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Rab GEFs and GAPs

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Abstract

Rabs are GTP-binding proteins with conserved functions in membrane trafficking. They are regulated by a diverse group of structurally unrelated GDP-GTP exchange factors (GEFs), and a family of GTP-hydrolysis activating proteins (GAPs) containing the conserved TBC domain. Recent structural and cell biological studies shed new light on the mechanisms of Rab GEF and GAP action, and the cellular trafficking pathways they act in.

Introduction

Rabs are a large group of GTP-binding proteins, comprising 11 members in budding yeast, where they are termed Ypts, and over 60 in humans with similar numbers in plants, worms and flies [1,2]. While not all have been studied, and relatively few in great detail, the current view is that Rabs function in controlling docking and tethering steps between membrane compartments and membranes and the cytoskeleton during diverse vesicle trafficking processes [3]. They do this by interacting with specific effector complexes often termed tethering factors, and thereby enhance the fidelity of vesicle trafficking and help to define organelle identity [4,5]. An important part of this model is the requirement for specific Rabs to be activated, switch from GDP to GTP form, and inactivated, hydrolyse GTP, at a specific membrane surface [6]. A further aspect is the need for temporal control of the initiation of Rab activation, and the length of time the Rab remains active. To do this Rabs need specific regulators, the GDP-GTP exchange factors (GEFs) to mediate their activation, and the GTP-hydrolysis activating proteins (GAPs) to terminate their activity. Here we discuss our current understanding of how distinct families of GEFs and GAPs control Rab function based on recent structural studies that shed light on their mechanism of action, and cell biological studies identifying the cellular trafficking pathways that they act in.

Rab regulation by GEFs in the secretory pathway

Trafficking within the yeast secretory pathway involves four Rabs, Ypt1p controlling ER-Golgi and intra-Golgi traffic, Ypt6p involved in recycling into the late-Golgi, and Ypt31–32p which together with Sec4p control late-Golgi to cell surface traffic. Yeast genetics combined with biochemistry and careful cell biological analysis has provided many of the foundations underpinning our understanding of Rab regulation, and has identified GEFs for all these Rabs.

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The two best examples are the conserved TRAPP complex and Sec2p which act as GEFs within the secretory pathway [7–9], and for which there is both cell biological and structural data (discussed later) on how they function. TRAPP-I is a 7-subunit complex recruited by interactions with the Sec23 subunit of the COPII coat to form ER-Golgi transport vesicles [10]. Once recruited TRAPP-I promotes activation of Ypt1p at the vesicle surface and tethering of the vesicles to one another via the Ypt1p effector protein Uso1p. A similar pathway exists in mammalian cells involving Rab1 and the Uso1p homologue p115. A second form of TRAPP with 10 subunits, termed TRAPP-II, exists in budding yeast where it exerts GEF activity towards Ypt1p and Ypt31–32p and plays a role in post-Golgi trafficking events [11]. While the TRAPP-II specific subunits are conserved in higher eukaryotes recent evidence suggests that TRAPP-II lacks GEF activity towards Rab11, the Rab most closely related to Ypt31–32p. Rather mammalian TRAPP-II interacts via its TRS130 subunit with the COP I coat and has GEF activity towards Rab1 [12]. The two forms of TRAPP therefore appear to integrate Rab1-dependent tethering on the COP II anterograde and COP I retrograde trafficking pathways operating from ER to Golgi and within the Golgi. On exiting the Golgi transport vesicles destined for the cell surface are first marked by Ypt31–32p that bind and recruit Sec2p, the GEF for the yeast Rab Sec4p [13]. Sec4p and its effectors promote first vesicle movement along actin cables towards the cell surface, then docking with specific sites on the cell surface at which growth occurs. It is important to note that both TRAPP and Sec2p have clearly defined mechanisms for recruitment to a defined membrane compartment, which is a requirement for any GEF if it is to promote Rab activation in a specific tethering or organelle identity pathway. Finally, Ypt6p is activated by the GEF Ric1p/Rgp1p which plays a role in recycling components of the exocytic machinery such as SNAREs from endosomes back into the late-Golgi for re-use [14]. Homologues of Ric1p and Rgp1p can be found in a wide range of eukaryotes, however to date none have been tested for GEF activity towards Rab6 the equivalent of Ypt6p.

