Interface analysis of small GTP binding protein complexes suggests preferred membrane orientations

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Abstract: Crystal structures of small GTP binding protein complexes with their effectors and regulators reveal that one particularly flat side of the G domain that contains helix α4 and the C-terminal helix α5 is practically devoid of contacts. Although this observation seems trivial as the main binding targets are the switch I and II regions opposite of this side, the fact that all interacting proteins, even the largest ones, seem to avoid occupying this area (except for Ran, that does not localize to membranes) is very striking. An orientation with this ‘flat’ side parallel to the membrane was proposed before and would allow simultaneous interaction of the lipidated C-terminus and positive charges in the α4 helix with the membrane while being bound to effector or regulator molecules. Furthermore, this ‘flat’ side might be involved in regulatory mechanisms: a Ras dimer that is found in different crystal forms interacts exactly at this side. Additional interface analysis of GTPase complexes nicely confirms the effect of different flexibilities of the GTP and GDP forms. Besides Ran proteins, guanine nucleotide exchange factors (GEFs) bury the largest surface areas to provide the binding energy to open up the switch regions for nucleotide exchange.

Keywords: buried interface; crystal structures; effector complexes; G domain dimerization; GTPase; membrane interaction.

Introduction

Most small GTP binding proteins except Ran are post-translationally modified with prenyl groups at either an N-terminal glycine (Arf family, myristoylation) or at the C-terminal CAAX box (Ras, Rho, Rab families, farnesylation or geranylgeranylation). Some G proteins are additionally modified by palmitoyl groups at cysteines preceding the CAAX box (e.g. H-Ras, N-Ras, K-Ras4A, Rap2A-B), whereas in others the palmitoylation site is replaced by a polybasic stretch (e.g. K-Ras4B, Rap1) (Colicelli, 2004; Tsai et al., 2015). As the prenyl groups are irreversibly attached, the membrane affinity conferred by those groups can be regulated only by helper proteins that bind to and shield the prenyl group from solvent, like the GDI-type proteins. In contrast, the palmitoyl groups are reversibly attached by DHHC proteins at the Golgi apparatus and can be removed by thioesterases. Candidate human thioesterases are APT1 and APT2 (Görmer et al., 2012). The closely related human protein LYPLAL1 has been shown to be unable to cleave palmitoyl chains (Bürger et al., 2012). The involvement of APTs in depalmitoylation is unclear, e.g. the orthologous protein to APT1/APT2 in toxoplasma can be knocked out without major consequences (Kemp et al., 2013), and APT1/2 inhibition/knockdown did not show a significant effect on N-Ras localization in NRAS mutant melanoma cells (Vujic et al., 2016), but inhibition of APTs (Rusch et al., 2011; Zimmermann et al., 2013) indeed altered Ras localization (Dekker et al., 2010). Efforts to exploit the palmitoylation machinery for therapeutic purposes are ongoing (Cox et al., 2015). Recently, other candidates for palmitoyl cleavage have been proposed, the ABDH17 family proteins (Lin and Conibear, 2015), and more might be found since the serine hydrolase family has many uncharacterized members.

From a structural viewpoint, the G domain (GTP binding domain) of the family of small GTP binding proteins is a versatile module that has evolved from an ancestral fold, the P-loop containing nucleoside triphosphate binding domain (Leipe et al., 2002; Ma et al., 2008). This fold is characterized by the presence of more or less flexible molecular ‘switch’ regions. According to the ‘loaded spring’ hypothesis (Vetter and Wittinghofer, 2001) the two switch regions are restrained in their dynamics by...
the γ-phosphate of a GTP molecule, so that the entropic penalty for binding of effector molecules and GTPase activating proteins (GAPs) especially to the switch I region is decreased. Consequentially, GDP binding increases the flexibility of the switches and attracts different interaction partners, the nucleotide exchange factors (GEFs) and the guanine nucleotide dissociation inhibitors (GDIs), using different additional contact regions.

The functionality of the G domain has expanded during evolution by introduction of various insertions (Wittinghofer and Vetter, 2011). The Rho/Rac/Cdc42 family has a small insertion of approx. 13 residues forming a short α-helix between helix α4 and β-strand β4 that does not change the conformation upon nucleotide hydrolysis and is usually not directly involved in effector binding. Arf/Arl/Sar proteins undergo a large conformational change of the G domain core where a register change of two β-strands causes the N-terminus, usually an amphiphilic, myristoylated helix, to detach from the protein core (therefore called ‘N-terminal switch’) so that it can interact with membranes or effectors (Pasqualato et al., 2002; Gillingham and Munro, 2007). Ran GTPases have an additional C-terminal helix that is destabilized if GTP is bound (called ‘C-terminal switch’; Vetter et al., 1999b).

