of Rap2A that differ from Rap1A are shown in bold characters, those that differ from Ras are underlined. α-helices and β-strands (defined with DSSP) are shown as hatched and open boxes respectively.

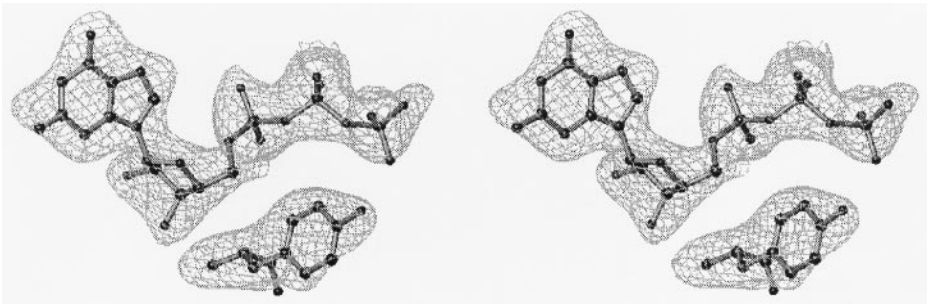

Fig. 2. $|F_o|-|F_c|$ electron density map of Rap2A–GTP at 2.5 Å resolution with GTP and Tyr32 omitted from the calculation, showing that the γ-phosphate of GTP and the position of Tyr32 are clearly identified in the electron density.

have weak densities, in particular Ile36. This region runs close to, but does not interact with, the GDP phosphates nor with Mg^{2+}. In this conformation, Tyr32 and Thr35 point towards the solvent. On the other hand, there is no interpretable density for residues 60–63, which form the N-terminal part of the switch II region. The rest of switch II forms a regular α-helix from Phe64 to Gly75, labelled α2, whose density is well-defined.

The GDP/GTP cycle of Rap2A can be analysed by comparing its GDP and GTP complexes (Figure 7). The γ-phosphate of GTP stabilizes an ordered conformation at the switch I and switch II regions. Tyr32 and Thr35 flip to the inside, while Ile36 is now exposed to the solvent. There is little movement of the Mg^{2+} ion as it gains an interaction with the γ-phosphate. The switch II region, in addition to forming an organized structure at residues 60–63, also rearranges helix α2 into a 3_{10} helix from Thr61 to Ser66, while the α-helix from Met67 to Gly75 is retained. The orientation of the α-helix at residues 67–75 is unaffected by the GDP/GTP alternation, and retains its

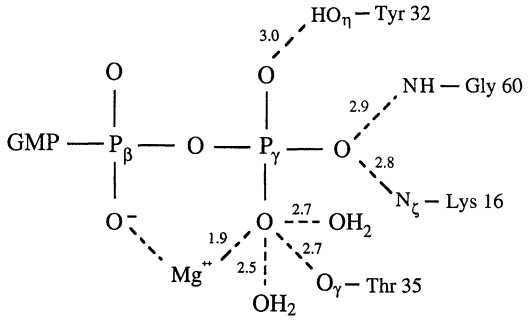

Fig. 3. Interactions of Rap2A with the γ-phosphate of GTP.

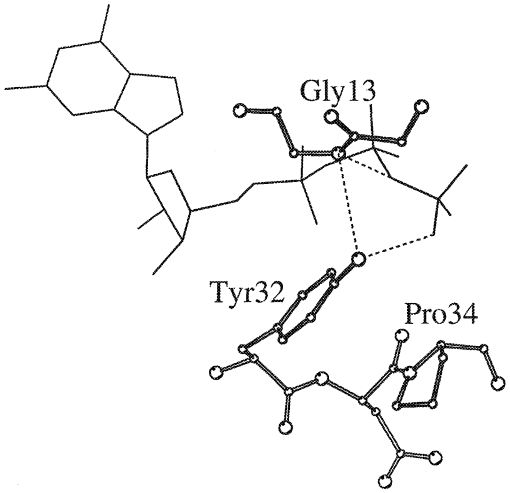

Fig. 4. The network of hydrogen bonds (dashed lines) at Tyr32, Gly13 and GTP. The nucleotide is shown as lines, protein atoms in ball-and-stick. Gly13 interacts with the β–γ bridge oxygen and Tyr32 with a γ-phosphate oxygen

packing on helix α3. The response of Rap2A to GTP binding is thus characterized by a disorder-to-order transition at residues 32–36 (switch I) and 60–63 (switch II), probably driven by the interactions of Tyr32, Thr35 and Gly60 with the γ-phosphate of GTP, and by a rearrangement of the helical conformation from residues 64–66 of helix α2. An eventual influence of crystal packing forces on the overall conformation of switch II cannot, however, be ruled out, since helix α2 forms lattice contacts in both crystal forms. In addition to these changes, Rap2A–GDP and Rap2A–GTP also differ in the L3 loop, which undergoes a rigid body movement of ~2 Å amplitude from residues 47 to 51. The L3 loop connects two β-strands, one of which follows switch I while the other precedes switch II, but it is not in direct contact with the guanine nucleotide. If its structural change is a consequence of GTP binding, it is thus indirect.