Polarised trafficking in multicellular eukaryotes

It has long been accepted that if cells are to establish and maintain different domains of the plasma membrane then polarised trafficking must play a key role. This is best understood for the apical-basolateral polarization seen in epithelial cell layers. A series of studies have identified Rab8 and the closely related Rab10 as key players in apical and basolateral trafficking, respectively [15–18]. More recently it has emerged that in mammalian cells Rab8A is involved in trafficking to the primary cilium [19,20], a specialized membrane subdomain of the apical cell surface involved in signaling pathways important for normal organ development. Rab8 is most closely related to yeast Sec4p, and it is therefore perhaps not surprising that the mammalian homologues of Sec2p, the Rabin proteins act on Rab8 [21]. A proteomic study analyzing components of the BBSome, a complex involved in cilium biogenesis and function, identified Rabin proteins and subsequently went on to show that Rab8 plays a role in cilium formation [20]. Sec2-domain proteins therefore appear to be conserved regulators of the Sec4p/Rab8 GTPases involved in transport to the cell surface. However, there is a fly in this particular ointment since the fruit fly *Drosophila melanogaster* although having both Rab8 and Rab10 homologues lack an obvious Sec2p homologue. This suggests that there must be additional GEFs for the Rab8/10 family of GTPases.

Early endocytic trafficking and the Vps9-domain

Vps9-domain proteins form a large family of GEFs thought to be specific for the different members of the Rab5 subfamily, Rab5A-C, Rab17, Rab21, Rab22A, and Rab22B/31 in humans. The best characterized is mammalian Rabex-5 which cell biological data indicates acts on Rab5 at the early endosome, where it is critical for controlling the dynamics of membrane fusion. Rabex-5 is recruited to endosomes by interactions with ubiquitinated cargo molecules and the Rab-effector protein Rabaptin-5 [22–24]. These interactions provide a

means to restrict its GEF activity to the early endocytic pathway where Rab5 is required. Interestingly, in biochemical assays Rabex-5 can act on both Rab5 and the related GTPase Rab21 and not other Rab proteins [25].

Genetics screens for defective receptor mediated endocytosis in *C. elegans* have identified the Vps9-domain protein RME-6 as a regulator of Rab5 (RAB-5) at coated pits where it makes direct interactions with the clathrin adaptor complex. In *rme-6* mutants there is an accumulation of small vesicles consistent with an early block in endocytic trafficking. Mammalian RME-6, also known as Gapex-5, has been reported to regulate both Rab5-dependent endocytosis and Rab31 dependent insulin transporter trafficking [26–28]. Taken together with previous findings this suggests that specific Vps9-domain proteins might associate with different parts of the early endocytic pathway and target different members or groups within the Rab5 subfamily to control unique trafficking pathways.

Late endosomes and lysosomes

Rab7 has long been known to be a central regulator of lysosomes and diverse lysosome-like organelles. However, it still remains unclear how it is regulated in all these cases. Early studies in budding yeast implicated the Vam6p/Vps39p protein as a GEF for Ypt7p [29]. Later work in mammalian cells and zebrafish supported the idea that Vps39-family proteins function in endocytic trafficking, although did not directly test its GEF activity [30,31]. This picture was complicated when screens for endocytic traffic in *C. elegans* showed that SAND-1, a homologue of yeast Mon1, was essential for RAB-7 function [32]. Intriguingly, the Mon1p-Ccz1p complex in budding yeast had already been implicated in the delivery of components to the yeast vacuole, and to function in tethering which is typically seen as a Rab-dependent process [33]. New studies in *C. elegans* and mammalian cells now show that the complex of SAND-1/Mon1 and CCZ-1 are required for the maturation of early endosomes into late endosomes and lysosome, as well as for phagosome maturation [34,35]. These studies provide further evidence that the maturation of early to late endosomes is coupled to a Rab conversion process whereby Rab5 is replaced with Rab7 [36]. The SAND-1/Mon1 and CCZ-1 complex promotes Rab conversion by displacing Rab5 from early endosomes, and promoting recruitment of the HOPS complex with its associated Vps39 Rab7 GEF activity [35].