Summaries of the structural features of G domain containing proteins can be found in the following references: Vetter and Wittinghofer (1999), Paduch et al. (2001), Cherfils and Zeghouf (2011), Wittinghofer and Vetter (2011) and Vetter (2014). In the following, structural characteristics of complexes of small GTP binding proteins are highlighted, with a focus on the implications for orientation at the membrane.

**Post-translational modifications and other structural determinants of membrane orientation**

In the available crystal structures of small GTP binding proteins, the C-terminal hydrophobic modifications are rarely present as they render the proteins very poorly soluble. Thus, prenylated GTPases usually crystallize only in complex with GDI proteins that chaperone the hydrophobic group, like the RabGDI Ypt1 (1UKV; Rak et al., 2003), RhoGDIs [(1DOA; Hoffman et al., 2000), 1HH4 (Grizot et al., 2001), 4F38 (Tnimov et al., 2012), 5FR2; Kuhlmann et al., 2016)], PDE6δ (3T5G; Ismail et al., 2011) or REP (Rab escort protein, 1VG0; Rak et al., 2004). The lipid modification site is separated from the G domain by a hypervariable region (HVR, 167-189, Figure 1B) that is assumed to be flexible and is normally deleted for crystallization, so that there are only a few crystal structures of full-length GTPases. For Ras proteins, one structure is H-Ras-GDP 1-189 (4Q21; Milburn et al., 1990); the second one is K-Ras4B 1-189 (GDP, 4DSU, GSP, 4DSO; Maurer et al., 2012). In the H-Ras structure (4Q21), the C-terminal helix (α5) is visible up to Leu168, so this structure does not contribute much additional information compared to the truncated structures (usually comprising residues 1-166). In the K-Ras structures (4DSU, 4DSO), the C-terminal helix continues up to residue Asp173, which is the last residue with unambiguous electron density (Maurer et al., 2012). The remaining residues 174-180 that contain the polybasic region, mostly lysines, have only weak electron density, indicating their intrinsic flexibility, and

![Figure 1: Proposed interaction of the three Ras proteins with the plasma membrane (orange).](image)

H-Ras in gray (5P21, GppNHP-bound), K-Ras in blue (4DSU, GDP-bound, 4DSO, GSP-bound, both look very similar), N-Ras in violet (GDP-bound, 3CON). (A) Positions carrying positive charges (128, 135, 161) are labeled and side chains shown as sticks, and residue 47 of the β-hairpin loop is indicated. (B) Orientation 110° rotated compared to (A). The hypervariable region (residues 167-189) forms an α-helix for residues 167-174 in the K-Ras structure (blue) and is probably flexible after residue 174, thus, the lysine side chains of the polybasic region were modeled according to geometrical constraints.
are not always conserved: Rab proteins have R/K at the position corresponding to Ras R161, but positions 128 and 135 are not conserved (at 128, e.g. R,K,E,Q,D, at position 135, e.g. R,K,D,Y). In the Rho family, positions corresponding to R161 and R135 in Ras are conserved as R/K, but position 128 has no positive charge, but is often E,Q or D. In Arf proteins, position 135 is mostly histidine, but the other two positions often have negative charges. A summary of the charge distributions in helices α4 and α5 can be found in a recent review (Prakash and Gorfe, 2016). In addition, Arf proteins, the major traffic regulators in the cell besides Ran and Rab proteins, have their lipid modification at the N-terminus instead of the C-terminus, and therefore will be described in some more detail in the following.

Similar to Ran, the Arf-family GTPases feature an additional switch mechanism that drastically remodels a part of the G domain. Where Ran has the C-terminal helix attached to the G domain core in the GDP form and detached in the GTP form, Arf proteins exhibit a shift of one β-strand in the G domain core upon replacement of GDP with GTP that pushes out the myristoylated N-terminus, the ‘interswitch toggle’ (Pasqualato et al., 2002). The N-terminus often forms a short helix that, in addition to myristoylation, is usually amphipathic (Huang et al., 2001; Vetter and Wittinghofer, 2001; Gillingham and Munro, 2007; Donaldson and Jackson, 2011; Lin and Conibear, 2015). The linker following this helix, as well as the C-terminus, often carries positive charges to facilitate membrane attachment (Liu et al., 2010). In some cases, the N-terminal helix can provide sufficient membrane affinity even without myristoylation, e.g. in Sar1 and Arl6, where the lack of the lipid group is compensated by additional hydrophobic residues at the N-terminus (Huang et al., 2001; Gillingham and Munro, 2007). The two attachment sites, i.e. the hydrophobic/amphiphilic N-terminus and the charged C-terminus, can restrain the orientation of Arf proteins at the membrane. Interestingly, NMR studies have led to a model where the C-terminal helix of Arf (that contributes to the plane of the ‘flat’ side) again lies almost parallel to the membrane (Liu et al., 2010). It is thus tempting to assume that the N-terminus of Arf proteins takes over the role of the positively charged positions 128 and 135 in Ras proteins as membrane anchor point.

