

# Chapter 2

## The Structure of the G Domain of the Ras Superfamily

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**Abstract** Since the first three-dimensional structure of H-Ras has been determined in 1990, the number of solved structures of small GTP-binding proteins has increased tremendously. As of February 2014, 555 structures of Ras-superfamily proteins have been deposited in the protein databank (PDB), either in uncomplexed form or bound to effectors or other regulatory proteins. The 751 chains contain either GTP or a GTP analogue (431 chains) and GDP (320 chains), respectively. This chapter summarizes the most important structural features of single-domain GTP-binding proteins of the Ras superfamily and focuses on the comparison of the solved structures, especially the switch loops, i.e., the regions that change conformation upon nucleotide exchange from GTP to GDP. In particular, the pitfalls of the crystal structure interpretation will be emphasized since flexible protein segments like the switch regions of the G domain are especially prone to crystallization artifacts. Regions that are mobile in solution are commonly “frozen out” into relatively arbitrary conformations that often are dictated by the specifics of the packing against neighboring molecules in the crystals. It requires very careful analysis to decide if the conformations populated in the crystals have any physiological relevance.

**Keywords** G domain structure • Dynamics of switch regions • Crystallography • Crystal structure interpretation

### 2.1 Introduction: Structural Elements of the G Domain

The Ras superfamily of small GTP-binding proteins is characterized by the so-called “G domain” that is unique for this superfamily and is one of the most ancient protein domains. Attached to it is a hypervariable C-terminus that can be

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posttranslationally modified to achieve membrane attachment of the G domain via prenyl-, or palmitoyl groups and/or by positive charges. This C-terminus is deleted in most of the crystallized constructs or, if present, disordered in all structures, unless complexed with a stabilizing protein partner. In Arf proteins, the N-terminus can be myristoylated.

The G domain belongs to the fold-family “P-loop containing nucleotide hydrolases” (Saraste et al. 1990, SCOP fold c.37) and is the most common fold in all kingdoms (bacteria, archaea, and eukaryotes) (Wolf et al. 1999). It is also called a “mononucleotide-binding domain” since it is a remote relative of the Rossmann fold. Both fold types might have originated from a common ancestor and are sometimes classified as “Rossmannoids.” The CATH database treats the “P-loop containing nucleotide triphosphate hydrolase” superfamily (3.40.50.300) as a subgroup of the Rossmann-fold proteins and accordingly lists it under the “Rossmann fold” topology. The genuine Rossmann fold proteins also have a glycine-rich phosphate-binding loop that is slightly longer than in the “P-loop containing nucleotide hydrolase” fold. In contrast to the genuine Rossmann fold proteins that bind NAD, the “P-loop containing nucleotide triphosphate hydrolases” have to compensate for the charges of the three phosphates, so they feature a magnesium ion and a conserved lysine side chain that contacts the phosphate oxygens. Both are crucial to enable high-affinity nucleotide binding (Kessel and Ben-Tal 2012).

The Ras superfamily is most commonly divided into five major families having related sequences and function as molecular switches in different biological systems: The Ras branch, involved in cell proliferation, gene expression, differentiation, and apoptosis, the Rho family that is involved in the dynamics of the cytoskeleton, the Rab and Arf/Sar families that regulate vesicular transport, and finally the Ran family that determines the direction of nucleocytoplasmic transport and is involved in mitotic spindle organization, with the only member being Ran itself [Table 2.1, (Goitre et al. 2014; Wennerberg 2005; van Dam et al. 2011)]. Since structural data is most abundant for the Ras subfamily, the “extended” Ras family (Di-Ras, Rap, Ral, RheB/RheB-like, Rerg, and the RGK-family (i.e., Rad, Gem/Kir, Rem) will be discussed separately from the core Ras group (formed by H-, K-, N-, M-, and R-Ras). The structurally best-characterized families are the Ras and the Rab families with 139 structures (190 structures including the extended Ras family) and 137 structures, respectively. The Arf and Sar proteins are sometimes assigned to separate families.

There are many more distantly related G-domain structures that contain a wide variety of inserts and deletions, and can also bind adenine nucleotides instead of guanine nucleotides, but they have been extensively reviewed elsewhere (e.g., Vetter and Wittinghofer 1999; Leippe et al. 2002; Wittinghofer and Vetter 2011) and are not covered in this chapter.

This chapter focuses on the single-domain G-proteins of the Ras superfamily, so some multidomain proteins with known structures that have G domains closely related to the Ras superfamily, e.g., the Miro, Roc, Centaurin- $\gamma$ , and Rag proteins, are also omitted here. The Miro and Rag proteins are discussed in other chapters of this book.

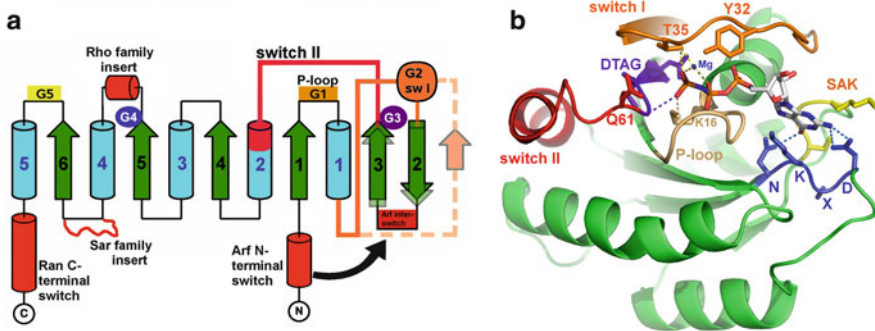
**Table 2.1** Structurally characterized families of single-domain small GTPases of the Ras superfamily. PDB accession codes are given where only one or few structures exist

Family	Subfamily	Members (PDB accession codes)
Ras	Ras	H-Ras, K-Ras, N-Ras (3con), M-Ras, R-Ras (2fn4), R-Ras2/TC21 (2ery)
Ras extended	Ral	RalA, RalB
	RheB	RheB, RheBL1 (3oes)
	Rap	Rap1A, Rap1B, Rap2A, Rap2B Ras-3 (4ku4, Ras-like protein from <i>Cryptosporidia parvovora</i> ) Di-Ras1 (2gf0), Di-Ras2 (2erx)
	RGK	Rad, Gem/Kir, Rem1 (2nzj), Rem2 (3cbq, 3q85, 4aai)
	Rerg	Rerg (2atv), RasL12 (3c5c)
Rho	Rho	RhoA, RhoB, RhoC, EhRho1 (3ref, 3reg, complex with Diaphanous-Protein: 4dvg)
	Rac	Rac1, Rac2, Rac3, Rop4, Rop5, Rop7, Rop9
	Cdc42	Cdc42, RhoUA (2q3h), TC10 (2atx)
	RhoD	RhoD (2j1l), Rnd1 (2cls, complexes with plexin: 2rex, 3q3j), RhoE/Rnd3
Rab	Rab	Rab1,2,3,4,5,6,7,8,9,11,12,14,18,21,22,23,25,26,27,28,30,31,33,35,43
	RasEF	RasEF (2p5s), IFT27 (2yc2/2yc4)
	Ypt	Ypt1,7,8,32,51
	Sec	Sec4
Arf	Arf	Arf1,2,4,5,6,8, Arl2,3,5,6,8,10,13
Sar	Sar	Sar1
Ran	Ran	Ran, RanE (4djt, Nuclear GTP-binding protein from <i>Encephalitozoon cuniculi</i> )

## 2.2 G Domain Topology and Motifs

The G domain consists of a central  $\beta$ -sheet with six strands that is flanked by five  $\alpha$ -helices on both sides (Fig. 2.1). The affinity of the G domain to GMP is usually very low, in contrast to GDP and GTP (John et al. 1990) so that small GTP-binding proteins are most frequently found in complex with GTP or GDP in the cell or after purification in vitro (typical concentrations for GTP and GDP in cells and tissues are 0.3–0.5 mM and 0.03–0.06 mM, respectively (Traut 1994)). Exceptions are found in more distant relatives like hGBP1 that can bind GMP with a comparable affinity to the di- and triphosphate nucleotides by allowing the  $\alpha$ -phosphate to shift towards the P-loop so that the position of the  $\alpha$ -phosphate of GMP now occupies roughly the position of the  $\beta$ -phosphate (Ghosh et al. 2006). This is achieved by inducing an unusual conformation of the nucleotide.

Comparing the three-dimensional structures with either GDP or GTP bound, large conformational changes are observed in all members of the Ras superfamily. The affected regions are called “switch” regions accordingly since they can switch the interactions of the G-domain with other proteins, e.g., effector proteins, “on” or



**Fig. 2.1** (a) Topology diagram for the G-domain of the Ras superfamily. (b) Three-dimensional structure of the G-domain with positions of the conserved nucleotide-binding motifs [same color code as in (a)]. Side chains of important residues (Lys16, Tyr32, Thr35, and Gln61 of H-Ras) are shown as sticks

“off.” Usually, the GTP-bound state is the active one that in case of Ras allows activation of downstream kinases and triggers, e.g., cell division.