In addition to the lysosome, there is a group of more specialized lysosome-like organelles. However, almost nothing is known about the GEF regulation of Rab7-like Rabs such as Rab32 and Rab38 that are present on lysosome-related organelles such as the melanosome [37], and how this might relate to Rab7 regulation. It has however been reported that the Vps9-domain protein VARP which acts as a GEF for Rab21 is an effector protein for Rab32 and Rab38 [38,39]. This is reminiscent of the relationship between the Sec4p GEF Sec2p, which is recruited by Ypt31–32p, and underlines the potentially complex relationship between the different Rabs and their regulators on the same organelle. These observations suggest that both lysosomes and lysosome-like organelles arise from early endocytic compartments by a maturation or Rab conversion process in which Rab GEFs and their associated regulatory pathways play a key role.

Identification of higher eukaryote DENN domain Rab GEFs

The final family currently known to have Rab GEF activity is the DENN (differentially expressed normal versus neoplastic) domain proteins originally reported to have roles in diverse signaling pathways [40]. In humans this family comprises 7 subgroups DENND1-5, MTMR5/13 (myotubularin related proteins 5 and 13), and MADD (MAP-kinase activating death domain protein). They were originally implicated as Rab GEFs when the protein MADD was purified as a Rab3 GEF from brain [41,42]. More recently MADD and its *C. elegans* homologue AEX-3 have been found to also have activity towards Rab27 [43,44], which

intriguingly shares some common effectors with Rab3 and might indicate that some common feature is recognized by both GEF and effectors [45]. It is now emerging that DENN domains in general are likely to function as Rab GEFs. Pioneering genetics screens for endocytosis in *C. elegans* found that the receptor mediated endocytosis defective mutant *rme-4* encodes a DENN domain protein most closely related to mammalian DENND1 [46]. RME-4 was shown to interact specifically with the GDP form of Rab35, and then subsequently the mammalian protein was found to have Rab35 GEF activity [46–48]. Importantly, like other specific GEFs DENND1 has a specific mechanism to target to its site of action, in this case by interacting with components of the clathrin coat at the plasma membrane [46–48]. These findings suggest that other DENNS are also likely to be specific GEFs. Consistent with this view mutations in CRAG the fly homologue of DENND4 show defects in polarized sorting reminiscent of defects seen when Rab8 and Rab10 are inactivated in human cells [18,49]. However, to date none of the other DENN proteins has been tested for GEF activity.

Unifying themes from structures of distinct GEF-Rab complexes

Over the last several years, structures of five distinct GEFs in complex with nucleotide free Rab GTPases have been determined. Below and in Figure 1, we highlight key insights from each and then consider unifying themes. Additional discussion can be found elsewhere [50].

The Rabex-5 catalytic core consists of a helical bundle-Vps9 domain tandem [51]. In the complex with Rab21 [25], the Vps9 domain engages the switch/interswitch regions, props switch I in an open conformation, and inserts an ‘aspartate finger’ to disrupt the Mg^{2+} /phosphate binding site and stabilize a collapsed P-loop by mimicking interactions made by the γ phosphate of GTP. Mss4 has weak activity for Rab1, Rab3, Rab8, and Rab10 [52,53], and has been suggested to act as a form of Rab chaperone rather than a GEF in cells [54]. In the complex with Rab8 [55], Mss4 engages the interswitch region and sequesters switch I in a conformation that conflicts with the normal ordered structure of the $\alpha 1$ helix, thereby stabilizing a completely open/disordered nucleotide site. Sec2p deploys an asymmetric coiled coil [56] that interfaces with the switch/interswitch regions of Sec4p, contorting switch I so as to displace its N-terminus from the base region of the nucleotide site while simultaneously forcing an isoleucine from its C-terminus into the Mg^{2+} /phosphate region [57,58]. Unlike typical GEFs, the catalytic core of the TRAPP complex is distributed over multiple subunits [59] that engage the N-terminus, $\beta 1$, interswitch and switch regions [60]. The Bet3p C-terminus plays a prominent role by trapping switch I in an open conformation as it extends to occupy the Mg^{2+} /phosphate region. Interestingly, TRAPP accelerates GDP/GTP association as well as GDP release and consequently does not lower nucleotide affinity as much as other GEFs [61].