In summary, the available structural information for small GTP binding proteins contributes to explain the experimental evidence for the different modes of membrane binding. Besides the properties of the G domain itself, analysis of the overall shape of structures of effector complexes that often form in direct vicinity of the membrane might give some additional information about the membrane orientation of those complexes, as described in the following.
Effector complexes of small GTP binding proteins – implications for membrane orientation and comparison of interaction surfaces

Effector proteins of small GTP binding proteins bind – per definition – to the GTP-bound form of the G domain. Superimposition of the available complex structures of membrane-interacting GTPases with their effectors reveal the interesting picture that one side of the G domain is left completely free (Figure 2A). This could be attributed to the fact that the switch regions that indicate the presence or absence of the γ-phosphate are at the opposite side of the G domain, and the effector molecules naturally do cluster around the switch regions of the G domain (Figure 2B). On the other hand, is still striking that even the largest effector molecules would not clash with the putative membrane position, and that – probably not coincidentally – this ‘flat’ side corresponds exactly to the orientation predicted for the H-Ras GTP form (Figure 1).

This orientation would allow simultaneous interactions of all arginines and the C-terminal lipid modification with the membrane while still bound to the effector molecule. It also corresponds to one of the major two orientations observed for the oncogenic mutant G12D of K-Ras, called ‘OS1’ in Prakash et al. (2016). There, it was already noted that the RafRBD effector domain would clash with the membrane in a second predicted orientation (denoted as ‘OS2’), in contrast to ‘OS1’. A third position features helices α3 and α4 parallel to the membrane, as predicted e.g. for K-Ras and Arf (Prakash and Gorfe, 2016). In this orientation (Figure 2C), the main clashes with the membrane would be with the formin- and the LidA-complexes (the latter not shown in Figure 2C for clarity). Figure 2D depicts the same orientation, but with the formin complexes omitted, now revealing potential membrane clashes of the Arf6-effector JIP4 (2W83; Isabet et al., 2009), and the Rab6-effector R6IP1 (3CWZ; Recacha et al., 2009), that is recruited to Golgi membranes by active Rab6 (Recacha et al., 2009). The coiled coil helix of JIP4 was proposed to be oriented parallel to the membrane (Isabet et al., 2009), which would also argue against membrane

![Figure 2](image-url)
interaction of the α3/α4 side in those complexes. In addition, it is evident that the α4/α5/β-hairpin interface (the ‘flat’ side) would bury a slightly larger interface with the membrane compared to the α3/α4 side, namely 2460Å compared to 1847Å, i.e. an about 25% larger surface area of the G domain, indicating a potentially more stable membrane association of the former.

Looking at the distribution of positive charges in those complexes, the arginine/lysine clusters of the three positions corresponding to H-Ras R128, R135 and R161 are clearly visible as an acute triangle (Figure 3B). As discussed by Prakash and Gorfe (2016), orientational restraints might also be relevant for G-domains of other small GTP-binding protein families, and this idea is corroborated by several crystal structures as highlighted in the following. A distinctive basic cluster is notable in a short helix close to the putative membrane that belongs to the F2 lobe of the FERM domain of KRIT1 in complex with Rap1B [(4DXA; Li et al., 2012), 4HDO/4HDQ (Gingras et al., 2013)] with the motif ‘KKHK’ (Figure 3A). This motif was postulated to be a NLS (Zawistowski et al., 2005), but in C2 domains, similar motifs are thought to confer affinity to membranes (Thomas et al., 1999). Mutation of a lysine (K570) in the KKKH motif reduced the affinity to Rap1B as it is involved in a salt bridge to E45 of Rab1B (Gingras et al., 2013), but that would not exclude a possible additional contribution to membrane interaction. The FERM domain has other interaction sites for the membrane as well that would not be compatible with the orientation in Figure 3, so it would be interesting to obtain experimental support for this speculation, and, furthermore, to check if similar motifs in other effector molecules might also facilitate membrane attachment. In case of Rho proteins, the function of the insert helix (Figure 3B) is still unclear. It usually does not interact directly with effectors, with the exception of some minor contacts at the tip of the helix in some complexes (e.g. in Cdc42-FMN2L2, 4YC7/4YDH; Kühn et al., 2015), but the insert has been found to be essential, e.g. for activation of the Rho kinase (Zong et al., 2001). Interestingly, the insert helix lies almost parallel and very close to the membrane in the orientations shown in Figures 2 and 3, and also features some positive charges (Figure 3B). Indeed, it was speculated that the insert could also be important for fine-tuning the orientation of Rho proteins at the membrane (Vidal, 2010). Fittingly, effector complexes of Ran – that does not interact with membranes during cargo transport through the nuclear pore – show the ‘flat’ side of the G domain completely blocked and buried by the huge helical solenoids of the transport proteins (karyopherins) that cover almost the entire surface of the Ran protein.