Since the main characteristic of the G domain is guanine nucleotide binding, it is not surprising that the five conserved fingerprint motifs are located in loops clustered around the nucleotide-binding site and are therefore often called G1-G5 (Fig. 2.1). G1 or the “Walker A motif” is the glycine-rich phosphate-binding loop (“P-loop,” GxxxxGKS/T) that gives the fold its name. The P-loop wraps around the phosphates allowing the main chain nitrogen atoms to interact tightly with the negatively charged phosphates. The P-loop lysine directly interacts with the  $\beta$ - and  $\gamma$ -phosphate oxygens and is crucial for nucleotide binding. The hydroxyl group of the serine or threonine contacts the  $\beta$ -phosphate oxygen and the magnesium ion. G2 (“switch I,” residues 32–38 in H-Ras) contains a threonine (Thr35 in H-Ras) that is conserved in all members of the Ras superfamily except the RGK family (see also Sect. 2.9). The switch I is one of the regions that changes its conformation upon exchange of GTP and GDP and is also called “effector region” since it is often involved with effector binding when in the GTP state. The conserved threonine is crucial for sensing the presence of the GTP  $\gamma$ -phosphate, and it also contacts the magnesium ion. G3 or the “Walker B motif” is the “DxxG” motif close to the “switch II” region where the D usually sits close to the magnesium ion, but does not necessarily contact it directly. Switch II (residues 59–67 in H-Ras) has no conserved sequence motif besides a glycine (G60 in Ras, conserved in the Ras superfamily except in the RGK family) and also senses the presence of the  $\gamma$ -phosphate. It is often involved in effector interactions also, and plays an important role in nucleotide exchange by GEFs (guanine nucleotide exchange factors) and in stimulation of GTP hydrolysis by GAPs (GTPase activating proteins). G4 is the N/TKxD motif where the aspartate contacts the nitrogen atoms of the base with a bifurcated hydrogen bond, and the asparagine can contact the oxygen of the purine, thus conferring specificity for the guanidinium base. The lysine of this motif stacks along the plane of the base. G5 is the weakly conserved SAK motif: the

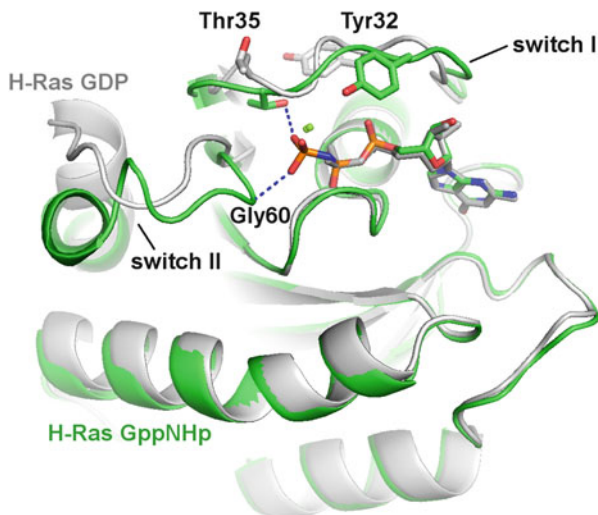
backbone amine interacts with the oxygen of the guanine base and the serine side chain helps to stabilize the adjacent loop in a tight turn. The Rho-family insert between  $\beta$ -strand 5 and  $\alpha$ -helix 4 (Fig. 2.1a) is missing among the members of this family only in the structure of Rho1 of *Entamoeba histolytica* (see Sect. 2.11). Typically it forms a short helix sticking out from the remainder of the G domain and does not change its position upon nucleotide exchange.

### 2.3 Structural Changes Upon Nucleotide Exchange

The structural change of the two Ras switch regions can be interpreted as a “loaded spring” mechanism (Vetter and Wittinghofer 2001): The presence of the GTP  $\gamma$ -phosphate causes the switch I and switch II regions to preferentially assume positions close to the nucleotide. The crucial contacts are from the side chain hydroxyl group of the conserved threonine in switch I and the main chain nitrogen of the conserved glycine in switch II to the  $\gamma$ -phosphate oxygens (Fig. 2.2). When the  $\gamma$ -phosphate is cleaved off, the switch regions are thought to become mobile and disordered so that the “open” (GDP) state usually does not have a defined conformation (with the exception of the Arf and Ran families). Instead, the switch regions are dynamic and assumed to fluctuate on a pico- to nanosecond timescale. The “closed” conformation of the switch regions confers a higher affinity to effector molecules when compared to the “open” form since no binding enthalpy has to be expended to fix a highly flexible region. Indeed, if the binding energy of an effector protein is sufficiently high, as, e.g., in case of the A85K mutant of the Ras-binding domain of Raf kinase, it can form a complex even with the GDP form. The switch I region is then forced into the closed conformation by the effector domain (Filchtinski et al. 2010). Generally, in the GDP-bound forms of uncomplexed G domains, the switch regions (and specifically the threonine and the glycine) are distant from the  $\gamma$ -phosphate since the contacts to the threonine and glycine are lost. However, as detailed below, even the GTP state shows intrinsic flexibility.

In the Ras, Rho, and Rab families, the release of the switch regions after GTP hydrolysis commonly leads to a less well-defined position of the switch I region as evidenced by NMR solution studies as well as by the numerous X-ray structures (Figs. 2.2 and 2.3). This situation is different in the Ran and Arf families where the switch I region in the GDP form changes its position and secondary structure in a defined way by forming an additional  $\beta$ -strand that extends the central  $\beta$ -sheet (Fig. 2.1a, orange/dotted position of the switch I loop). In Ran, the position of  $\beta$ -strands 2 and 3 (also called the “interswitch” since they are located between the two switch regions (Pasqualato et al. 2002)), is relatively similar when comparing the GDP and GTP forms. In contrast, in the Arf and Sar proteins  $\beta$ -strands 2 and 3 undergo a register shift of two residues relative to the rest of the  $\beta$ -sheet (Fig. 2.1a). This is probably mediated by strand number 3 moving into the direction of the  $\gamma$ -phosphate, and strand number 2 then adjusts to this movement. The location of this interswitch- $\beta$ -hairpin in the GDP form opens a hydrophobic groove opposite the nucleotide-binding pocket where the amphipathic N-terminal helix can

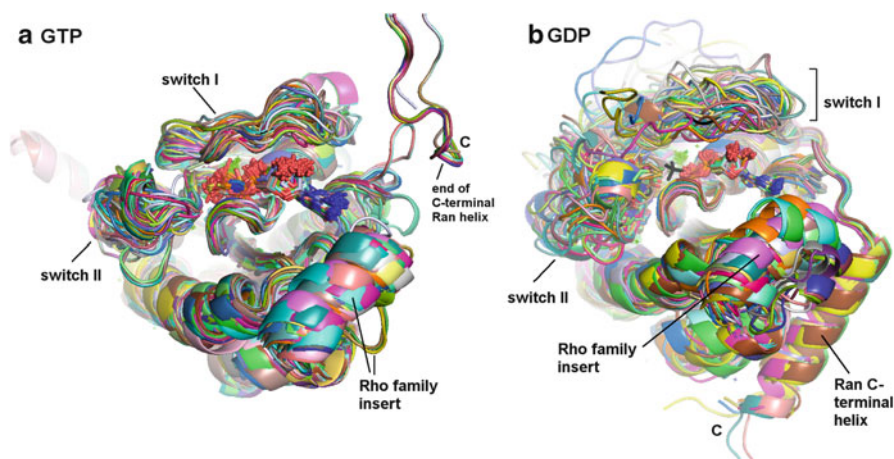
**Fig. 2.2** Change of the switch regions in the H-Ras G domain upon nucleotide exchange. H-Ras-GppNHp in *light green* (5p21), H-Ras-GDP in *gray* (4q21). The positions of Thr35, Gly60, and the hydrogen bonds of the switch regions to the  $\gamma$ -phosphate oxygens in the GTP state are indicated. The side chains of Thr35 and Tyr32 are shown as *sticks*, and the magnesium ions as *spheres*



bind (Fig. 2.1a, black arrow). This additional helix is a hallmark of the Arf family. Formation of this pocket in Arf-GDP is only possible because of a much shorter  $\beta 2$ - $\beta 3$ -hairpin as compared to Ran and the other members of the Ras superfamily (Pasqualato et al. 2002). In the GTP-bound form, the N-terminal helix (that is myristoylated in most of the Arf proteins at the second glycine) is “pushed away” from the core domain and can then mediate interactions with membranes. This additional switch mechanism is reminiscent of the Ran proteins, where a third switch region has been described that again consists of a helix, but in this case C-terminal to the G-domain (Fig. 2.1a). In the GDP form, the helix is associated with the G domain (Fig. 2.3), whereas in the GTP form the altered position of the switch I region leads to a destabilization of the linker region that precedes the helix, causing the C-terminal helix to detach completely from the core domain. Since the helix is now freely accessible, it can be captured, e.g., by Ran-binding proteins. In addition, the dislocation of the helix uncovers the binding site for karyopherins and allows formation of the high-affinity Ran-karyopherin complexes, thus triggering cargo release in the nucleus. The flexibility of the C-terminal helix apparently interferes with crystallization, so no structures of uncomplexed Ran in the GTP form are available. To date, the Ran-GTP structure has been solved only in complex with other proteins like karyopherins or Ran-binding proteins.

