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Structural Clues to Rab GTPase Functional Diversity*

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Rab GTPases are key regulators of membrane trafficking in eukaryotes. Recent structural analysis of a number of Rabs, either alone or in complex with partner proteins, has provided new insight into the importance of both conserved and non-conserved features of these proteins that specify their unique functions and localizations. This review will highlight what we have learned from crystallographic analysis of this important protein family.

Human cells contain almost 70 Rabs and Rab-like proteins (1, 2), and members of this large family of Ras-like GTPases are localized to distinct membrane-bound compartments (2). For example, Rab5 is on early endosomes, Rab6 is on the Golgi complex, and Rab7 and Rab9 are on late endosomes. Although some of the Rabs are tissue-specific, many are ubiquitous in their expression. Rabs are versatile catalysts; they participate in receptor cargo collection during transport vesicle formation, they enable motor proteins to interact with membranes to drive vesicle motility, and they interact with additional components to mediate the complex events of accurate docking and fusion of transport vesicles with their targets (2).

The yeast protein, Sec4, was the first Rab family member to be discovered (3). It is located on secretory vesicles and is needed for their delivery to the plasma membrane and subsequent fusion. Sec4 recruits cytosolic docking factors onto the secretory vesicle surface that enable the vesicle to find its target and engage the fusion machinery (4). Studies of Sec4 have provided a paradigm for Rab function in vesicle docking. In mammalian cells, Rab5 is required for fusion of early endosomes with other early endosomes during the processes of receptor-mediated endocytosis, recycling, and down-regulation (2). Like Sec4, Rab5 also recruits proteins to the early endosome membrane to facilitate endosome-endosome fusion (2). Rab9 functions in the transport of proteins from late endosomes to the Golgi complex (5). Rab9 recruits a cytosolic adaptor protein onto the surface of late endosomes that then binds to mannose 6-phosphate receptor cytoplasmic domains. In this manner, Rab9 participates in the collection of cargo into newly forming transport vesicles that bud from late endosomes (5). A final example is Rab27, which in melanocytes links melanosomes to the actin-based motor protein, myosin Va, via an adaptor protein named melanophilin (6, 7).

To catalyze these very different processes, Rabs interconvert between active, GTP-bound forms and inactive GDP-bound forms; the active GTP-Rabs interact with a large number of different so-called effector proteins. Effectors are defined as proteins that interact preferentially with Rabs when they are in their active, GTP-bound conformations. The number of effectors for an individual Rab protein is growing rapidly. It has been estimated that there are as many as 30 distinct effectors for Rab5A, and multiple effectors are being reported for other members of the Rab family (2). The effectors that have been identified to date show highly restricted interaction with one or a small number of specific Rabs. However, as larger numbers of Rabs are studied, we may find that Rab effectors interact with multiple, closely related Rabs in different tissues. For example, the protein Rabphilin interacts with Rabs 3A, 3B, 3C, and 3D as well as Rab8, Rab15, and Rab27A/B (8). These Rabs are close relatives and have evolved to retain this effector interaction. Much work is in progress to understand how Rabs interact with effectors to mediate their diverse cellular functions. Recent structural clues to these interactions are summarized here.

Understanding the Molecular Switch

Rab proteins closely resemble other Ras-related GTPases in their overall core fold; they contain a six-stranded β sheet, with five parallel strands and one anti-parallel strand, flanked by five α helices (Fig. 1). The structural basis of the Rab molecular switch can be defined as the portions of the Rab protein that are unique to and therefore specify the GDP and GTP-bound conformations. As for Ras, these elements are called "Switch regions." The first direct comparison of the structures of a Rab protein (yeast Sec4) bound to the GTP analog, GppNHp,[1] and to GDP revealed the precise identity of the Rab Switch elements that distinguish active from inactive forms (*yellow* strands in Fig. 1) (9). In analogy to Ras, there are two Switch elements, termed Switch I and Switch II. These regions appear to be the only elements of the protein that change conformation upon nucleotide binding.