Analysis of the contact areas between the G domain and the effectors also reveals some characteristic differences between membrane-bound GTPases and Ran proteins: Effector complexes of the former typically show relatively small buried surface areas, corresponding to the transient nature and moderate affinities of those interactions (on average 785 Å² for 74 structures containing 139 sub-complexes (i.e. heterodimeric complexes), analyzed with PISA, (Collaborative Computational Project, Number 4, 1994). Values range from 422 Å² (Rab6-golgin-GCC185 complex; Burguete et al., 2008) to 1860 Å² (Rab27-SLAC2-A/melanophilin complex; Kukimoto-Niino et al., 2008). In contrast, the karyopherins are snail-like α-helical solenoids that wrap around the Ran G domain and bury up to 2503 Å² in the interface (Crm1-RanQ69L

![Figure 3](attachment:image.png)

**Figure 3**: Superimposition of effector complexes of small GTP binding proteins, except Ran complexes, with positive residues highlighted as thin lines/blue color. The positions of the arginines R128/R135 and R161 and the C-terminus in Ras are indicated. ‘H’ is the β-hairpin loop at residue 47 (compare to Figure 1). (A) Side view with the putative position of the membrane in orange and the KKHK motif of KRIT1 (4DXA, 4HDO, 4HDQ) highlighted. (B) View as seen from the membrane side.
complex 3NC1; Güttler et al., 2010), leading to picomolar affinities. On average, 1997 Å² are occluded in the interfaces of Ran-effector-complexes (45 structures containing 65 sub-complexes). In these complexes, nucleotide hydrolysis is almost completely abolished, which is essential for Ran function, as otherwise the karyopherin complexes would dissociate in the wrong cellular compartment or during transit through the nuclear pore. Other effectors can have different effects on the GTP hydrolysis rate, they can either leave the rate unchanged or even accelerate it slightly (Vetter, 2014). In karyopherins, hydrolysis suppression is achieved by remodeling switch II so that the catalytic glutamine is in a hydrolysis incompetent position (Vetter et al., 1999a). Later it has been shown that the conserved Tyr39 in switch I of Ran proteins also contributes to the slow hydrolysis in Ran effector complexes by interfering with the optimal position of the catalytic water molecule (Rudack et al., 2015). In all Ran GTP forms, the (additional) C-terminal helix (α6) is detached from the core G domain, and in case of the karyopherin complexes, it is usually disordered and not visible in the structures. In contrast, another type of Ran effectors, the Ran binding domains (RanBD), are designed to capture the (flexible) C-terminus of Ran-GTP and wrap it around themselves (Vetter et al., 1999b). Although the Ran binding domains are quite small with a molecular weight of approx. 16 kDa, the ‘molecular embrace’ of Ran with a RanBD buries almost as much surface as the large karyopherin complexes (2350 Å²), along with having a very high affinity in the low nanomolar range. Thus, the type of effector complexes formed by Ran is fundamentally different from the membrane-attached GTPases as Ran effectors cover much larger surface areas of the G domain.

GAP (GTPase activating protein) and GEF (guanine nucleotide exchange factor) complexes

Similar to the effectors (Figures 2 and 3), the superimposition of the GAP complexes leaves the ‘flat’ side of the G domain free (Figure 4A). Since many GAPs are membrane-bound or recruited to the membrane to stimulate

![Figure 4: Superimposition of GTPase-GAP and GTPase-GEF complexes.](image)

(A) GTPase-GAP complexes with the G domain in green, and RanGAP/Ran/RanBD1 in white/pink. (B) GTPase-GEF complexes with the G domain in green in the same orientation as in (A). The protruding pink and cyan coiled coils belong to the Sec2p GEF of a Rab protein (2EQB, 2OCY). RCC1-Ran is yellow, overlaps with the ‘membrane’ (orange rectangle) are seen in the complexes with DOCK9 and DOCK8 that are depicted in (D). (C) RhoGAP complexes 1GRN, 1OW3, 1TX4, 2NGR, 3MSX,5C2J, 5HPY, 5IRC, 5JCP. (D) Cdc42-DOCK9 (2WMO, 2WMN, 2WM9), Rac1-DOCK9 (3B13, 2YIN), the minor overlaps with the ‘membrane’ could be resolved by a small rotation.
GTP hydrolysis and thus create the GDP-bound form of the G domain, it would again seem logical to allow for simultaneous interaction of the G domain with the GAP and the membrane. The only exception is again the Ran-RapGAP-RanBD complex (Figure 4, gray and pink) where the linker of the C-terminal helix of Ran and the N-terminus of RanBD covers the ‘flat’ side of the G domain, but this complex is most likely not attached to membranes anyway.