## 2.4 Dynamics of the Switch Regions

The now numerous crystal structures of the G domain tend to confer the misleading picture that the switch regions are preponderantly in fixed conformations, either in the “closed” conformation that can bind to effectors or in an “open” one that disfavors effector binding.



**Fig. 2.3** Superimposition of GTP/GppNHp/GppCH2p/GTP- $\gamma$ S-bound structures (a) and GDP-bound structures (b) of the Ras superfamily, highlighting the difference in variability of the positions of the switch I and II regions. H-Ras GppNHp (5p21) is shown in *black* for comparison. The “Rho family insert” is in similar positions in the GTP- and GDP states; it appears to be more variable in the GDP forms since the plant Rop proteins are included here which have a shorter insert helix with a tilted axis relative to the canonical position. The C-terminal helix of Ran is assumed to be flexible in the GTP state in solution

NMR spectroscopy has shown that in solution the switch regions of H-Ras-GDP are disordered and that they show intrinsic mobility on the nanosecond timescale (Kraulis et al. 1994). Likewise, the switch regions of Cdc42-GDP displayed a high level of disorder (Feltham et al. 1997). There were also indications in H-Ras that the helix following the switch II region ( $\alpha 2$ , Fig. 2.1a) changes its position slightly compared to the X-ray structures of H-Ras-GDP (Kraulis et al. 1994). These observations are nicely corroborated by today’s plethora of crystal structures: The “open” conformations of the GDP-bound forms show a much larger variation compared to the GTP forms (Fig. 2.3). Additionally, the switch regions are frequently disordered in the crystals, i.e., they do not show interpretable electron density. In cases where well-defined electron density is observable, they often pack against neighboring molecules in the crystal, so one has to be very careful in interpreting those structures. If a specific conformation of an open switch region is stabilized just because of crystal packing forces, it should be regarded as a crystallization artifact. This is evidenced by the many crystal structures with bound GDP whose switch regions do have well-defined density. For example, in H-Ras-GDP (4q21), there are extensive contacts between the switch regions and neighboring molecules, and, accordingly, both switch regions are reasonably well defined, whereas in a different crystal form of H-Ras-GDP (1ioz), switch II is located next to a relatively wide solvent channel and does not show electron density for residues 61–67. Switch I in 1ioz has crystal contacts only at its ends and correspondingly shows relatively high temperature factors at the tip between



residues 25 and 36. It is evident that the crystallization conditions and the crystal environment have a significant influence when a protein with intrinsically flexible regions is investigated via X-ray structure analysis.

Even in the GTP-bound state, the switch regions are not completely locked down in the “closed” form as evidenced by numerous NMR studies. Phosphorus NMR with H-Ras bound to GppNHp can differentiate between the conformational states as defined by the chemical shifts of the nucleoside phosphates. Especially the  $\gamma$ -phosphate shows a split resonance, indicating the presence of (at least) two different chemical environments of this phosphate, called “state 1” and “state 2,” respectively (Geyer et al. 1996). It is likely that the chemical shift change of the  $\gamma$ -phosphate is mainly influenced by a tyrosine in the switch I loop (Tyr32 in H-Ras, Fig. 2.2). The situation with tyrosine distant or close to the  $\gamma$ -phosphate would then correspond to “state 1” and “state 2,” respectively. Usually it is assumed that “state 2” is the closed conformation that facilitates effector binding, and that “state 1” consists of a rather undefined ensemble of various “open” conformations (Spoerner et al. 2001). This is also confirmed by a H-Ras-GppCH2p structure (6q21) where the switch I region shows a disordered “open” conformation (see next section).

Only one of the nine crystal forms of H-Ras, [the P3<sub>2</sub>21 space group of the first H-Ras structures, e.g., 5p21 (Pai et al. 1990), does not show the tyrosine 32 in contact with the  $\gamma$ -phosphate, in spite of having a “closed” switch I region. Again, most likely the crystal packing forces are causing the tyrosine to be flipped outwards so that it interacts with the  $\gamma$ -phosphate oxygen of a symmetry-related molecule. In 2007, a new crystal form of H-Ras was published [2rge, in a R32 space group, (Buhrman et al. 2007)] that showed the tyrosine 32 in a position very similar to the effector-bound position [e.g., 1gua, (Nassar et al. 1995)]. In this crystal form, there are no crystal contacts close to the Tyr32, indicating that this might be the most reliable equivalent to the “state 2” observed via NMR spectroscopy. These observations reaffirm the importance of being aware of crystal packing effects.

The equilibrium between the different states of the switch conformations in the GTP form is a delicate balance, fine-tuned to transiently stabilize the active Ras sufficiently to allow activation of downstream effectors without switching it off again via intrinsic GTP hydrolysis, while allowing stabilization of the switch regions into the catalytically competent conformation by the GAP proteins.

## 2.5 Authentic GTP Versus GTP Analogues

At 298 K, “state 2,” corresponding to the “closed” state of truncated H-Ras-GppNHp (residues 1-166), is only slightly preferred with a ratio of approx. 56:44 (Ye et al. 2005), whereas H-Ras with authentic GTP shows a strong preference for the closed state (92:8) (Spoerner et al. 2010). The GTP analogues GppNHp and GppCH2p apparently tend to shift the equilibrium towards the open state, whereas GTP and GTP $\gamma$ S are more efficient in fixing the switch I region (Spoerner



et al. 2010; Long et al. 2013). The equilibrium is strongly temperature dependent; the interconversion rate between the two states increases from  $130\text{ s}^{-1}$  at  $5\text{ }^{\circ}\text{C}$  to  $1,900\text{ s}^{-1}$  at  $25\text{ }^{\circ}\text{C}$  (Spoerner et al. 2001). This is consistent with the H-Ras-GppCH2p structure 6q21 where one of the four chains in the asymmetric unit (chain D) shows an “open” conformation with a disordered stretch between residues 33 and 36 even in the crystal, whereas chains C and B are in the canonical conformation. Chain A has the threonine 35 in the canonical position, but the preceding part of the switch I region is in a more detached position compared to the other chains. The inherent flexibility of switch I even in the GTP form is usually obscured in other crystal forms since dynamic regions are commonly “frozen out” and artificially stabilized by neighboring molecules in the crystals. NMR studies of H-Ras-GppNHp (Araki et al. 2011) indicate that the switch regions in state 1 move rapidly on a picosecond to nanosecond timescale, and that the mobility is drastically reduced (but still present) in the closed state (state 2).

Of the 65 Ras-superfamily structures with bound GTP in the PDB database, three quarters are in complex with effector molecules or toxins, and three structures involve hydrolysis-impaired mutants [Arf1 Q71L (1o3y), Rab5a A30P (1n6l), Rab7 Q67L (1t91)]. The remaining nine structures are mostly of naturally slow GTPases like the RhoE/Rnd3 constitutively active core domain that has two serines in place of Q61 and A59 of Ras, the extremely slow GTPase Rab6a, and the slow GTPase RheB that has an extension in the switch II region that places the catalytic glutamine away from the  $\gamma$ -phosphate (see also Sect. 2.9 and Fig. 2.6a). The remaining structures are freeze-trapped H-Ras (1qra), the slow GTPase Rap2 (2rap and 3rap), an unpublished structure of the GTPase-deficient Rnd1 (2cls), and an unpublished Arl6 structure (2h57). Detailed analysis of H-Ras-GTP and H-Ras-GppNHp structures at different temperatures has revealed that the nucleotides bind in identical manner to the protein with only slight differences around the bridging NH group (Scheidig et al. 1999), indicating that tiny changes in the structure can still lead to drastic changes in the dynamics of the switch regions.