Comparison of Rap2A with H-Ras and Rap1A

Our set of structures provides the first opportunity to determine to what extent the features of the structural switch of H-Ras are common to other small G proteins. Rap2A is related to H-Ras by 46% sequence identity (Figure 1B). The regions where Rap2A and H-Ras have diverging sequences, essentially loops, display different conformations, but outside switch I and switch II these regions are not affected by the GDP/GTP alternation.

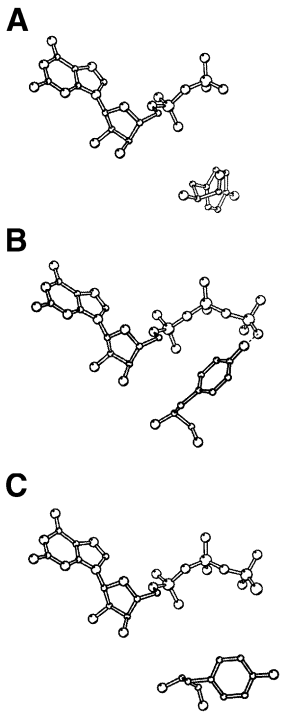

Fig. 5. The position of Tyr32 relative to the nucleotide in (**A**) Rap2A–GDP, (**B**) Rap2A–GTP and (**C**) Rap2A–GTPγS. The hydrogen bond of Tyr32 to the γ-phosphate of GTP is in dashed lines. Tyr32 is exposed to the solvent in the Rap2A–GDP complex, and its distance to the sulfur of GTPγS is 7 Å.

Both H-Ras and Rap2A undergo structural transitions at switch I and switch II and a small amplitude movement at loop L3 upon binding GTP (or a GTP analogue). Rap2A differs from H-Ras by three residues in switch I, but both proteins have Glu62, Met67, Arg68 and Asp69 in common in switch II. In the GDP structures, the disordered regions extend to the same residues: 31–36 in switch I, 60–63 in switch II. The switch I regions are essentially similar, except that the ribose 2′-hydroxyl of the nucleotide forms a hydrogen bond to the main chain carbonyl of Glu30 in Rap2A, while it is exposed to the solvent in H-Ras. Binding of GTP organizes an ordered conformation at switch I that is comparable for both proteins, with Thr35 binding to the γ-phosphate and to Mg^{2+}. The additional interaction of Tyr32 with the γ-phosphate that we see in Rap2A–GTP is not present in the complexes of H-Ras with GTP analogues, but we expect that it also exists in the H-Ras–GTP complex (see Discussion). Switch II residues 60–63, at the end of loop L4, undergo a disorder-to-order transition in both proteins, which brings the conserved Gly60 in contact with the γ-phosphate. Surprisingly, the structural rearrangements beyond residue 61 are markedly different (Figure 8). In H-Ras, the conformational change extends from residues 64 to 75, with a rigid body motion of helix α2 which rotates by over 60° perpendicular to its axis. The hinge is located near Gly75, a residue that both proteins have in common. In Rap2A, helix α2 does not rearrange beyond residue 66. In the GDP form, the Cα–Cα distances between H-Ras and Rap2A amount to 10 Å in switch II. These discrepancies vanish in the triphosphate conformations as the rotation of helix α2 in H-Ras superimposes it onto helix α2 of Rap2A. Yet the 3_{10} helix of residues 62–66 of Rap2A is