When comparing all of the to date-identified Rab Switch elements (from Sec4, Rab5, Ypt7, Rab11), they overlap significantly in terms of their overall lengths and boundaries (9–12). However, in some cases, additional differences between GDP and GTP forms are detected just beyond the classical Rab Switch I domain. For example, Ypt7 and Rab11 Switch I domains comprise residues 36–45 and 39–46, respectively (analogous to Sec4 residues 48–56), but GDP/GTP differences are also seen for residues 50–54 in Ypt7 and 53–60 in Rab11 (11, 12). Similarly, the Switch I region of Rab5 seems longer than that of Sec4 (10).

The GDP-bound structure of Sec4 revealed disorder of Switch I residues 48–56 and rearrangement of Switch II residues 76–93 (9). Yet two conformations were detected for the Switch II domain; in one form, residues 76–83 were poorly ordered, and in the other form, residues 86–93 were poorly ordered. Sec4 binds magnesium with lower affinity than other GTPases, and the structures were obtained in cobalt chloride

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[1] The abbreviations used are: GppNHp, guanyl-5′-yl imidodiphosphate; CDR, complementarity-determining region; GDI, GDP dissociation inhibitor; RabSF1, -SF2 etc., Rab subfamily sequence 1, etc.

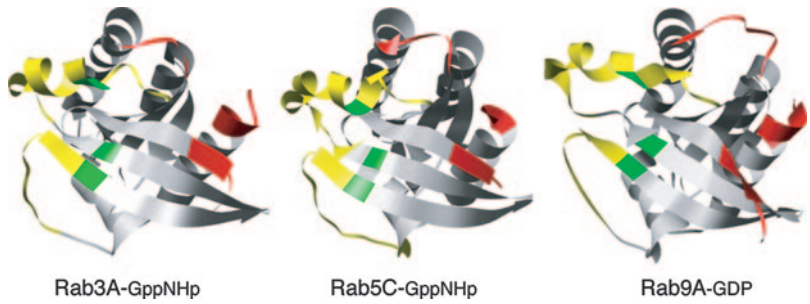

FIG. 1. **Comparison of the structures of three Rab proteins.** Drawings were generated using available coordinates (15, 18, 19) and UCSF Chimera software (31). Shown in *yellow* are Switch regions and in *red* are domains that correspond to the complementarity-determining regions identified in Rab3a (see Ref. 18). *Green* positions represent the hydrophobic triad residues in which side chains have different orientation in Rab structures despite their identical sequences (19).

(with cobalt bound to the enzymes). It is possible that *in vivo*, with magnesium present, one of these two conformations is favored.

Similar heterogeneity of the Switch II domain was seen in Rab5-GDP (10). Switch II would be expected to be contributed by residues 76–94, but these residues were essentially unchanged in a Rab5 "form B" and Rab5-GTP. In the form "A" structure, however, a significant conformational change was detected. Which structural form best represents Rab5-GDP? The structure of a GDP-bearing yeast Rab, Ypt1 bound to Rab GDP dissociation inhibitor (GDI) (13), revealed a structure closer to form A (with significant Switch domain disorder) suggesting that this is the GDP conformer that is recognized in the cell. Binding to a protein such as GDI that interacts preferentially with GDP-bound Rabs may stabilize form A; nevertheless, the ability of a Rab to be distinguished by its bound GDP or GTP likely involves the complete Switch I and Switch II transitions.

Rab11 Switch domains were also recently identified, and the unstructured Switch regions of Rab11-GDP appear to dimerize in a crystal of this Rab protein (12). No magnesium was present in this crystal form, and dimers of this protein were not detected in solution; thus, the physiological significance of dimerization is unclear. Similarly, Rab9-GDP dimers were seen in one crystal form (14) but not another (15). Interestingly, in the former structure, Rab9 bound strontium, and the Switch II domain was ordered as if in a GTP-bound form. Perhaps the variability in Rab-GDP Switch domain structures reflects conformational transitions between active and inactive forms and is influenced by the presence and nature of the bound metal ion. Indeed, the Switch regions of Ypt7-GDP containing a bound magnesium ion resembled more closely those of Sec4 than those of Ypt7-GTP in terms of their relative disorder (11).