## 2.6 Dynamics and Switch States in Other Subfamilies

The dynamic behavior of the switch regions varies drastically between the different members of the Ras superfamily: For example, the Rap proteins (Rap1A and Rap2A) are between 86 and 94 % in the closed form even with GppNHp, whereas RalA-GppNHp is only 40 % closed, and M-Ras-GppNHp is almost completely open (93 % open conformation) (Liao et al. 2008). The corresponding NMR experiments also allowed dissection of the importance of certain residues for the dynamics: Mutation of the conserved switch I Thr35 in Ras [not conserved only in the RGK-family Rad, Gem/Kir, Rem, and in a Ras-like protein (RasL21, 3c5c, unpublished)] to alanine leads to an almost complete shift of the equilibrium towards the open state (>96 % open), and even the mutation T35S shows >78 % open form, suggesting that not only the hydroxy group but also the methyl group of

threonine is important to favor the closed side. The effect of the T35S mutation has been confirmed by solving the X-ray structures of H-Ras T35S, where the switch I regions are either disordered, as expected [1iaq, (Spoerner et al. 2001)], or packing tightly against neighboring molecules, thereby being fixed in a most likely artificial position [3kkn, 3kkm (Shima et al. 2010)]. The effect of the mutation G60A in H-Ras is less drastic: about one-third of H-Ras-G60A-GppNHp is still in the “closed” form (Spoerner et al. 2010).

All crystal structures of members of the RGK family (lacking the T35 and G60) show either completely disordered switch regions or some arbitrary open conformation [Rem-GDP (3cbq, unpublished), Rem-GDP (4aii, (Reymond et al. 2012))]. In some cases the switch regions are only partially visible (Gem-GDP (2cjl, (Slingard et al. 2007), 2ht6, (Opatowsky et al. 2006), 2g3y, unpublished).

A Ras-like protein (RasL12) determined in a structural genomics initiative (3c5c, unpublished) also lacks the conserved threonine and glycine. Interestingly, the switch regions are (even in the GDP form) not too far away from the Ras-GTP position (see Sect. 2.9).

## 2.7 Influence of Structural Elements on the Intrinsic Hydrolysis Rate

The intrinsic hydrolysis rate of small GTP-binding proteins has to be sufficiently slow so that the biological activity can be accomplished while in the GTP state. GAP proteins accelerate hydrolysis by several orders of magnitude. Interestingly, the intrinsic hydrolysis rates are quite diverse as well, ranging over three orders of magnitude from extremely slow proteins like Ran with  $1.8 \cdot 10^{-5} \text{ s}^{-1}$  [25 °C, (Klebe et al. 1995)] or one of the slowest Rabs, Rab6a, with  $5 \cdot 10^{-6}$  molecules GTP per second (Bergbrede et al. 2005) to, e.g., Cdc42 with around  $1 \cdot 10^{-3} \text{ s}^{-1}$  [20 °C, (Zhang et al. 1997)]. In general, spontaneous hydrolysis of GTP in water is slower than the intrinsic hydrolysis of GTPases. Thus, the presence of the catalytic machinery of the G domain has an accelerating effect. One obvious candidate responsible for this accelerating effect is the catalytic glutamine (Q61 in H-Ras) in the switch II region which, when mutated to, e.g., leucine, reduces the intrinsic hydrolysis of H-Ras by a factor 22–80 (Frech et al. 1994; Smith et al. 2013). But, even if the catalytic glutamine is present, the flexibility of the switch regions implies that only a small fraction of the ensemble of conformational states is in the catalytically competent form at any time. This is strikingly evident from the structure analysis of 151 uncomplexed G-domain structures in the GTP-bound state (222 chains), of which only four (seven chains) show the catalytic glutamine close to a  $\gamma$ -phosphate oxygen (distance cutoff 3.5 Å). Of 120 structures complexed with effector molecules (205 chains), 7 structures (22 chains) show a distance <3.5 Å. In contrast, all transition state structures complexed with GAP and GDP-AlF<sub>3</sub> (19 structures, 47 chains) show a close contact between the glutamine and the

$\gamma$ -phosphate, with exception of the Gyp1-TBC domain-GAP/Rab33 complex where the catalytic glutamine is supplied by the GAP [2 g77, (Pan et al. 2006)] and the functionally similar complexes of Rab1 with the bacterial GAP proteins VirA and EspG [4fmb, 4fmc, 4fmd, 4fme, (Dong et al. 2012)]. Superimposing the non-transition state structures shows the glutamine side chain pointing in all possible directions. The presence of multiple conformations of the glutamine side chain at room temperature is also supported by the data from non-frozen H-Ras crystals [3tgp, (Fraser et al. 2011)]: here, glutamine 61 is found in two conformations, with one rotamer pointing away from the nucleotide (the major conformation), and one closely resembling the optimal catalytic position found in the transition state complexes, i.e., close to the  $\gamma$ -phosphate oxygens. This highlights a general problem of collecting structural data from cryo-cooled xtals: The cooling leads to a shrinkage of the crystal lattice and thus a compaction of the proteins, resulting in the freeze-out of a particular state of proteins (Halle 2004; Juers and Matthews 2004). Another pitfall are the positions of the (catalytic) water molecules that might change between physiological temperatures and cryo conditions (Scheidig et al. 1999). Since nowadays practically all X-ray structures are solved at low temperatures (around 100 K), one has to be very careful in interpretation of the data, especially from proteins with flexible regions.

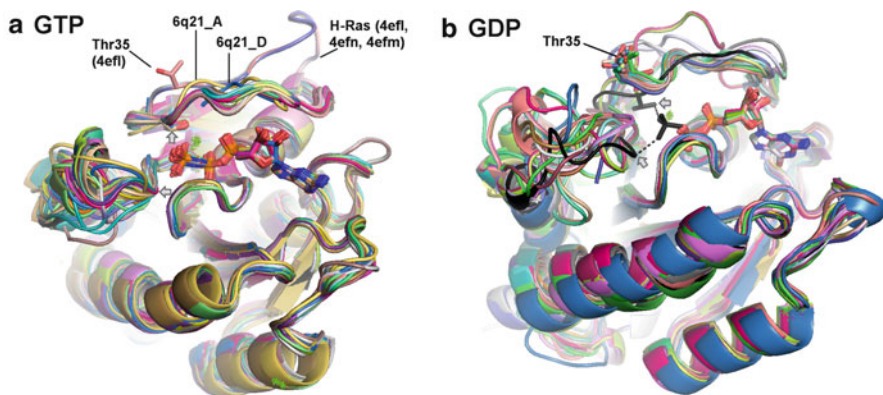
Usually, the binding of effector/helper molecules has a stabilizing effect on the switch regions. GTP hydrolysis might be either slightly faster (e.g., in Ran-RanBP1 complexes (Bischoff et al. 1995), remain unchanged [Ras-RafRBD complexes (Spoerner et al. 2010; Herrmann et al. 1995)], or can be completely abolished [e.g., in Ran-karyopherin complexes (Gorlich et al. 1996)]. This has been attributed to the particular position in which the catalytic glutamine is “trapped” by the effector binding (Seewald et al. 2002). The closer the glutamine is to the catalytic position, the faster the hydrolysis. An additional observation concerns the tyrosine of the switch I region (Tyr 32 in Ras): In very slow GTPases like Rab6 and Rab7, this tyrosine covers the phosphates of the triphosphate nucleotide in all known structures, even if not complexed to an effector molecule. The same is observed for the slow GTPases Rap, Rab28, RheB, RheB-like, and the GTPase-deficient RhoE/Rnd1. In complex with effector molecules, the tyrosine is in a similar position in all Ran-GTP complexes and in some Cdc42 (3eg5, 1nf3), Rap (1c1y, 4hdo), Ral (1zc3, 1zc4), and Rho (3a58, 1z2c, 1e96) complexes. Mutating the tyrosine to alanine significantly speeds up hydrolysis in Ran Y39A (Brucker et al. 2010) as well as in RheB Y35A (Mazhab-Jafari et al. 2012). Some of the Rabs with faster intrinsic hydrolysis rate [e.g., Rab3a ( $3 \cdot 10^{-4} \text{ s}^{-1}$ ) (Clabecq et al. 2000)] have a phenylalanine instead of the tyrosine, and RheB Y35F is almost as fast as RheB Y35A (Mazhab-Jafari et al. 2012), indicating that the hydroxy group interaction might be more important than the shielding effect. It is thus tempting to assume that the hydroxyl group of the tyrosine unfavorably interferes with the position of the catalytic glutamine. It would seem that the slow GTPases Rab4 and Sec4 would prove this hypothesis wrong since instead of the tyrosine they have a phenylalanine or a serine, respectively. However, Rab4 has a histidine (His39) that occupies exactly the position of the hydroxyl group of the tyrosine, and might thus also

block access for the catalytic glutamate (Huber and Scheidig 2005). Sec4 has a serine (Ser29) in the P-loop that is thought to interfere with the intrinsic hydrolysis rate via a hydrogen bond to the  $\gamma$ -phosphate oxygens (Stroupe and Brunger 2000), which might have an influence either on the positioning or the electronic properties of the  $\gamma$ -phosphate oxygens.