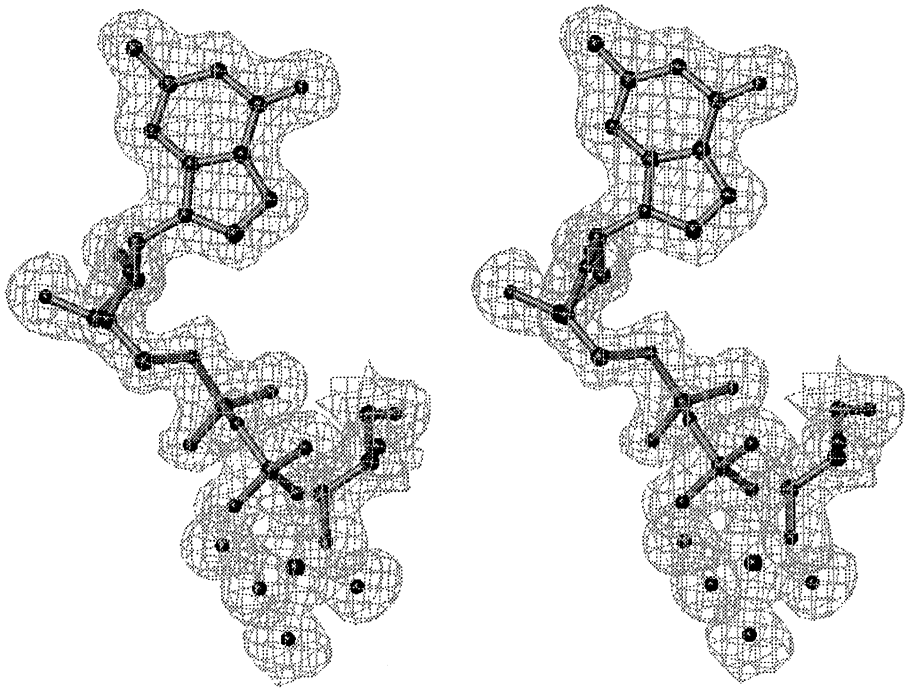

Fig. 6. $2|F_o|-|F_c|$ electron density map of Rap2A–GDP at 1.7 Å resolution, showing the GDP nucleotide, Mg^{2+} and its ligands Ser17 and four water molecules.

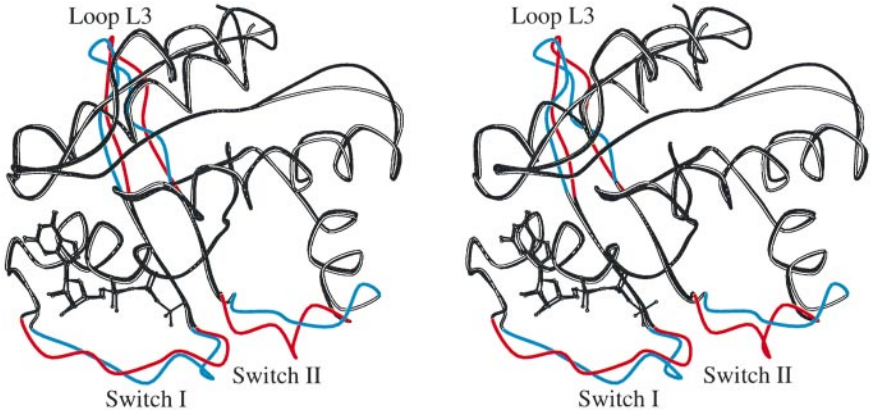

Fig. 7. The GDP/GTP structural changes of Rap2A. Rap2A–GDP is shown in white with the regions that undergo structural changes upon GDP/GTP alternation in blue. The corresponding regions of Rap2A–GTP are shown respectively in black and red.

not present in H-Ras. Thus, although the GDP/GTP structural changes of Rap2A and H-Ras are located in similar regions and the triphosphate structures display close similarities, Rap2A and H-Ras differ markedly in their GDP conformations, especially at switch II, which undergoes a smaller motion and is nine residues shorter in Rap2A than in H-Ras.

The structure of Rap2A–GTP can be further compared with that of Rap1–GPPNP in the complex with RafRBD. In contrast to Rap1A, Rap2A is unable to exert a Ras-antagonist activity (Jimenez *et al*., 1991). Rap1A and Rap2A carry two substitutions in the docking site for RafRBD as compared with H-Ras, Glu30Asp and Lys31-Glu. Replacement of these two residues in Rap1A with their corresponding amino acids in H-Ras endows the mutant protein with the ability to activate ERK signalling (Nassar *et al*., 1996). Rap2A has only one additional substitution, Ser39Phe. Whether the effect in signalling can be attributed to these differences in sequence can be investigated by comparing the binding site for RafRBD on Rap1A to its counterparts in H-Ras–GPPNP and Rap2A–GTP. Not surprisingly, H-Ras–GPPNP fits readily in the RafRBD interface, but this is also true for Rap2A. Ser39, in Rap1, forms a hydrogen bond with the main chain of RafRBD, which cannot be established by Phe39 in Rap2A. Yet Phe39 can be accommodated in the interface by a small rotation of its side chain. This interaction seems to have limited functional consequences, since a Ser39Phe substitution in oncogenic Ras only reduces its transforming ability by one order of magnitude (J.de Gunzburg, unpublished observation). Thus, from the comparison of the triphosphate structures at the RafRBD interface, Rap2A appears very close to both H-Ras and Rap1A. The present study shows that the structural differences between Rap2A and H-Ras are larger in the diphosphate conformations. This may also be the case between