The *Legionella pneumophila* effector SidM/DrrA is required for Rab1 recruitment to the *Legionella*-containing vacuole [62,63], catalyzes exchange for Rab1 [63], and can displace prenylated Rab1 from Rab GDI [64,65]. The SidM/DrrA catalytic core interacts with $\beta 1$, the switch/interswitch regions and the P-loop of Rab1 in order to disrupt the Mg^{2+} /phosphate region and induce a helical conformation of switch I that is prominently displaced from the nucleotide site. The extensive interface with the switch regions precludes simultaneous binding of Rab1 to RabGDI [66,67]. The GDI displacement activity evidently results from competitive binding to Rab1 and is facilitated by the relatively weak affinity of Rab1 for RabGDI [66]. These studies also definitively resolve confusion over the location of the GEF core, which corresponds to the central region [65] and not the C-terminal region [63].

Switch I displacement occurs in all of the GEF/Rab complexes and plays a key role by dislodging the broadly conserved phenylalanine/tyrosine that caps one face of the guanine ring, thereby facilitating nucleotide egress. In addition, the GEFs disrupt the Mg^{2+} /phosphate site, either through direct occlusion or by promoting conformational rearrangements. Whereas

switch I displacement might plausibly accompany formation of the initial GEF-Rab-GDP intermediate, insertion of residues into the Mg^{2+} /phosphate region presumably occurs after or in concert with GDP release. Considering that Mg^{2+} dissociation is relatively rapid, some if not most of the GEFs may prevent Mg^{2+} rebinding, perhaps in conjunction with switch I displacement, to increase the rate of GDP-dissociation. Subsequent interactions that stabilize a collapsed or distorted P-loop conformation in the nucleotide free intermediate may decrease the probability of GDP rebinding. A more complete picture of the initial reaction pathway, as achieved for Sec7 domain Arf GEFs [68], requires structures of GEF-Rab-GDP complexes.

A conserved domain identifies a family of Rab GAPs

GAPs are thought to limit Rab activity, either temporally or spatially [4,69]. The simplest examples of these two possibilities can be found in the endocytic pathway. Hydrolysis defective Rab5 mutants prolong the time of Rab5 activity and thus cause more membrane fusion and grossly enlarged early endosomes. Normally Rab5 activation is tightly controlled to specific subdomains within early endosomes to refine compartmental boundaries important for the various endocytic sorting-events needed to discriminate cargo for recycling to the cell surface and degradation in the lysosomes [70]. Another function may be to attenuate Rab activation to prevent accumulation in undesirable locations, and thus help refine compartment boundaries. Because of these requirements multiple GAPs may act on a single Rab to help refine its site of action, and as we will discuss evidence exists for this. It is important to mention that GAPs are possibly less critical than GEFs, since many Rabs hydrolyze GTP at a reasonable rate and thus may not have an absolute requirement for a GAP.

Rab GAPs were first identified using yeast genetics and share a common TBC1 (Tre-2/Cdc16/Bub2) domain [71–77]. In yeast they are referred to as GAPs for Ypts (GYPs), and as TBC1 domain proteins in other species. Frustratingly, the initial biochemical data on yeast GyPs is inconclusive and they seem to show little substrate specificity *in vitro* [73,74]. However, genetics and cell biological screens have implicated various GYPs in discrete trafficking pathways suggesting they do have specificity *in vivo*, either due to their localization or additional regulatory factors. Gyp7p provides a good example of this latter possibility [77]. While *in vitro* Gyp7p has only partial specificity towards Ypt7p, *in vivo* it acts to control Ypt7p function during vacuole fusion but acts in concert with a protein kinase Yck3p [78]. Mammalian TBC1D20, Gyp8p in yeast, is a GAP where localization may be important for achieving absolute specificity. It has good specificity towards Rab1 and Ypt1p in biochemical assays but due to its trans-membrane domain is restricted to ER membranes and can thus only target Rabs that are present at this location [79,80]. Overexpression of TBC1D20 in animal cells inactivates Rab1 and causes collapse of the Golgi [80], while a genome-wide RNA interference screen in insect cells identified fly TBC1D20 as a physiologically relevant regulator of secretion [81]. In budding yeast there is a second GAP reported to act on Ypt1p at the Golgi, called Gyp1p [79,82]. Gyp1p is a peripheral membrane protein found at the Golgi, perhaps indicating that ER and Golgi pools of Ypt1p are controlled by discrete GAPs for specific purposes.