Analysis of the interfaces in GAP complexes reveals that ArfGAPs tend to have the smallest solvent excluded areas of all GAPs, e.g. the ArfGAP/ankyrin domain of ASAP3 (3LVR/3LVQ; Ismail et al., 2010) buries only 562/715 Å², corresponding to a relatively low affinity, and thus had to be crystallized as a fusion protein (Ismail et al., 2010). As in most other GAP complexes, additional domains (like e.g. a PH domain that would facilitate membrane attachment) are missing in the ArfGAP construct that could compensate for the low affinity by e.g. increasing the local concentration of the GAP at the membrane. RhoGAPs bury larger interfaces in their complexes, ranging from 715 to 1184 Å² (15 sub-complexes in 11 PDB structures), similar to RabGAPs, which have related sequences and structures amongst each other (TBC domains), and bury 1036–1233 Å² (6 sub-complexes in the two structures 4HLQ and 2G77). Several RabGAPs have regions with positive charges following their catalytic domains that are thought to be involved in membrane localization (Kuhlmann et al., 2016).

Bacterial GAP mimics like the Legionella protein LepB are in the same range of interface areas [1098-1305 Å², 8 sub-complexes of 3 structures: 4JVS (Yu et al., 2013), 4110 (Gazdag et al., 2013), 4IRU (Mishra et al., 2013)] although they are mechanistically very different: they stabilize the catalytic glutamine of the switch II region in analogy to RanGAP, in contrast to the TBC GAPs that provide this glutamine in trans. Another bacterial GAP is the MglB protein that regulates cell polarity in the bacterium Myxococcus xanthus (Miertzschke et al., 2011) together with the GTPase MglA. MglA forms a complex with a MglB homodimer (3T12; Miertzschke et al., 2011) and buries 1188 Å², which is in the lower range. As mentioned above, the RanGAP-Ran complex does not form at the membrane, but probably at the filaments of the mammalian nuclear pore where the RanBP2 protein is located. RanGAP has been crystallized in form of a ternary complex with Ran and a Ran binding domain of RanBP2 (1K5G/1K5D; Seewald et al., 2002). The Ran binding domains help to sequester the Ran proteins that exit the pore in form of karyopherin-complexes (Sarić et al., 2007) and subsequently facilitate GTP hydrolysis (Seewald et al., 2003). In this complex, the Ran-RanBD1 buries 2730 Å² whereas the interface between Ran and RanGAP buries 1300 Å², which is average for a GAP. The largest GAP-G domain interfaces are found in RasGAPs (1420 Å², 1WQ; Scheffzek et al., 1997) and RapGAPs (Rap1-RapGAP 1094 Å² 3BRW; Daumke et al., 2004; Scrima et al., 2008), with the record holder Rap1-Plexin C1 with 1610–1734 Å² (4M8N; Wang et al., 2013). Probably due to the fact that they do not have to unfold parts of the G domain, the GAP proteins tend to be slightly less ‘engulfing’ than the GEFs described in the following. (GAPs bury on average 1205 Å² in 36 structures with 77 sub-complexes, GEFs 1407 Å² in 69 structures with 115 sub-complexes).

As expected from proteins that have to open the nucleotide binding site for nucleotide exchange by binding to the often completely unfolded switch regions, GEF complexes tend to bury the largest interface areas observed in small GTPase complexes, up to 2259 Å² in the Cdc42-DOCK9 complex (2WM9; Yang et al., 2009). In contrast to all complexes described so far, superimposition of the GEF complexes (Figure 4B) reveals that some GEF domains would clash with the membrane in the depicted orientation of the G domain, but not by a large amount. Interestingly, they all belong to GEFs of the DOCK family, in contrast to the DH-PH-domain containing GEF family. The largest clash is seen for the helical domain of the Cdc42-GEF DOCK9 (2WM9/2WMN/2WMO; Yang et al., 2009), followed by the Rac1-DOCK9 complexes [(2YIN; Kulkarni et al., 2011), 3B13 (Hanawa-Suetsugu et al., 2012)], where DOCK9 is slightly rotated compared to the Cdc42 complex, and lastly the Cdc42 complex with the DHR-2 domain of DOCK8 (3VHL; Harada et al., 2012). In all structures, the DHR-1 domain is missing that directly interacts with the membrane via its C2 domain fold, but the complete DOCK1-Rac1 has been modeled (Premkumar et al., 2010). In the model, the C-terminal helix of Rac is again almost parallel to the membrane, and the β-hairpin (Figure 1A, position 47) is close to the membrane. Thus, only a slight rotation is needed from the orientation in Figure 4 to avoid the clash with the membrane. In summary, also the GAP and GEF complexes would argue for the possibility of a preferred membrane interacting side of the G domain.