In summary, the flexibility of the switch regions might determine the probability of the catalytic glutamine being in the correct position, and any residue that hinders access of the glutamine to this optimal position is then expected to reduce the intrinsic hydrolysis rate.

## 2.8 Ras Family

Based on sequence homology, the Ras family in its more narrow definition can be divided into the two subgroups H-Ras/K-Ras/N-Ras and M-Ras/R-Ras, respectively. Among the “canonical” Ras proteins, H-Ras is by far the structurally best-characterized protein, whereas there is only one structure of N-Ras, and until recently there were only two structures of K-Ras. This changed in 2012 and 2013 due to the revived interest in binding small molecules to K-Ras. Still, there is only one wild-type structure of K-Ras in the database. In contrast, the 67 structures of uncomplexed H-Ras in the GTP state have been obtained by growing nine different crystal forms from 31 wild-type and 36 mutant proteins. This large number of structures allows a quite detailed comparison of the switch regions that exemplify various features of crystallization artifacts as explained above. The switch I region tyrosine (Y32 in H-Ras, conserved in the extended Ras subfamily) is in a position close to the  $\gamma$ -phosphate, i.e., in “state 2” in all crystal forms except the trigonal one. The switch regions are in the “closed” position with Thr35 and Gly60 forming the anchor points as expected, with the exceptions of the intrinsically “open” M-Ras, two chains of H-Ras-GppCH2p (6q21) and three structures of H-Ras-GppNHp [4efl (wild type), 4efn (Q61L) and 4efm (G12V)] (Fig. 2.4). The latter were obtained by seeding a H-Ras solution with crystals of the H-Ras-T35S-GppNHp form, causing the H-Ras wild type to crystallize in the orthorhombic I222 space group of the mutant crystals that is otherwise not accessible. Since the crystal form (space group) and thus the crystal packing is now exactly the same, it is not surprising that the switch I region of H-Ras-GppNHp wild type is in exactly the same position as in the H-Ras-T35S-GppNHp crystals. In both mutant and wild type the open state is stabilized by switch I packing tightly to a symmetry-related molecule and pulling away the threonine 35 in the process [2efl compared to 3kkn, (Muraoka et al. 2012)]. Again, this particular manifestation of a “state 1” conformation appears to be an artifact caused by the specifics of the crystal packing. The reverse experiment, i.e., crystallizing H-Ras-T35S-GppNHp in the space group of wild-type H-Ras-GppNHp [rhombohedral crystal form, e.g., 2rge (Buhrman et al. 2007)], illustrates once more the delicate balance of the switch I conformation: in contrast to wild type, only part of the switch I region of the T35S mutant is in the

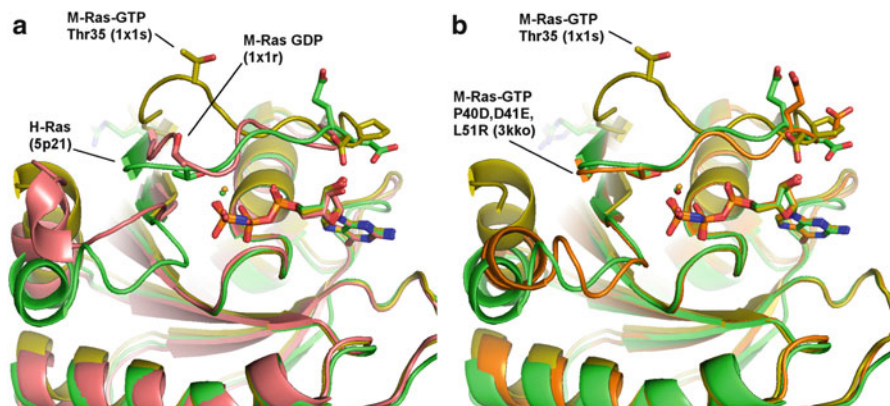


**Fig. 2.4** (a) Superimposed structures of the GTP state of the H- and K-Ras families (the GTP forms of N-Ras and R-Ras are not available), (67 PDB files, 82 chains). M-Ras-GppNHp is intrinsically in the “open” form of switch I and has been omitted for clarity. Similarly, two “open” H-Ras-Y32F mutants are not shown (3k9l, 3k9n). (b) Superimposition of the GDP states, also except M-Ras, and except the K-Ras mutants with bound inhibitors (4luc... and 4m1o... series) (24 PDB files, 31 chains). H-Ras in the GTP-form (5p21) is shown in *black* for comparison. The hydrogen bonds from the  $\gamma$ -phosphate oxygens to Thr35 and Gly60 are highlighted by *dashed lines*. The *arrows* point to Thr35 of H-Ras (*upper arrow*) and glycine 60 of H-Ras (*lower arrow*). In (b), the molecule is rotated slightly upwards and to the right to allow a better view on the threonine (Thr35 in Ras) and the glycine (Gly60 in Ras)

“closed” conformation (i.e., the stretch between residues 32 and 37 slightly deviates in direction of a neighboring molecule), corroborating that the T35S mutant is more predisposed towards the open state than wild type (Shima et al. 2010).

The switch I regions of the GDP state (Fig. 2.4b) appear to be relatively homogeneous which might seem contradictory to the idea that the variability should be higher compared to the well-ordered GTP form. However, there are fewer crystal forms (five) available, and in two of them the switch I regions are disordered and thus not visible. In the other three, switch I is again stabilized to various degrees by neighboring molecules.

M-Ras (also called R-Ras3) has been solved in both nucleotide states, and interestingly, the switch I is open in both nucleotide forms (Fig. 2.5a), with no contacts of the threonine and the glycine to the  $\gamma$ -phosphate at all, corroborating the NMR results which found an almost exclusively “state 1” open conformation for M-Ras-GppNHp (Ye et al. 2005). The sequence of switch I of M-Ras is very similar to H-Ras except for the “DE” (positions 30 and 31 in H-Ras) which is “PD” in M-Ras. Mutational analysis found that replacement of the proline in M-Ras with aspartate leads to a slightly larger percentage of the “closed” state in NMR experiments [13 % in P40D, compared to 7 % in wild-type M-Ras, (Shima et al. 2010)], and additional mutation of the aspartate in M-Ras to glutamate further increased the closed state fraction to about 30 %. Correspondingly, the affinity to the Ras-binding domain of Raf kinase (Raf-RBD) increased from a  $K_d$  of 5.6  $\mu$ M (M-Ras wild type) to 2.5  $\mu$ M (M-Ras P40D) (Ye et al. 2005). For comparison:



**Fig. 2.5** (a) M-Ras-GppNHp (yellow, 1x1s) and GDP state (pink, 1x1r) in comparison with H-Ras-GppNHp (green, 5p21). Both GTP- and GDP states of M-Ras show the “open” form of the switch I region. (b) M-Ras-GppNHp (yellow, 1x1s) in comparison with the M-Ras mutant P40D, D41E, L51R in complex with GppNHp (orange, 3kko) and H-Ras-GppNHp (green, 5p21). Residues D30, E31 in Ras (green), and P/E40, D/E41, and L/R51 in M-Ras (orange/yellow) are shown as sticks. The mutations in M-Ras lead to a “closed” switch I conformation (orange, 3kko)

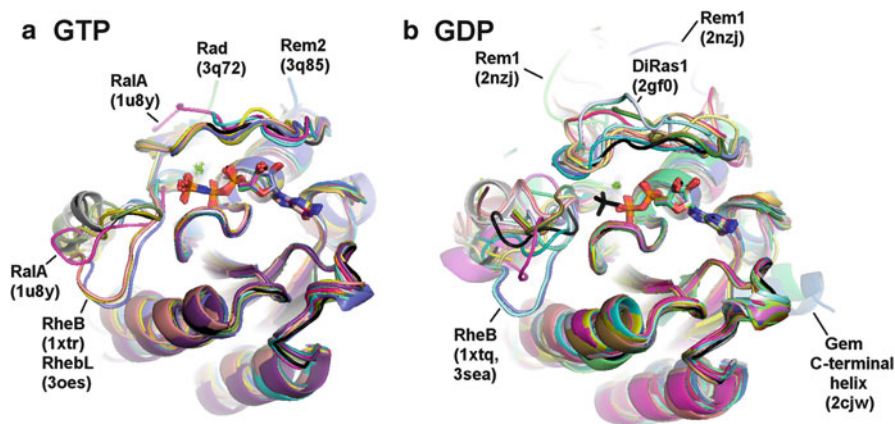
H-Ras - as expected—shows tighter binding to Raf-RBD with a  $K_d$  of approximately 0.5  $\mu\text{M}$  (Ye et al. 2005). The M-Ras double mutant P40D, D41E apparently did not crystallize, but the structure of a triple mutant P40D, D41E, L51R (3kko, with about the same closed state fraction as P40D, D41E of approx. 30 %) could be solved (Shima et al. 2010). Indeed, the triple mutant has Thr45 (equivalent to Thr35 in H-Ras) and Gly70 (=Gly60 in H-Ras) in the canonical positions (Fig. 2.5b, orange). Since H-Ras wild type complexed with GppNHp also shows only slightly more than 50 % closed form (see above), the 30 % closed form of M-Ras is not so different, and, accordingly, the mutation of two amino acids (M-Ras P40D, D41E) is sufficient to shift the equilibrium of M-Ras towards the closed form.