Endocytic roles for Rab GAPs

Multiple Rab GAPs have been implicated in the control of endocytic trafficking in multicellular eukaryotes, and the picture is somewhat confusing. In mammalian cells assays for the receptor mediated uptake of epidermal growth factor (EGF), and Shiga toxin transport from the plasma membrane to the Golgi have identified a series of GAPs controlling Rabs active early in endocytosis. RN-tre was first identified as a Rab5 GAP important for endocytosis [83]. Other studies have implicated it in the endocytic trafficking of Shiga toxin, where it controls the activity of Rab43, but not the Rab5-dependent receptor mediated uptake of EGF [84]. Biochemical GAP assays indicate that while Rab43 is the preferred substrate, Rab5A-C are

also substrates so it is possible that RN-tre may control both substrates *in vivo* and that additional regulatory partners come into play. RabGAP-5/RUTBC3 is another GAP acting on Rab5, for which it has a high degree of specificity [84,85]. A role for this protein in early endocytic trafficking is supported by both RNA interference and overexpression studies causing a specific block of EGF trafficking, while leaving other endocytic events untouched [84,85]. Historically, many studies have focused on the roles of Rab4, Rab5 and its related GTPases, and Rab11 in endocytic uptake and recycling. This narrow picture has begun to change due to the identification of other Rabs with “higher” numbers. Rab35 and its DENND1 GEF regulator have been found to be an important regulator of endocytosis in both human cells and *C. elegans* [46–48]. A GAP regulator TBC1D10A-C has been identified for human Rab35 and shown to function in controlling not only endocytosis but also trafficking events at the plasma membrane necessary for exosome formation [84,86,87].

Studies of phagocytosis in *C. elegans* found that mutations in TBC-2 cause an early block in phagocytosis due to altered regulation of RAB-5 [88]. Subsequently, a mammalian homologue TBC1D2/Arms was found to function in the control of cell adhesion by acting as a GAP for Rab7 [89]. While this apparent conflict may be due to differences in the substrate specificity of the human and *C. elegans* TBC1D2 proteins, it seems more likely that a more careful biochemical analysis of this protein is required before any firm conclusions are made. Similar concerns can be expressed for most of the characterized Rab regulators so this is not a specific criticism of this data.

Rab GAPs controlling polarised transport

As mentioned above Sec4p and Rab8 and their GEF regulators Sec2p and Rabin3 are important for polarized growth into the bud, and polarized trafficking and transport to the cilium in yeast and mammalian cells, respectively. In both cases GAPs have been identified to complete these regulatory systems. The polarized growth of budding yeast requires two TBC domain proteins Msb3/Gyp3p and Msb4/Gyp4p that act as GAPs for Sec4p *in vivo* [76,90]. Two other TBC proteins, Gyp11/App2p and Gyp5p, have also been reported to be required for polarized exocytosis and may also function as a GAP for Sec4p [91], however it is unclear how their function relates to that of Gyp3p and Gyp4p. A complementary study analyzing the TBC domain Rab GAPs in human cells found that TBC1D30 was a GAP for Rab8A, and showed that Rab8A was the sole Rab enriched on primary cilia [19]. This study also identified EVI5-like as the GAP for Rab23, which known to have a function at cilia during development, and TBC1D7 as the GAP for Rab17, which belongs to the Rab5-subfamily. Two closely related GAPs TBC1D1 and TBC1D4 have been implicated in the control of GLUT4 glucose transporter recycling via Rab10 [92,93]. One might imagine that this indicates redundancy between these two GAPs, however there is good evidence that this reflects tissue specific control of the GLUT4 recycling pathway. TBC1D4 is the predominant Rab10 GAP and is controlled by the Akt signaling pathway [93], except in muscle where TBC1D1 is more important and under the control of the AMP-activated kinase [92].