Interactions with the GDP state

As expected from the more dynamic state of the switch I and switch II regions in the GDP form, where they are not restrained by the γ-phosphate of GTP, there are relatively few proteins that bind to the GDP form of GTPases: in nuclear transport, NTF2 facilitates trafficking of the
‘empty’ RanGDP through the FG-repeat meshwork of the nuclear pore (Zahn et al., 2016), and the zinc finger domains of Nup153 at the inner ‘basket’ of the nuclear pore might form a storage site for RanGDP waiting for its GEF RCC1 to exchange the nucleotide (3CH5; Schrader et al., 2008). The buried areas are rather small, on average 765 Å² for NTF2 (two structures with six sub-complexes), and 513 Å² for the zinc fingers, respectively, (seven structures with 11 sub-complexes) indicating a weak interaction consistent with the transient nature of the sequestration at the nuclear basket for the zinc finger domains (Schrader et al., 2008). The interacting proteins are very different for membrane-localized G proteins: GDI proteins (GTP dissociation inhibitors) and the related REP protein bind to the Rho and Rab families, and the Arf effector Arfaptin binds to Rac1 in a GDI-like way both in the GTP and GDP form (Tarricone et al., 2001). GDIs come in three structural classes: RhoGDI, RabGDI and PDE6δ/UNC119 (Cherfils and Zeghouf, 2013). GDIs usually bind similarly to GDP and GTP forms of the G domain and therefore have to avoid contact to the switch I region (Figure 5A and B). Instead, they concentrate their interactions to the vicinity of the switch II region and to the C-terminal region close to their main binding target, the prenyl moiety at the C-terminus.

RhoGDIs have a loop with positive charges (Figure 5B) for membrane attachment, in addition to a negatively charged stretch at the N-terminus that is thought to bind to the C-terminal basic region of Rho/Cdc42/Rac proteins close to the lipid modification site that provides additional membrane affinity for the complex (Nitov et al., 2012). This would allow orienting the complex again with the ‘flat’ side of the G-domain close to the membrane (Figure 5B). In contrast, the RabGDIs can not be positioned in a way that the mobile effector loop (residues 225–228; Ignatev et al., 2008) close to the prenyl binding site (Figure 5D, highlighted in red) and the ‘flat’ side of Rab can reach the membrane at the same time. But it would be feasible for the RabGDIs to bind to Rab if it were in the position depicted in Figure 5D since there would be no clashes with the membrane, and subsequently extract Rab by binding to the lipid anchor and the membrane as postulated (Ignatev et al., 2008).

Interestingly, some toxins and pathogens use a similar mechanism to GDIs for binding to the GDP forms of G proteins: SopB (4DID; Burkinshaw et al., 2012) and

![Figure 5: Superimposition of GTPase-complexes with GDIs and related proteins. (A) GDIs and GDI-like proteins including toxins and pathogens. (B) RhoGDIs (1CC0, 1DOA, 1DS6, 1HH4, 4F38) in yellow, cyan and magenta, with the G domain in green, switch I in blue and switch II in orange. The blue plus/minus signs indicate the charged regions close to the C- and N-termini of the GDI, the green plus sign represents the charges of the C-terminus of the G domain. (C) Fic domains of adenylylating proteins (3N3V, 4ITR). (D) RabGDIs (1UKV, 2BCG, 3CPH, 3CPJ). The prenyl binding site in the back is indicated in red.](image)
Interaction of effectors with the GDP state forced by high affinity