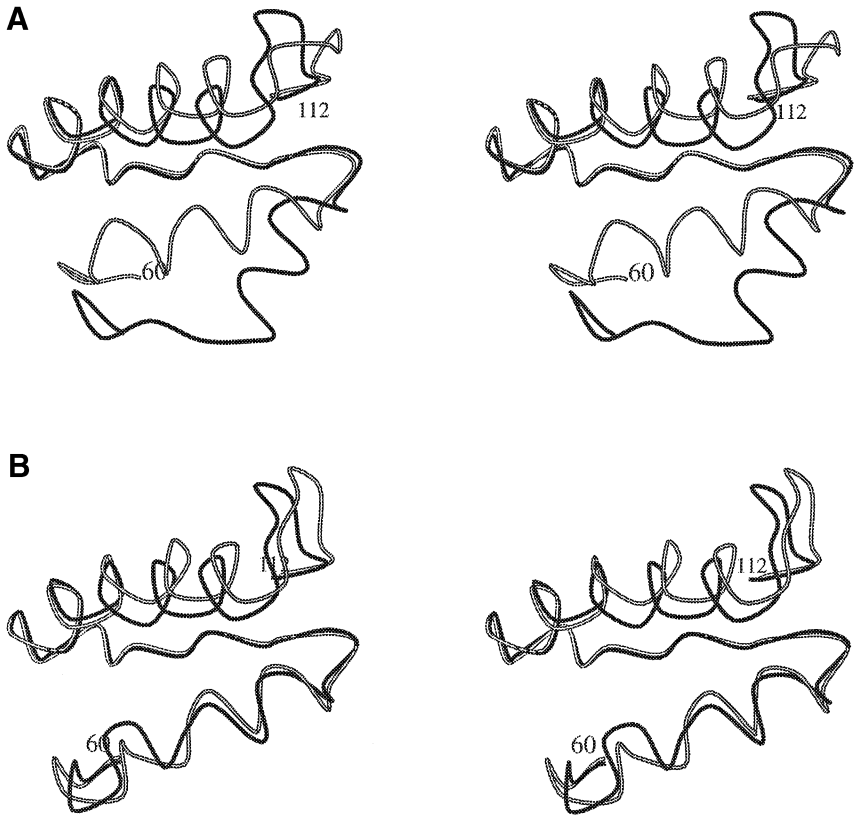

Fig. 8. The GDP/GTP structural changes at switch II in Rap2A (white) and H-Ras (black) from residues 60 to 112 (see Figure 1B for secondary structure elements). Comparison of (**A**) Rap2A–GDP with H-Ras–GDP and (**B**) Rap2A–GTP with H-Ras–GPPNP. Helix α2 has a different orientation in the GDP structures, but the triphosphate conformations are very similar. The shift at helix α3 is probably a consequence of the dissimilar GDP/GTP transitions at switch II.

Rap2A and Rap1A, suggesting that, besides non-structural features, such as their subcellular localization (Béranger *et al*., 1991), or other effector-binding regions, such as the as yet unidentified region that interacts with the zinc finger domain of Raf (Luo *et al*., 1997), differences between their GDP forms may contribute to their functional differences via the interaction with GDP-specific partners, for instance exchange factors.

Discussion

We have determined the structures of Rap2A in complex with GTP, GDP and GTPγS, from which we analysed the GDP/GTP structural changes and, for the first time, the interactions of a small G protein with its natural substrate GTP. Our structures show that the GDP/GTP conformational changes are located at switch I and switch II as previously described for H-Ras, but the mobile segment in switch II is nine residues shorter and the amplitude of its movement markedly smaller than in H-Ras. On the other hand, the Rap2A–GTP structure resembles the Rap2A–GTPγS complex and previously determined structures of H-Ras, but it displays a novel interaction of Tyr32 with the γ-phosphate of GTP and with Gly13. The results we have obtained for Rap2A on the GDP/GTP switch and the Tyr32–GTP interaction can now be discussed in the context of the superfamily of small G proteins.

Switch II may be responsible for the discrimination of GDP-specific partners

The ability to segregate 'upstream' from 'downstream' partners by means of their dissimilar GDP and GTP structures is the hallmark of proteins bearing G domains. In addition to the present structures of Rap2A, GDP and GTP forms are available for H-Ras, for the heterotrimeric G-proteins transducin-α and Giα, and for the bacterial elongation factor EF-Tu (Kjeldgaard *et al*., 1996 and references therein). All these proteins undergo structural transitions at their switch I and switch II regions. Switch I has proved to be a general structural sensor ensuring the GTP-specific recognition of small G proteins by their effectors, and in many cases mutations in this region interfere with their proper function. Switch I indeed forms the central part of the docking site for $tRNA^{Phe}$ on EF-Tu–GPPNP (Nissen *et al*., 1995), for RafRBD on Rap1A–GPPNP (Nassar *et al*., 1995, 1996) and for RGS4 on Giα–GDP–AlF_4^- (Tesmer *et al*., 1997).