Non-TBC domain Rab GAPs

The sole known non-TBC GAP is the Rab3GAP1/Rab3GAP2 complex that as the name suggests is thought to act on Rab3 [94]. Mutations in Rab3GAP1 and Rab3GAP2 cause the related Martsolf and Warburg micro syndromes in humans [95,96]. These are characterized by neurological defects consistent with a function controlling Rab3. However, since plants have homologues of the Rab3GAP1 catalytic subunit but lack Rab3 it is possible that this GAP may also act on other Ras-superfamily GTPases. While no structural data exist for Rab3GAP, it does appear to use a similar arginine-finger catalytic mechanism to many other GAPs [97], the details of which we will now discuss.

Two fingers are better than one for TBC Domain Rab GAPs

TBC domain Rab GAPs conserve several signature sequences, including nearly invariant IxxDxxR and YxQ motifs [98]. Early on, mutational analyses implicated the IxxDxxR arginine as a catalytic ‘arginine finger’ by analogy with Ras and Rho family GAPs [74,82]. The structure of the Gyp1p TBC domain revealed an all helical fold with an N-terminal terminal subdomain containing most of the conserved motifs and a variable C-terminal subdomain [99]. Similar architecture and disposition of conserved residues has been reported for the mammalian proteins TBC1D22 and TBC1D14 [100].

Unexpected roles for the glutamine residues in the TBC domain YxQ and Rab DxxGQ motifs were revealed by the crystal structure of the Gyp1p TBC domain in complex with Rab33 (a fortuitous mammalian Rab substrate *in vitro*) and the transition state mimic AlF₃ [101]. As predicted, the arginine in the IxxDxxR motif inserts into the nucleotide site to stabilize the GDP-AlF₃-H₂O transition state mimetic (Figure 2). Remarkably, however, the glutamine in the DxxGQ motif does not contact the AlF₃-H₂O moiety as expected but instead engages the backbone of the TBC domain, allowing the YxQ glutamine to mediate polar interactions equivalent to those of the DxxGQ glutamine in Ras and Rho family GAP-GTPase complexes [102–104] as well as the G_α subunits of heterotrimeric G proteins [105,106]. In Rap, on the other hand, the DxxGQ glutamine is replaced by a non-catalytic threonine. RapGAP lacks an arginine finger but supplies a catalytic asparagine ‘thumb’ [107] analogous to the glutamine finger of TBC domains. Although the catalytic mechanism of TBC domains has been clarified, the determinants of Rab recognition remain largely unexplored. Even less is known about the catalytic mechanisms and recognition determinants for non-TBC domain Rab GAPs such as the Rab3GAP complex.

The gaps in our knowledge

While GEF and GAP regulators have been identified for many Rab proteins, there are still large gaps in our knowledge. A big concern, since it is central to our understanding of *in vivo* function, is the specificity of many of the GEF and GAP regulators. Structural studies have begun to elucidate the mechanisms of these regulators, yet it is still unclear what the key specificity determinants are and further structures are required. Many Rabs lack any known regulators, and it therefore seems important to search for the missing components.