If the affinity to the effectors is sufficiently high, they can bind the G domain even in the GDP-bound form. Examples are the Ran-karyopherin complexes (Villa Braslavsky et al., 2000), e.g. RanA181C-Kap95P (3EA5; Forwood et al., 2008) with a huge buried surface of up to 2182 Å² that provides sufficient binding energy to force the switch regions into the GTP conformation. Another interesting case are complexes of Ras with the Ras binding domain (RBD) of e.g. Raf kinase, where the RBD has been mutated (A85K) for higher affinity (Ras-RBDA85K/Rap1A E30DK31E, 3KUD/3KUC; Filchtinski et al., 2010). The increased affinity enabled crystallization of the H-Ras-GDP-RBD(A85K) complex that showed the switch I region forced into the GTP conformation by the RBD, but coinciding with high temperature factors of the switch I region and the attached RBD, consistent with the higher flexibility expected from switch I when the γ-phosphate is missing (Filchtinski et al., 2010). This illustrates the delicate balance between effector affinity and flexibility/dynamics of switch I. The dynamics of switch I can also be influenced by small molecules like cyclen (Rosnizeck et al., 2010), and some G domains have intrinsically increased dynamics, e.g. Rac1b, caused by a destabilizing insert (Fiegen et al., 2004), so that this protein has a drastically reduced affinity to the nucleotide and does not even require a GEF protein. The last two cases of GTPase-GDP complexes occur in Arl2, where the protein can be purified in the GTP-bound form if co-expressed with its effector PDE6δ, but where the GTP is partially hydrolyzed in one of the crystal forms so that it contains 3 : 1 GDP/GTP as confirmed by HPLC (Hanzal-Bayer et al., 2002), and in the complex of Rab1 with the coiled-coil domain of LidA (3SFV; Cheng et al., 2012), an effector mimic, that is able to bind both the GTP and GDP and renders Rab persistently active (Cheng et al., 2012).

Analyzing the self-interaction of G domains

Dimerization of Ras proteins at the membrane has been predicted by several groups now (e.g. Inouye et al., 2000; Gültenhaupt et al., 2012; Muratcioglu et al., 2015; Jang et al., 2016). Besides e.g. molecular dynamics simulations, crystal structures were also consulted to find putative dimerization interfaces, and a Ras dimer with an interface formed...
by helices $\alpha_4$, $\alpha_5$ and the $\beta$-hairpin (i.e. the ‘flat’ side) was described in (Güldenhaupt et al., 2012) that occurs in four different space groups and in the majority of the available crystal structures (50 of 71 structures). It is important to note that these dimers are mostly formed by crystallographic neighbors in the crystals, i.e. there are G domain monomers in the asymmetric unit. An analysis of the buried interface areas in the available crystal structures of solitary GTPases with PISA (Collaborative Computational Project, Number 4, 1994) indeed reveals that the largest interface areas are buried in this $\alpha_4/\alpha_5/\beta$-hairpin dimer, and that the same dimer actually occurs not only in seven different crystal forms of H-Ras (2RGC; Buhrman et al., 2007), 2QUZ/4L9S (Denayer et al., 2008; Ostrem et al., 2013), 3LO5 (Nassar et al., 2010), 1QRA/2RGG (Scheidig et al., 1999), 1AGP (Franken et al., 1993), 1IAQ (Spoerner et al., 2001), 3KKM (Shima et al., 2010), but also in one crystal form of K-Ras (4EPR; Sun et al., 2012) and one form of RhoE (1GWN; Garavini et al., 2002). K-Ras 4EPR has the same crystal parameters as H-Ras 3KKM, with the one of the monomers very slightly rotated compared to H-Ras (Figure 6). RhoE (1GWN) shows a similar dimer, although rotated some more, in yet another crystal form. In Ran, the C-terminal helix and the linker blocks the ‘flat’ side in the GDP form, but side chains in the interface are so different that this dimer could not form anyway, not even in the GTP form.

Although the superimposed dimers are very similar (Figure 6), the buried surface areas vary quite a bit due to slight differences in loops and side chain orientations in the wild type and mutant crystals in the different space groups. The largest area is $918\text{Å}^2$ in 2RGC (H-Ras Q61V-GppNHp; Buhrman et al., 2007), the smallest $772/766\text{Å}^2$ in 1QRA(H-RasGTP)/2RGG(H-RasQ61I-GppNHp). N-Ras has very few amino acid substitutions in this dimer interface compared to H-Ras and K-Ras, only His131, Lys135 and Tyr166 are different to both H- and K-Ras. Although it should therefore be theoretically possible for N-Ras to form the same dimer, a different packing with a very small interaction area ($335\text{Å}^2$) is found in the only available crystal structure of N-Ras (3CON, unpublished). The very different packing might have been caused by the in situ partial proteolysis with chymotrypsin during crystallization that left the switch II region either completely disordered or even digested.