Switch II, at least beyond residues 60–63, is generally not critical for the interaction with effectors. It is involved in protein–protein interactions in the complex of EF-Tu with its nucleotide exchange factor EF-Ts (Kawashima *et al*., 1996), and between Gα–GDP and the β subunit (Wall *et al*., 1995; Lambright *et al*., 1996). In Ras proteins, mutations of this region impair the recognition of guanine nucleotide exchange factors (reviewed in Polakis and McCormick, 1993). The comparison of the GDP/GTP

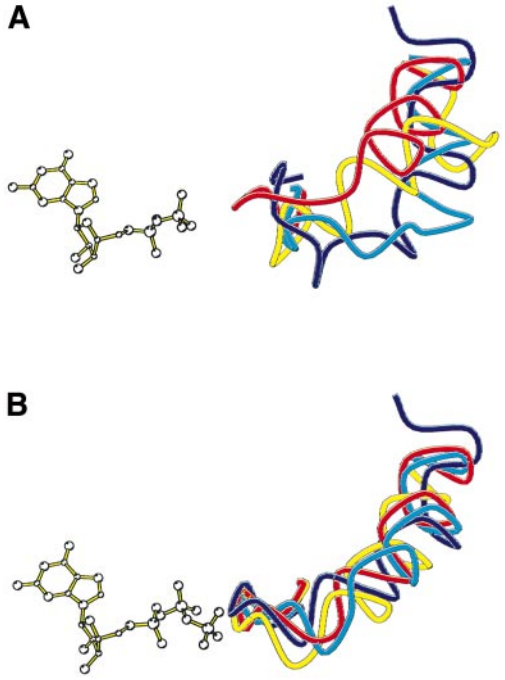

Fig. 9. The GDP/GTP structural transition at switch II in Rap2A (red), H-Ras (blue), transducin α (green) and EF-Tu (yellow). The orientations correspond to an overall superposition of the G domains onto Rap2A–GTP, excluding switch I and switch II. (**A**) The GDP complexes (H-Ras–GDP, file 4q21; transducin α-GDP, file 1tag; EF-Tu–GDP, file 1etu). (**B**) The triphosphate complexes (H-Ras–GPPNP, file 5p21; transducin α-GTPγS, file 1tnd; EF-Tu–GPPNP, file 1eft). The GDP complex of EF-Tu is from *E.coli* and the GPPNP complex from *Thermophilus aquaticus*, but their switch II sequences are identical. The GDP to GTP motion of the switch II helix is different in all structures, but their GTP conformations are very similar. In Rap2A, the helix retains the same orientation in the GDP and GTP complexes; in H-Ras it undergoes a rigid body motion perpendicularly to its axis coupled with the unwinding of its N-terminus (Stouten *et al*., 1991), a movement that also describes the transition at switch II in EF-Tu (Polekina *et al*., 1996). In Gα proteins, the corresponding helix rotates around its own axis but has no hinge motion (Noel *et al*., 1993; Coleman *et al*., 1994; Lambright *et al*., 1994; Sondek *et al*., 1994).

structural changes from available crystal structures suggests that switch II may consist of two subregions. One is directly related to the binding/hydrolysis of GTP, including Gly60 and two or three following residues, and the second is helix α2. In the GDP-bound forms, the orientation of helix α2 relative to the overall fold of the G domain varies markedly from one protein to the other (Figure 9A). On the other hand, its axis and position coincide in all triphosphate complexes (Figure 9B), as was previously observed by Berghuis *et al*. (1996). This variability may be a general property of helix α2 in switch II of G domains, and may be involved in the recognition of their GDP-specific partners. The consensus configuration of helix α2 in the GTP form would then promote the dissociation of GDP-specific targets and prevent their subsequent binding as long as the GTP state is maintained. The existence of two switch regions, each dedicated to the recognition of one set of partners and the segregation of the other, may ensure an optimal signal-to-noise ratio in the response of G proteins to cellular stimulations. Candidate GDP-specific partners of small G proteins are guanine nucleotide exchange factors (Boguski and McCormick, 1993), guanine dissociation inhibitors (Wu *et al*., 1996) and lipid transferases (Sanford *et al*., 1993; Schiedel *et al*., 1995). It is of course likely that the binding site for GDP-specific partners extends beyond helix α2, thus providing additional affinity and/or specificity regardless of the nucleotide state.