## 2.9 Extended Ras Family

The closest relatives to the core Ras family are Ral, RheB, Rap, Ras-3, Di-Ras, Rerg/RasL12, and, somewhat more distant, the RGK family (Rad, GEM/Kir, Rem).

The Rap (Ras-proximal) structures are very similar to their Ras counterparts, and the first complexes of a small G protein with a Ras-binding domain of an effector have been solved with (mutated) Rap proteins. The Di-Ras proteins are closely related to Rap as indicated by sequence homology and by sharing the same family of activating GAP proteins (Gasper et al. 2010). Like the Rap proteins, they lack the catalytic glutamine; it is replaced by a threonine in Rap and a serine in Di-Ras. The two available structures (2gf0 and 2erx, both unpublished) contain GDP, but, interestingly, 2erx has a phosphate ion bound close to the position where the





**Fig. 2.6** Extended Ras family GTP (a) and GDP forms (b). All GTP forms are in “state 2,” i.e., the switch tyrosine corresponding to Tyr32 in H-Ras is close to the  $\gamma$ -phosphate of the triphosphate nucleotide, except for RalA (1u8y) where the switch I region is in the “open” conformation even in the GTP state, consistent with solution NMR studies. H-Ras GppNHp (5p21) is shown in *black* for comparison

$\gamma$ -phosphate of a GTP would be located. The structure thus resembles a transition (or product) state, and indeed, the switch regions assume the canonical positions including the hydrogen bonds of the phosphate ion that mimicks the  $\gamma$ -phosphate of GTP to Thr39 and Tyr36 of switch I, and Gly64 of switch II (Thr35, Tyr32, and Gly60 in Ras).

RheB (Ras Homolog Enriched in Brain) proteins have low intrinsic GTPase activity and thus are mostly GTP-bound in the cell. The impaired hydrolysis can be attributed to the unusual conformation of the switch II region (Fig. 2.6) where the  $\alpha$ -helix preceding switch II is unraveled, leading to displacement of the catalytic glutamine (Yu et al. 2005). Upon nucleotide exchange, switch II remains in the same, extended conformation, whereas switch I undergoes the canonical rearrangement. The same conformation is observed in the RheB-like protein (structure 3oes, unpublished). RalA-GppNHp (Ras-like) has an open switch I (1u8y, Fig. 2.6), corresponding to the NMR findings that RalA's equilibrium of open/closed is slightly shifted towards the open form (Liao et al. 2008).

The RGK family proteins [Rad (Ras associated with diabetes), GEM/Kir (gene overexpressed in skeletal muscle), Rem (Ras and Gem-related)] have a *bona fide* G domain with N- and C-terminal extensions (between 29 and 90 residues) and are characterized by the unusual DXWE/D motif that replaces the canonical “DxxG” motif at the G3 position immediately before the start of switch II (Fig. 2.1). Although the members of the RGK family bind GTP and GDP, there are no indications of conformational changes upon nucleotide exchange. The tryptophan of the DXWE/D motif forces the switch II loop into a sharp turn so that it prevents switch I from assuming the canonical position close to the  $\gamma$ -phosphate. Consequently, the switch I region is disordered in all of the available structures. The

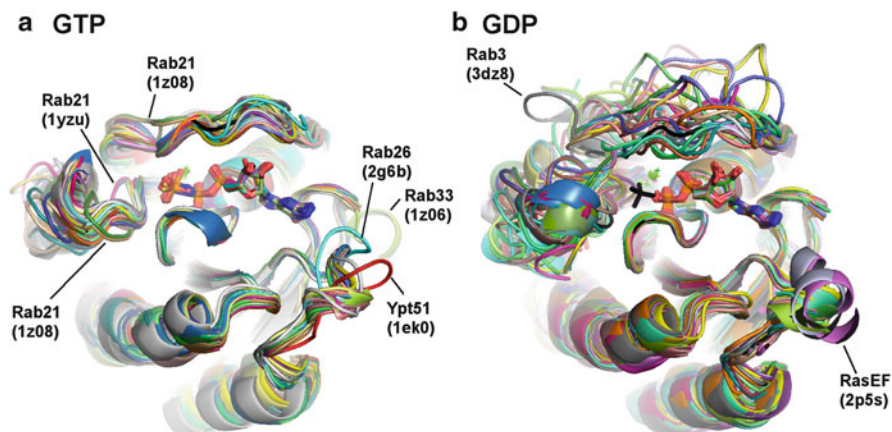


switch regions are generally not conserved within the RGK family, including the residues equivalent to Thr35, Tyr32, Gly60, and Gln61 in Ras (Splingard et al. 2007; Sasson et al. 2011). Rem1, Rad, and GEM are thought to regulate Ca<sup>++</sup> channels via interactions of their  $\beta$ -subunit (Colicelli 2004). Gem proteins seem to have a generally lower nucleotide affinity (Splingard et al. 2007), probably due to the missing phenylalanine in the switch I region (Phe28 in Ras). This phenylalanine is conserved in the Ras, Rab, Rho, and Ran families, but, besides the RGK family, not in Sar and Arf proteins and in some Arls.

Rerg (Ras-related and Estrogen-Regulated Growth inhibitor, 2atv, unpublished) and RasL12 (also known as Ris or RasLC, 3c5c, unpublished) are related between each other (43 % sequence identity) and to the RGK family members (38 % sequence identity to Rem2). Both are crystallized in the GDP form and do not have the tryptophan of the DXWE/D motif, leading to positions of the switch regions that resemble the canonical conformations. In contrast to RasL12, Rerg does have the canonical residues Thr35/Gly60/Gln61 and has been shown to function as a *bona fide* molecular switch (Key et al. 2006).

## 2.10 Rab Family

The Rab proteins are structurally relatively homogeneous, especially in the GTP form: the structures of 44 chains from 28 (uncomplexed) structures covering 19 different Rab families superimpose very well (Fig. 2.7a). The only exceptions where the conserved glycine and threonine of the switch regions are not contacting the  $\gamma$ -phosphate oxygen are some chains of Rab21 (1z08 and 1yzu) where switch II is poorly ordered even in the active conformation [Fig. 2.7a (Eathiraj et al. 2005)]. Three insertions are noteworthy in Rab33, Rab26, and Ypt51, the first one in the loop preceding the C-terminal helix (G5 region), the latter two in the same loop that contains the Rho family insert helix (Figs. 2.7 and 2.1). The switch I tyrosine corresponding to Tyr32 in Ras is only partially conserved in Rab proteins (e.g., in Rab1, Rab35, and Ypt1), in some of them it is replaced by a phenylalanine (e.g., in Rab3, Rab8, Ran26) or by other amino acids. If it is a tyrosine, it does not always contact the  $\gamma$ -phosphate oxygens but shows an even greater variation in positions as compared to the genuine Ras family. The Rab-homology domain of RasEF (also called Rab45, 2p5s, unpublished) is mentioned here only because it has an  $\alpha$ -helical insert remotely resembling the canonical Rho-family insert at the same position of the structure (Fig. 2.7b). The Rab3B-GDP-structure 3dz8 (Zhang et al. 2012) is the only Rab structure that features an extra  $\beta$ -strand in the switch I region (Fig. 2.7b) as well as a shift of the two “interswitch”- $\beta$ -strands relative to the GTP form (interswitch toggle). These conformational changes are typical only for the GDP form of Arf- and Ran proteins (Fig. 2.1). Interestingly, the 86 % identical Rab3D does not show a  $\beta$ -strand conformation of the switch I region in its GDP form (2gf9), although practically all residues of the involved regions (strands  $\beta$ 1,  $\beta$ 2,  $\beta$ 3 and the switch I region) are conserved between the two proteins. A closer look at the