As described above, it would be interesting to know if full-length Ras proteins would be able to form the same dimers as the truncated forms that are used for the overwhelming majority of crystal structure determinations. The closest to full length structures in the PDB are K-Ras 4DSU and 4DSO (Maurer et al., 2012). Both GDP and GSP forms crystallize as monomers and in the same crystal form, with the C-terminal basic lysine stretch (residues 175–180, Figure 1) mostly disordered and loosely packing into a neighboring, symmetry-related molecule, burying only $427\text{Å}^2$. No interface in the crystals resembles the $\alpha_4/\alpha_5/\beta$-hairpin dimer interface described above. One reason could...
be a potential clash of the (extended) C-terminal helix: superimposing 4DSU on top of the 1QRA dimer would cause Arg135 to clash with Glu168 of the second monomer. The C-terminal helix seems to be relatively rigid, the temperature factors are only slightly increased, and only the polylysine tail is probably very flexible, so this could be a possible explanation why the α4/α5/β-hairpin dimer is not seen in the crystals. On the other hand, the clash is not severe, and might be circumvented by minor side chain adjustments or domain rotations. Another reason for the absence of the α4/α5/β-hairpin dimer in full-length K-Ras could be that the charged lysine tail causes this specific packing as it might accidentally fit better into the neighboring molecule this way.

In summary, the crystallographic α4/α5/β-hairpin dimer that shows a direct face-to-face interaction of the H-Ras/K-Ras/RhoE G domains might be physiologically relevant, although it probably has a very low affinity and would need to be enhanced by other components, e.g. the lipid modification. Indeed, an intact CAAX box was shown to be necessary for dimerization of K-Ras (Nan et al., 2015). One could envision both a negative regulation of the interaction with effectors by the dimer blocking the ‘flat’ side of the G domain, or, on the other hand, a dimerization could tilt the G domains away from the membrane while staying anchored via the lipid modified C-terminus as proposed, e.g. for the Ras GDP form (Gorfe et al., 2007) or for K-Ras G12D in the GTP form (Prakash et al., 2016), and two effector molecules could then bind at the same time. A dimerization of effectors is thought to be required for activation of, e.g. Raf-1 kinase (Freeman et al., 2013), and Ras dimers were shown to form to activate MAPK (Nan et al., 2015), so a direct dimerization via the ‘flat’ face could be a possible mechanism. Other dimers have been postulated (Muratcioglu et al., 2015; Jang et al., 2016; Sayyed-Ahmad et al., 2016), but from the crystallographic point of view the interaction that buries the largest surface area is the most likely one. A dimer via the effector interacting β2-strand has just been regarded as most likely not physiologically relevant (Sayyed-Ahmad et al., 2016).

Conclusions and future directions

It is evident that the crystal structures of small GTP binding proteins and their complexes have contributed tremendously to elucidate the detailed interactions with their effectors and regulators. The defining feature of this protein family is a delicate balance between entropic repulsion by the switch regions that are more flexible in the GDP form as compared to the GTP form, and the enthalpic binding energies. Large interaction surfaces are needed to provide sufficient affinity especially for the GEF proteins that have to unfold the nucleotide-binding site to facilitate nucleotide exchange, and for the Ran proteins to achieve high affinity binding to karyopherins for secure transport through the nuclear pore.

A superimposition of all available complexes of GTPases revealed the quite surprising picture that the ‘flat’ side of the G domain formed by the C-terminal helix (α5) as long axis and a second axis from R128/R135 in helix α4 of H-Ras to the β-hairpin at residue 47 does not participate in any interactions with effectors, GDIs, GAPs or GEFs, except in case of Ran. Ran proteins use their C-terminus not for membrane attachment, but for shielding the binding site for its effectors in the GDP-bound form. Helix α4 is the least conserved part of small GTPases, it was found to be the major membrane interacting element (together with the C-terminal part of helix α5) in K-Ras (Prakash et al., 2015; 2016; Prakash and Gorfe, 2016), and it was speculated that helix α4 is an important determinant of specificity for cellular localization (Abankwa et al., 2010). As this helix is practically never involved in direct contacts with other proteins, it could achieve this function by influencing the interaction with the membrane.

Analysis of potential dimerization interfaces of small GTPases confirmed a possible interaction site exactly at the ‘flat’ surface of the G domain that is never (except in Ran proteins) covered by interacting proteins.

Most publications, original work as well as reviews, contain only comparisons to a limited set of related structures, so it might be fruitful in the future to look more often at the larger picture of the structures of this extended family of small GTP binding proteins. In this way, crystal structures can help to understand even the membrane interactions of these most versatile and ancient signaling proteins, in spite of the impossibility to crystallize them at a membrane.

Acknowledgments: The review summarizes the work that was achieved within the SFB 642. The German Science Foundation (DFG) is kindly acknowledged for continued financial funding. I would also like to thank all colleagues who helped with data collection, as well as the beamline staff of the synchrotrons ESRF, Grenoble, France, SLS, Vil- ligen, Switzerland and PETRA, Hamburg, Germany.

References