Interaction of Tyr32 with the γ-phosphate of GTP in small G proteins

Up to now, the GDP/GTP conformational cycle of small G proteins has been inferred only from structures with GTP analogues such as GPPNP, GPPCP or caged GTP. Our study shows that the complex with the natural ligand GTP is similar to the complex with GTPγS, and thus justifies most conclusions obtained from previous structures of G proteins with GTP analogues. However, the structure of Rap2A with GTP reveals a novel interaction of Tyr32 with an oxygen of the γ-phosphate of GTP and with the amide group of Gly13. In the GTPγS complex, Tyr32 is shifted away from the corresponding atom. The Rap2A–GTP and Rap2A–GTPγS complexes are otherwise identical, and form isomorphous crystals. Thus, the interaction of Tyr32 with GTPγS is prevented solely by the presence of the γ-sulfur, but the conformation of switch I and II is similar whether GTP or a GTP analogue is bound. GTPγS probably introduces a different charge distribution on the γ-(thio)-phosphate and/or modifies the polarizability of the β–γ P–O bonds as compared with GTP. This is probably also true for other analogues, and may account for the shifted position of Tyr32 in previously reported H-Ras and Rap1A triphosphate complexes. Some conclusions obtained with GTP analogues, in processes where Tyr32 would be a critical component, might therefore be questioned. The structure of H-Ras–GTP, investigated before in Laue experiments using caged GTP, did not identify the Tyr32–GTP hydrogen bond (Schlichting *et al*., 1990). The method, however, has important limitations due to the incompleteness of the diffraction data and to structural changes associated with the binding of the caged compound (Scheidig *et al*., 1995). The two different configurations of Tyr32 in Rap2A–GTP and Rap2A–GTPγS may correspond to the two configurations of H-Ras suggested by ^{31}P NMR in the presence of GPPNP, while only one was detected with GTP (Geyer *et al*., 1996). It is thus likely that in H-Ras and Rap1A also, Tyr32 forms a similar interaction with the γ-phosphate of GTP. Together with Ras and Rap, the small G proteins Rho, Ran and several Rabs have an equivalent in their sequence to tyrosine Tyr32. We surmise that their tyrosine also forms a hydrogen bond with GTP.

The interaction of Tyr32 with GTP suggests that its general conservation in small G proteins may have structural or functional advantages. Tyr32 is located three amino acids upstream of a threonine (Thr35 in Ras) which is extremely conserved in most G domains. The α subunits of heterotrimeric G proteins have a conserved arginine equivalent in sequence to Tyr32, which was shown from crystal structures to interact with the γ-phosphate of GTPγS (Noel *et al*., 1993; Coleman *et al*., 1994), and with the transition state analogue AlF_4^- in the presence of GDP (Coleman *et al*., 1994; Sondek *et al*., 1994). This arginine was suggested to stabilize the transition state of the GTPase reaction, and indeed its mutation to Cys or His

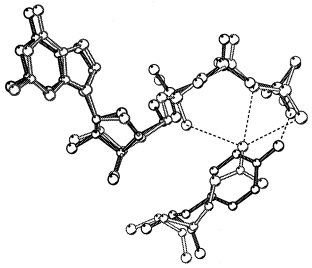

Fig. 10. Comparison of Tyr32 in Rap2A–GTP (black) and Arg174 in transducin α-GTPγS (white, PDB file 1tnd). Tyr32 and Arg174 occupy a similar location relative to the nucleotide, and both form a hydrogen bond to the γ-phosphate of the guanine nucleotide, but only Arg174 interacts with the β–γ bridging oxygen.

reduces the GTPase activity of Gs (Landis *et al*., 1989). Because small G proteins lack this arginine, it was proposed that their lower GTPase rates were due to the impossibility of forming such an interaction (reviewed in Maegley *et al*., 1996). The Rap2A–GTP structure rules out this hypothesis, since Tyr32 occupies a location relative to GTP and forms a hydrogen bond with the γ-phosphate equivalent to that of Arg174 in transducin-α (Figure 10). The tyrosine of small G proteins and the arginine of trimeric G proteins may therefore play similar roles in GTP binding and/or hydrolysis. However, although tyrosine residues can in principle stabilize negative charge build-up in transition states, the mutation of Tyr32 into Phe, which abolishes the hydrogen bond, leaves the intrinsic GTPase of Ras essentially unchanged and decreases its activation by GAP only moderately (Yamasaki *et al*., 1994). In addition, whereas mutations of the arginine are oncogenic in certain trimeric G proteins (Landis *et al*., 1989), Tyr32 has not been reported as an oncogenic site in small G proteins. Thus, although the hydrogen bond to the γ-phosphate can form equally well with the tyrosine or the arginine, further differences between these residues must be investigated.

A first difference concerns the GDP/GTP rearrangements at these residues. In Gαs, the arginine interacts with the β-phosphate of GDP, and does not have far to move to bind to the γ-phosphate of GTP. In contrast, Tyr32 flips outside-in on alternating the GDP and GTP conformations. The energetic cost of this movement may therefore moderate its contribution to catalysis. On the other hand, Tyr32 in Rap2A–GTP forms another hydrogen bond with the amide of Gly13, an interaction that the guanidinium of Arg174 cannot establish. Both Arg174 in transducin and Gly13 in H-Ras interact with the oxygen that bridges the β- and γ-phosphates, and have recently been proposed to stabilize the negative charge that develops on this oxygen at the transition state (Maegley *et al*., 1996). In Rap2A–GTP, Gly13 is within distance of forming a hydrogen bond to either Tyr32 or to the bridge oxygen of GTP. Since the amide nitrogen can act as only a single hydrogen bond donor, the presence of Tyr32 may modulate the polarity of the Gly13–GTP interaction. In addition, the Rap2A–GTP maps lack electronic density corresponding to the attacking water molecule, suggesting that Tyr32 may disfavour the binding of the nucleophilic water molecule.