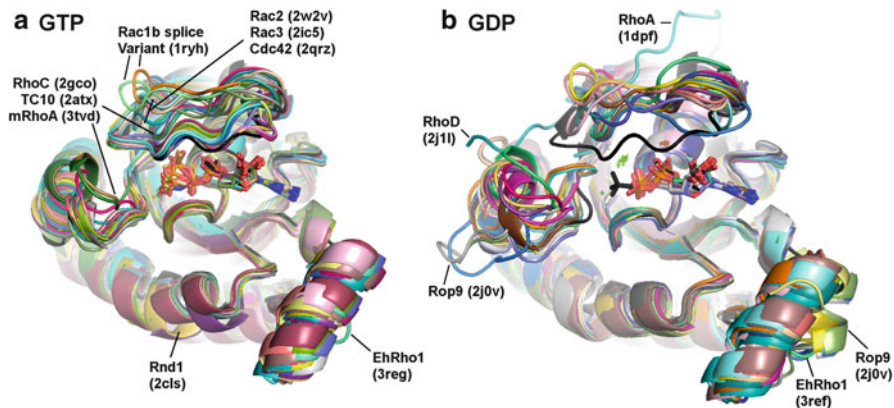
**Fig. 2.7** Rab family GTP (a) and GDP forms (b). H-Ras GppNHp (5p21) is shown in *black* for comparison. Rab21 (1z08) shows canonical threonine/glycine contacts only in chains A and B, whereas chains C and D are distant from the  $\gamma$ -phosphate. Another crystal form of Rab21 (1yzu) has two chains with a distant and disordered switch II region, respectively, but shows a canonical position of the threonine. Rab3B (3dz9) is the only Rab-GDP structure with switch I forming the extra  $\beta$ -strand that is characteristic for Arf- and Ran proteins

structure 3dz8 reveals that there are additional residues ( $^{10}\text{RENLYFQG}^{17}$ ) at the N-terminus of residues 18–190 of Rab3B, and those extra residues form a  $\beta$ -strand that interacts tightly with the unusual “extra” switch I  $\beta$ -strand of a symmetry-related molecule. This suggests that this conformation might not be physiologically relevant, but it is still surprising that a proper “interswitch toggle” can apparently be triggered quite easily in the GDP form of a Rab protein by the relatively weak packing forces in a crystal.

## 2.11 Rho Family

In contrast to the quite homogeneous Rab family structures, the uncomplexed Rho-family structures bound to GTP or its analogues show 7 structures out of 20 with an “open” switch I region (Fig. 2.8). The “closed” forms show the canonical conformation with the switch I tyrosine that is conserved in Rho family proteins (Tyr 32 in Ras) interacting with the  $\gamma$ -phosphate. The seven “open” structures can be divided into three groups according to the most likely reasons for the specific switch I conformation:

1. “Disturbed by disorder of neighboring region”: The splice variant of Rac1b (1ryh) has a 19 amino acid insertion close to the end of the switch II region that is not visible in the electron density. This flexible insert might cause the adjacent switch II region to be disordered as well (Fiegen et al. 2004). The lack of stable



**Fig. 2.8** Rho family GTP (a) and GDP (b) forms. H-Ras GppNHp (5p21) is shown in *black* for comparison. EhRho1 is Rho1 of *Entamoeba histolytica* that lacks the insert helix typical for the Rho family. In (b), the tilted insert helix of the Rop proteins is indicated (Rop9)

interactions of switch II with the switch I region might lead in turn to the destabilization of the switch I region. The affinity of the Rac1b splice variant for the nucleotide is drastically reduced by a factor of 57, and nucleotide hydrolysis is 30 fold slower as compared to Rac1b that lacks the 19 amino acid insertion (Fiegen et al. 2004), highlighting the importance of conformationally stable active site residues for the hydrolysis reaction. In contrast, the Rac1-GppNHp wild-type structure (1mh1) shows a “closed” switch I region although there are no obvious contacts to neighboring molecules in the crystal, suggesting that in Rac1 wild type the closed form probably also exists in solution.

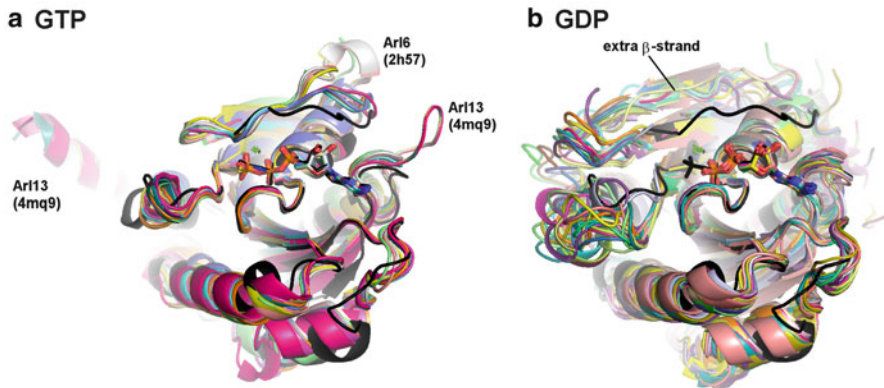
2. “Probable packing artifacts combined with a mobile switch I region” [Rac2-GTP- $\gamma$ S (2w2v), Cdc42-GppCH2p (2qrz), Rac3-GppNHp (2ic5)]: These three protein structures show a wide open switch I whose threonine is completely detached from the  $\gamma$ -phosphate. Thus, Rac2-GTP- $\gamma$ S looks very similar to the Rac2-GDP structure (Bunney et al. 2009). The space groups of the latter two structures appear to be different, but the unit cell dimensions are very similar, as is the packing of the molecules, thereby providing a possible explanation for the similar conformations. The Cdc42-GppNHp structure also has switch I packing against a neighboring molecule, and it is concluded that the switch I region is most likely mobile in both nucleotide states (Phillips 2008), a finding that is also supported by NMR data (Feltham et al. 1997). Similarly, crystal contacts appear to stabilize the switch I region in the unpublished Rac3-GppNHp structure (2ic5).
3. “Partially open conformation of switch I” (mouse RhoA-GppNHp (3tvd), TC10-GppNHp (2atx), and RhoC-GTP- $\gamma$ S (2gco)): Those three structures show a position of their “open” switch I regions that is different from the proteins above (group 2), but very similar among each other, especially in the region

around the conserved threonine. In the canonical “closed” state the threonine contacts the magnesium ion via its hydroxy group. Here, it flips by 180° and now contacts the magnesium position with its carbonyl group. RhoA from mouse (3tvd, only one residue in the CAAX box differs from human RhoA (Jobichen et al. 2012)) is the only uncomplexed RhoA-wild-type-GTP-form structure available. Two other RhoA structures, one with GppNHp (1kmq) and one with GTP- $\gamma$ S (1a2b) both have “closed” switch I regions. Since mouse RhoA (3tvd) was crystallized at low pH (4.6) and as a dimer, in contrast to the other two RhoA structures (1a2b, 1kmq), the partially open switch I could be seen as an artifact of the crystallization conditions. However, the structure of TC10 [2atx, (Hemsath et al. 2005)], a close relative of Cdc42, has switch I in a very similar position to 3tvd. In this case the crystal packing shows no tight contacts that could hold the switch I regions in the closed state, and the switch I tyrosine packs only loosely against a neighboring molecule, suggesting that this “open” position of the switch I region is not induced by packing against neighboring molecules. This hypothesis is corroborated by RhoC that was crystallized with either GppNHp (2gco, “open”) or GTP- $\gamma$ S (2gcp, “closed”) in two different crystal forms (Dias and Cerione 2007). The “open” form of 2gco again showed the effector loop in a very similar conformation to 3tvd (mouse RhoA) and 2atx (TC10) and is not altered by any crystal contacts. It was speculated that this conformation might represent a “partially activated” state of Rho-family proteins (Dias and Cerione 2007) that is stabilized by the unique conserved phenylalanine of the Rho family switch I region (Phe39 in RhoA and RhoC). This phenylalanine also mediates hydrophobic contacts with effector molecules. The “partially activated” conformation has the potential to be a major form also in solution and would be one of the few examples where at least one of the “open” or “state 1” conformations of the switch I region shows a defined structure instead of being another crystallization artifact. This exemplifies how the availability of many crystal structures can help to distinguish between “real” intermediates of the open forms and crystal artifacts.

The short helix forming the typical insert of the Rho family is absent in the structures of Rho1 of *Entamoeba histolytica* (3reg, 3ref, 4dvg) where it is replaced by a loop that is only slightly longer than in Ras proteins (Fig.2.8). Otherwise, the position of the insert helix is remarkably conserved among Rho family proteins and not influenced by the type of bound nucleotide. Only the Rop proteins seem to be an exception with a shorter insert helix whose axis is rotated relative to the “canonical” position (Fig. 2.8b).