Structural Clues to Rab Interactions

Because Rabs are structurally similar, their functional specificity and unique properties must be determined by sequences that distinguish one Rab from another in addition to Switch regions that indicate the nature of the nucleotide bound. Careful analysis of Rab sequences led to the identification of Rab-specific features that distinguish Rabs from other members of the Ras GTPase family (17). Pereira-Leal and Seabra (17) identified five so-called Rab family sequences, F1–F5, that are conserved among Rabs but not Ras or Rho GTPases. (F1, F3, and F4 are in Switch domains.) Moreover, four Rab subfamily-conserved (RabSF) regions were also identified and proposed to represent effector-interaction motifs (17). Indeed, the three Rab subfamily-conserved elements that are not in Switch regions correspond to domains shown to represent an interaction interface between Rab3A and its effector, Rabphilin (18) (see below). In addition, Rab proteins can be distinguished from Ras in terms of the orientation of parts of both Switch 1 and 2 domains (9–12).

By definition, effectors must recognize Switch domain determinants as they are proteins that interact preferentially with the GTP-bound Rab proteins. The structure of Rab3A bound to its effector, Rabphilin, confirmed this notion and revealed three additional Rab determinants (termed complementarity-determining regions or CDRs) that are important for Rabphilin binding (18). The CDRs (Fig. 1, *red* strands) are located just N-terminal to the first β sheet (β1), between α helix 3 and β sheet 5 (loop 7 or SF3), and near the C terminus (near the end of helix 5 and slightly beyond (SF4)). The CDRs correspond roughly to RabSF1, -SF3, and -SF4.

Comparison of the active conformations of multiple Rabs (Sec4-GppNHp, Rab3A-GppNHp, and Ypt51-GppNHp (9)) and (Rab5C, Rab3A, and Ypt51 (19)) revealed that the greatest differences between these Rabs were located in the loops between strands β2/3 and in loop 7. These correspond to RabSF2 (part of Switch 1) and to RabSF3. This independent approach thus revealed Rab-specific distinctions that are likely recognized by different effector proteins.

An unexpected structural anomaly was seen in the crystal structure of Ypt7 (11). Unlike the closely related Rab7 protein (16), Ypt7 seems to lack a well defined α helix that results in a long extended loop L4 in the protein (11). The significance of this structural distinction is not yet clear but may provide another unique determinant for effector recognition.

Structural Differences despite Sequence Conservation

Although CDRs are important for Rab3A binding to the effector Rabphilin, Switch regions also contribute to effector recognition *beyond* providing information regarding the identity of the bound nucleotide. Conserved hydrophobic elements within Switch regions appear to show conformational heterogeneity and contribute to effector interaction. Specifically, key residues in the Rab3A Switch region are important for Rabphilin binding, and although these residues are conserved in Rab5C, they are structurally unavailable for effector binding by that protein (19). Lambright and co-workers (19) studied a triad of invariant hydrophobic residues located at the interface between the Switch regions that were shown to be important for Rab3A interaction with Rabphilin (*green* positions in Fig. 1: Phe-59, Trp-76, and Tyr-91). In their independent Rab5C and yeast Rab5 relative, Ypt51 structures, they found that the conformation of this amino acid triad is dramatically altered by non-conserved sequences found between the Switch domains (19).

Cell biologists often assume that conserved elements in related proteins will assume a similar structure. Yet the conserved triad residues point their side chains at different angles in relation to the β strands of the core. These angle shifts create very distinct surfaces of related GTPases that are surely important for their ability to interact specifically with distinct effector proteins. Thus, Rabs have a related overall shape, but they are very different in subtle ways that will be recognized by binding partners.

Differences between Rabs can be seen when comparing the structures of Rab3A and Rab5C (Fig. 1). In addition to angle differences among helices, the lengths of helices also differ. In