Besides its role in binding and/or hydrolysing GTP, Tyr32 is also important for the quality of the recognition of the GTP-bound form of small G proteins by their partners (Stone *et al*., 1988; Yamasaki *et al*., 1994; Nassar *et al*., 1995; Akasaka *et al*., 1996; Wittinghofer and Nassar, 1996; Li and Zheng, 1997). The Tyr32Phe mutant Ras, for instance, binds to Raf but fails to activate it; on the other hand, it does not recognize Byr2, an effector of Ras in *Schizosaccharomyces pombe* (Akasaka *et al*., 1996). Our results suggest that Tyr32 may also be important for the activation of GTP hydrolysis by GAP. Recent results suggest that p120GAP supplies Ras with active site residues (Mittal *et al*., 1996; Scheffzek *et al*., 1996). The Rap2A–GTP structure shows that Tyr32 covers the γ-phosphate of GTP and precludes a direct contact of a GAP side chain with Pγ oxygens. If the above hypothesis were verified, Tyr32 should therefore be displaced in order to promote a productive GAP association. Various structural approaches, including NMR (Kraulis *et al*., 1994; Geyer *et al*., 1996) and X-ray crystallography (Milburn *et al*., 1990; Pai *et al*., 1990; this work), have indeed confirmed that Tyr32 is a mobile residue (Figure 5). The finding that the hydrogen bond is disrupted when GTP is replaced by GTPγS suggests that it is not very strong, and could thus be easily displaced upon interaction with GAP.

In conclusion, our study has emphasized the importance of elucidating the role of Tyr32 in catalysis, stabilization of the GTP conformation and binding/dissociation of protein targets. New mutational studies at position 32, in particular with smaller amino acids, are now needed to settle these issues. It has also been shown that differences between closely related small G proteins, and all the more so between different families, may affect not only the nature of the chemical groups that they present to their targets, but also the location and the amplitude of the structural switch. This may turn out to be an important parameter limiting the apparent promiscuity of recognition of small G proteins, such as Ras, Rap1 and Rap2, for their potential targets.

Materials and methods

Expression and purification of the Rap2A protein

The Rap2A protein was truncated at residue 167 by site-directed mutagenesis on a single-stranded M13 template by introducing a stop codon with oligonucleotide 5′ CGC GAG TAC TAA GTG GTG GTG. The *Eco*RI–*Hin*dIII fragment containing the Rap2A-coding sequence was introduced into the expression plasmid ptac32 as described (Lerosey *et al*., 1991). The recombinant vector was transformed into competent cells of an *E.coli* K12 strain (JS218) containing plasmid pEMR602 that overexpresses GroEL/GroES chaperones and provides tetracycline resistance (Bergès *et al*., 1996). Expression of Rap2A was induced by IPTG. Cells were harvested by centrifugation, resuspended in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM NaCl, and broken with alumina in 50 mM Tris–HCl, pH 7.5, 5 mM Mg acetate, 1 mM EDTA, 2 mM DTT and 50 μM GDP.

Rap2A was purified to homogeneity in three chromatographic steps, all performed at 4°C. The clear lysate was loaded onto a Matrex Red A column (Amicon) equilibrated with the extraction buffer. Rap2A eluted in the flow-through fraction, which was loaded on an ion-exchange QA Trisacryl column (BioSepra) and extensively washed with the same buffer. The protein was eluted by a linear gradient of 0–0.4 M NaCl. The positive fractions were concentrated and loaded on a gel filtration column of Ultrogel AcA54 (BioSepra). The protein preparation was shown to be pure by the presence of a single band on SDS–PAGE, and was stored at –80°C. The yield of 1 l of bacterial culture was 50 mg of pure protein, as compared with 0.5 mg in the absence of the chaperones.

Table I. Statistics for X-ray structure determination

Data set	Rap2–GDP	Rap2–GTP	Rap2–GTPγS
Space group	$P2_1$	$P6_1$	$P6_1$
Unit cell	$a = 36.8$ $b = 35.3$ $c = 58.3$ $\beta = 106.6$	$a = b = 64.5$ $c = 84.2$	$a = b = 65.4$ $c = 84.5$
Measured reflections	131 709	46 223	26 504
Unique reflections	14 661	8828	4126
Completeness (%)	92.8	98.5	99
Resolution range (Å)	25–1.7	30–2.5	28–3.0
R_{sym} (%)	7.0	6.5	6.8
R-factor (%)	18.3	21.6	18.4
R.m.s.d. bond lengths (Å)	0.008	0.014	0.009
R.m.s.d. bond angles (°)	2.1	2.9	1.6
Average *B* factor (Å^2)	13.0	43	46
No. of water molecules	76	25	2