## 2.12 Arf/Sar Family

Characteristic for the Arf/Arl proteins is the formation of an extra  $\beta$ -strand in the switch I region in the GDP state, similar to the Ran proteins (Fig. 2.9). The sequence of this extra strand (<sup>42</sup>IVTTIPTIGF<sup>51</sup>) is conserved in Arf proteins, and



**Fig. 2.9** Arf/Sar family GTP (a) and GDP forms (b). The Sar-GDP structures (2fmx, 1f6b, 2fa9, 2gao) and Arl10B-GDP (1zd9) are omitted for the sake of clarity. H-Ras GppNHp (5p21) is shown in *black* for comparison. All Arf/Arl structures lack the switch I tyrosine (Tyr32 in Ras)

Gly50 (equivalent to D38 in Ras) allows formation of the  $\beta$ -turn that is required for this conformation (Goldberg 1998). Indeed, this glycine is also conserved in Ran proteins. In Rho proteins, a phenylalanine (Phe39 in RhoA and RhoC) occupies this important position and is a determinant for switch I conformation and effector interaction as discussed in Sect. 2.10. Surprisingly, Arl10B-GDP (1zd9, unpublished) shows switch I in the “canonical” Ras position, i.e., without the extra  $\beta$ -strand, in contrast to two other Arl10-GDP structures (2h18 (Arl10B) and 2a17 (Arl10C), both unpublished), with a “normal” extra  $\beta$ -strand. All three structures have the conserved Gly50. Arl10B and Arl10C [also termed Arl8A and Arl8B (Kahn et al. 2006)] lack several canonical residues of other Arf/Arl GTPases, and it was even suggested to place them in a separate category (Neuwalde 2010). However, the observed difference in the switch I position between the two Arl10B-GDP structures (2h18 and 1zd9) might again be a crystal packing effect: In 2h18, the N-terminus of a symmetry-related molecule blocks the “GTP” position of switch I, whereas in 1zd9, switch I might be stabilized in the “GTP” orientation by the neighboring molecules, accompanied by the canonical “interswitch toggle” of strands  $\beta$ 2 and  $\beta$ 3. Like in case of the Rab3B (3dz8) described above, Arl10B can apparently easily interconvert between the GTP and GDP conformations even with the same nucleotide (GDP) bound.

Arl6 and Arl13 also show some noncanonical features: Arl13 has a long C-terminal helix that is added to the G-domain with an unusual  $90^\circ$  kink [Fig. 2.9a, (Miertzschke et al. 2014)]. The catalytic glutamine is replaced by a glycine, explaining the lack of intrinsic GTP hydrolysis activity. It also has an insertion of five residues with unknown function after the SAK motif (TAK in Arl13) forming a loop (Fig. 2.9a) that seems to be relative stable since it lacks crystal contacts at least in two of the three monomers in the crystal. Arl6 (2h57, unpublished) has an insertion of two residues in the effector loop that does not interfere with the threonine assuming the canonical position (Fig. 2.9a).

Although Sar proteins are sometimes classified as a separate family, they are functionally and structurally closely related to Arf, with a similar retracted interswitch region in the GDP-bound form. They are not posttranslationally modified like Arf proteins, but, like the Arfs, they also have a N-terminal extension (albeit in a different orientation relative to the G domain) that confers interactions with membranes of the endoplasmic reticulum via bulky hydrophobic amino acids in the so-called “STAR” motif (Huang et al. 2001). The N-terminus is important for the interaction with the Sec23/24 GAP and the cargo selection of the COP I coat. Amino acids 156–171 are an insertion relative to the Arf proteins that form a loop. The Sar1 switch I region lacks the conserved glycine 50 of the Arf/Arl proteins and, indeed, does not form the extra  $\beta$ -strand found in Arf and Ran proteins in the GDP conformation.

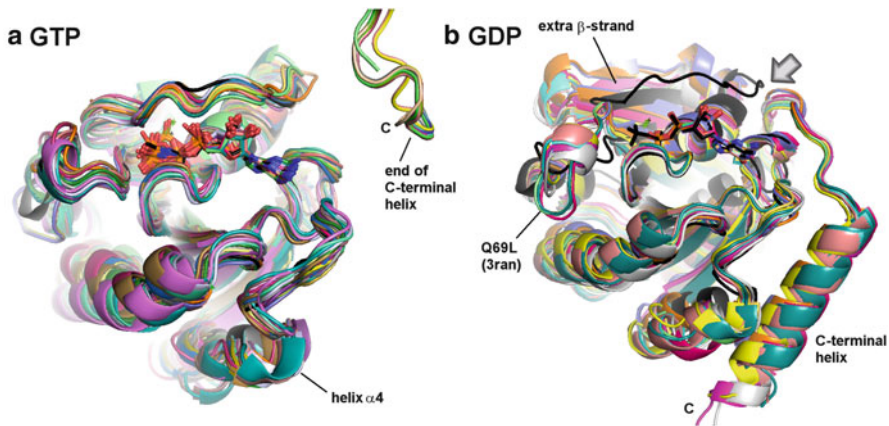
Switch I in the Sar1-GDP structures is in a position relatively similar to the “closed” position, but in different positions in each of the available structures as expected from the unattached threonine residue. The catalytic glutamine is replaced by a histidine but has a similar function in orienting the catalytic water molecule, similar to protein synthesis elongation factors (Huang et al. 2001).

## 2.13 Ran

The Ran family is characterized by a C-terminal helix extension (Fig. 2.1a) that is attached to the G domain in the GDP form (Fig. 2.10b) and detached in the GTP form (Fig. 2.10a). The switch I region in the “closed” form would clash with the stretch of residues preceding the C-terminal helix (arrow in Fig. 2.10b), causing the C-terminal helix to be dislocated (Vetter et al. 1999). As in case of the Arf proteins, the switch I region forms an additional  $\beta$ -strand in the GDP form (Fig. 2.10b), leaving the nucleotide exposed to the solvent. In complex with karyopherins that have a sufficiently large contact area to Ran proteins to be able to bind Ran even in the GDP form with significant affinities, the switch I region can be forced into the closed conformation [3ea5, (Forwood et al. 2008)]. The plasticity of the switches is highlighted by the structure of a Q69L mutant of canine Ran [3ran, (Stewart et al. 1998)] where conformational changes of up to 2 Å are observed (Fig. 2.10b). In the monoclinic crystal form (3ran), the switch II region in the conformation of the wild type would clash with a symmetry-related molecule which might be the cause of the altered conformation. The Ran-GTP complex structures appear quite homogeneous; the switch regions are relatively well defined. In contrast, helix  $\alpha$ 4 shows relatively large changes of the helix axis orientation compared to the GTP form. The area around helix 4 forms one of the main contacts with the  $\alpha$ -solenoids of the karyopherins, and might thus be important for lowering the affinity to those effector proteins in the GDP form of Ran.

The structure of an orthologue of Ran from *Encephalitozoon cuniculi* (4djt, unpublished) has all GTP-interacting motifs including the catalytic glutamine





**Fig. 2.10** Ran GTP (a) and GDP forms (b). The triphosphate structures are in complex with various Ran-binding proteins and karyopherins since there is no uncomplexed Ran GTP structure available. H-Ras GppNHp (5p21) is shown in *black* for comparison. The backbone of H-Ras-GppNHp in (B) emphasizes that the switch I region in the closed conformation would clash (indicated by an *arrow*) with the residues preceding the C-terminal helix, causing it to detach from the remainder of the G domain in the GTP conformation (a)

conserved. It was crystallized in the GDP-bound form. Interestingly, its C-terminus does not form a helix, but a random coil that is positioned roughly where the switch I region of the GTP form is located.

All Ran-GTP structures show the switch I Tyr39 (Tyr41 in yeast Ran) closed over the nucleotide, the hydroxy group contacting the  $\gamma$ -phosphate oxygens. The side chain of the catalytic glutamine (Gln69 in human and Gln71 in yeast Ran) shows again random rotamers except in the complex with RanGAP, indicating catalytically incompetent forms that are consistent with the slow or unmeasurable hydrolysis in the (non-GAP) complex structures.

## 2.14 Summary/Concluding Remarks

The G domain is a very versatile and evolutionary ancient structure whose dynamic switch regions can sense the nucleotide state and alter the outer shape of the molecule, allowing binding to specific effector and regulator proteins. The switch regions are in a delicate balance between a transiently stable GTP-bound form that often interconverts between an “open” and a “closed” form on a fast timescale, and a GDP form with even less well-defined switch regions (except for the Arf and Ran families). The dynamics of the switch regions influence the intrinsic hydrolysis rate by positioning the catalytic machinery more or less closely to the optimum position. Some of the subgroups of the Ras superfamily use additional secondary structure elements like N- and C-terminal helical extensions to achieve regulation of



transient membrane interaction (Arf/Sar) or enable the specific formation and dissociation of extremely high-affinity complexes to facilitate nuclear transport (Ran). Although the number of structures in the protein databank grows rapidly and more and more structures of G domains become available, the analysis especially of proteins with flexible regions has many pitfalls and should take into account that crystallization conditions, crystal packing, and freezing of the crystals for data collection can introduce artifacts into the structure.

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