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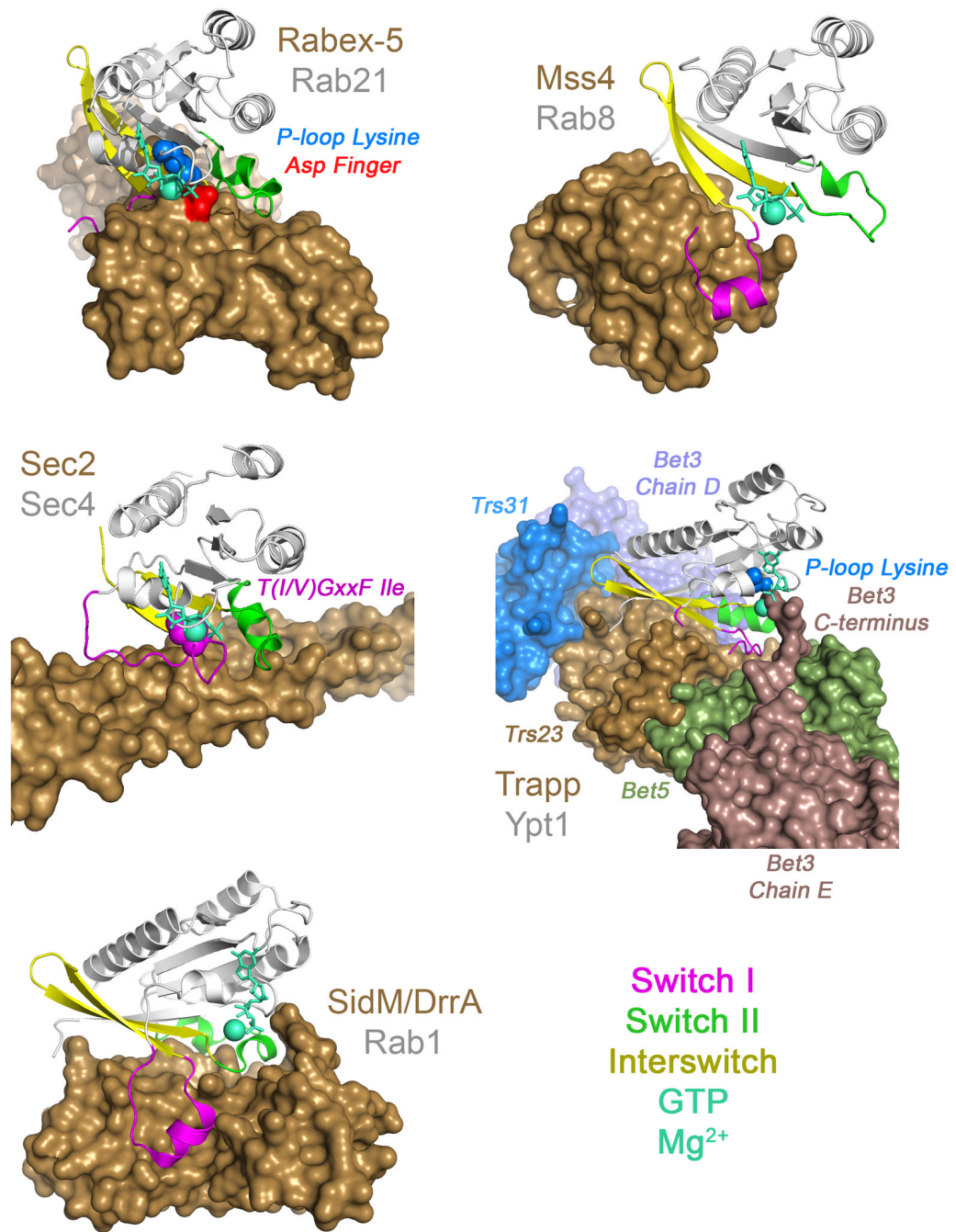


Figure 1. Comparison of GEF complexes with the nucleotide free intermediates of Rab GTPases
The nucleotide binding site is marked by GTP and Mg²⁺ from the structure of Rab5A after superposition. Images were rendered with PyMOL (www.pymol.org) using coordinates from crystal structures with the following PDB ID codes: 2OT3 (Rabex-5/Rab21), 2FU5 (Mss4/Rab8), 2OCY (Sec2/Sec4), 3CUE (TRAPP/Ypt1), and 3JZA (SidM/DrrA). See text for citations and discussion.