Crystallization of the Rap2A complexes

Crystals of Rap2A–GDP were obtained without exchanging the nucleotide bound to the protein as purified from *E.coli*. Crystals grew in hanging drops over wells containing 19% PEG 4000, 100 mM $MgCl_2$, 100 mM Tris, pH 7.5, at a protein concentration of 10 mg/ml. Crystals of Rap2A loaded with either GTP or with the GTP analogue GTPγS were obtained after thorough degradation of bound GDP, using the following procedure: 5 mM EDTA was added to chelate the Mg^{2+} ion, then the protein was incubated for 30 min with agarose-coupled alkaline phosphatase to digest GDP. GTPγS, which is not degraded by the phosphatase, was added at the beginning of the incubation, while GTP was added after the agarose beads had been removed by centrifugation. Both nucleotides were used at concentrations of 10–20 mM. Nucleotide exchange was stopped by excess $MgCl_2$, and remaining nucleosides and phosphate were removed by buffer exchange on Microcon 10. Crystals of Rap2A loaded with either GTP or GTPγS were grown by the hanging drop method, with 25% PEG 8000, 100 mM $LiSO_4$, 100 mM Tris, pH 8, in the wells. The nucleotide content of the protein stock was monitored on an FPLC system (Pharmacia) using a 0–1 M NaCl gradient in 100 mM Tris, pH 7.5, on a MonoQ anion exchanger column.

Data collection and structure determination

Diffraction data were collected at 4°C either on a Rigaku X-ray generator with *R*-axis image plates, or at the synchrotron beam lines W32 and W21 of LURE (Orsay) with Mar Research image plates. Intensities were integrated with Denzo and reduced and merged with Scalepack (Otwinowski, 1993). Molecular replacements were done with AMORE (Navaza, 1994). All structures were refined by several rounds of minimization and simulated annealing dynamics with X-plor including a model of bulk solvent (Brünger *et al*., 1989), and graphical building was performed with TURBO (A.Roussel, A.G.Inisan and C.Cambillau, AFMB and BioGraphics, Marseille, France). Maps were computed with the CCP4 programs (1994), using figure-of-merit weighted amplitudes.

Rap2A–GDP crystals belong to space group $P2_1$, with $a = 36.7$ Å, $b = 35.2$ Å, $c = 58.3$ Å, $\alpha = 90°$, $\beta = 106.8°$, $\gamma = 90°$, and contain one molecule per asymmetric unit and 30% solvent. The Rap2A–GDP complex was solved by molecular replacement using diffraction data collected on a single crystal on the laboratory X-ray generator. The search model was a modified H-Ras–GDP structure [Protein Data Bank (PDB) entry code 1q21], in which non-identical side chains (~50%) were turned into alanines. The initial *R*-factor after rigid body refinement was 52%, but the corresponding map clearly showed the classical overall fold with the six-stranded β-sheets, the guanine nucleotide and helices α1 and α4. Most loops, and helices α2, α3 and α5 were either not visible or poorly defined, and were progressively re-built. The structure was first refined to an *R*-factor of 16% at 2.3 Å resolution. Diffraction amplitudes to 1.7 Å were collected later at the synchrotron beam line on two crystals and merged with the Rigaku data, from which the Rap2A–GDP complex was further refined to an *R*-factor of 18.3% at 1.7 Å resolution.

Rap2A–GTPγS and Rap2A–GTP crystals were very sensitive to X-ray damage and did not withstand freezing, thus precluding data collection at the Cu wavelength (1.54 Å). Crystal damage was less critical near 1 Å on synchrotron beam lines, where data could be collected. This sensitivity to X-rays is reflected in the high overall *B* factor. Crystals of Rap2A–GTPγS and Rap2A–GTP are isomorphous and belong to space group $P6_1$, with $a = b = 65.3$ Å, $c = 84.4$ Å, and contain one molecule per asymmetric unit and 53% solvent. Diffraction data for Rap2A–GTPγS were collected on a single crystal. The Rap2A–GTPγS complex was positioned by molecular replacement using the modified H-Ras–GDP complex, but the refinement was done with Rap2A–GDP which had been partially refined in the meantime. The final *R*-factor for the refined Rap2A–GTPγS complex is 18.4% at 3 Å. Diffraction intensities for Rap2A–GTP were collected from two crystals and merged. Since these crystals were isomorphous to the crystals of Rap2A–GTPγS, and were obtained after the Rap2A–GTPγS structure had been refined, the structure could be solved by difference Fourier maps, and refined to an *R*-factor of 21.6% at 2.5 Å resolution. The free *R*-factor was used essentially to monitor the early stages of the molecular replacement procedure, but were not taken into account after additional data were collected at the synchrotron beam lines. For Rap2A–GDP, it was 26% for 10% missing data at 2.3 Å, and 26.1% for 5% missing data for Rap2A–GTPγS at 3 Å. Crystallographic statistics are summarized in Table I.

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