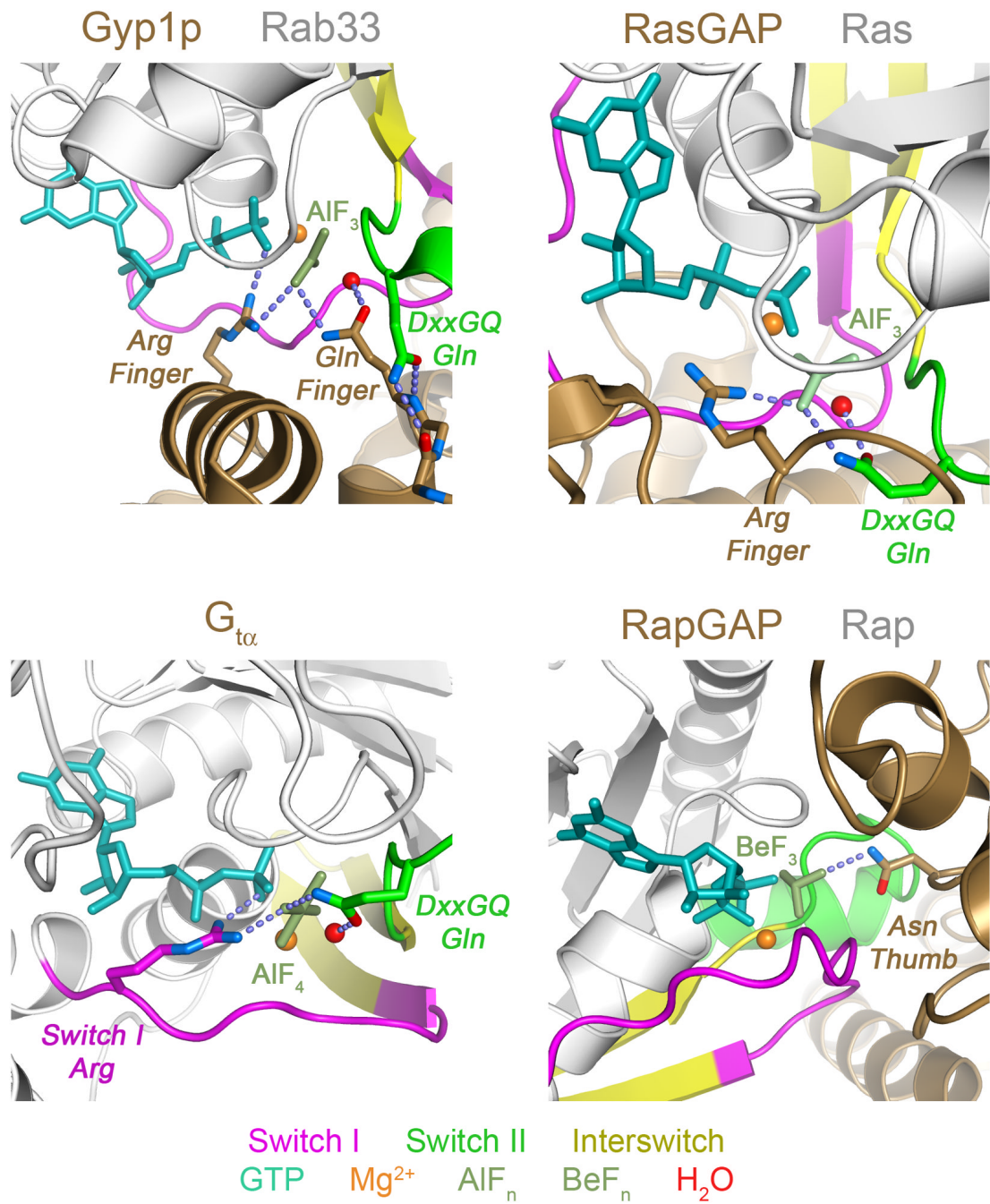


Figure 2. Comparison of the nucleotide sites of GAP-GTPase complexes with GDP and either the transition state mimic $\text{AlF}_3/\text{AlF}_4$ or the ground state mimic BeF_3

Images were rendered with PyMOL (www.pymol.org) using coordinates from crystal structures with the following PDB ID codes: 2G77 (Gyp1p/Rab33), 1WQ1 (RasGAP/Ras), 1TAD (G_{α}), and 3BRW (RapGAP/Rap). See text for citations and discussion.