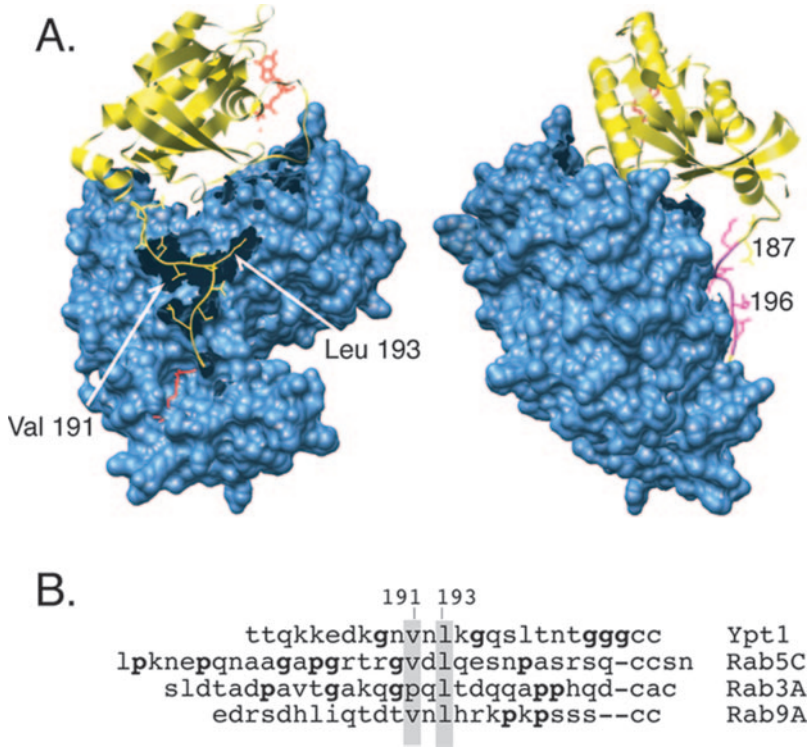

FIG. 2. *A*, structure of monoprenyl Ypt1 bound to GDI (13) drawn using UCSF Chimera (31). Shown in *yellow* is the yeast Rab, Ypt1 and in *blue*, GDI. At left, the Ypt-bound GDP is in *red* as is the GDI-bound, monogeranylgeranyl group. At right, the same structure is rotated 90° about the vertical axis; hypervariable domain residues are highlighted in *magenta*. *B*, comparison of Rab hypervariable domain sequences. Shaded in *gray* are hydrophobic residues predicted to be important for interaction of Rab hypervariable domains with GDI (13) and with geranylgeranyl transferase (16). Note the differences in hypervariable domain lengths and the abundance of helix-breaking amino acid residues.

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3A  icekmsesldtadpavtgakqgpqlsdqqvpphqd   cac  35/35 (100%)
3B  icdkmsdsldt-dpsmlgsskntrlsdtppllqqn   csc  16/35 (46%)
3C  icdkmseslet dpaitaakqntrlketpppqpn    cac  17/35 (50%)
3D  icekmneslepssssgsngk-gpavgdapapqpss   csc  13/35 (37%)
27A  krmercvdkswipegvvrsnghastdqlseekekga cgc   4/36 (11%)
8A  ikakmdkklegnspqgsn--qgvkitpdqqkrssffr cvll 10/35 (29%)
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FIG. 3. **Diversity of Rab hypervariable domains for Rabs that share effector interactions.** Identities to Rab3A are shown in *boldface*; the percent identity is shown at the *right*. *CXC* residues are prenylated. Note the abundance of proline and glycine residues in these sequences.

particular, the C-terminal α helix is truncated in Rab5C relative to those in either Rab3A or Rab9A. This is an important distinguishing feature among Rabs, as these sequences in Rab3A are important for Rabphilin interaction.

Rab Hypervariable Domains

Not shown in any of these three-dimensional Rab structures is the so-called C-terminal hypervariable domain, which is the most distinct element of Rabs. Rab hypervariable domains have attracted a great deal of interest as they represent the most divergent elements of Rab sequences (20). These domains are primarily unstructured (18, 21) and were cleaved off of (or engineered out of) Rab proteins to obtain the crystal structures shown in Fig. 1. Because of their unique signatures, hypervariable domains have been postulated to be key for Rab protein localization (22). Several subsequent studies suggest that Rab localization is likely to be more complex and involve additional, non-hypervariable domain determinants (23, 24).

Rab C termini differ in length (from 27 to 43 residues based on early sequence alignments (20)) and usually terminate with two cysteine residues that are geranylgeranylated. Sequence variability between Rab sequences begins within the last α helix and extends to the C termini of the proteins. As discussed above, the divergent sequences within the last α helix are important for Rabphilin recognition of Rab3A and led to speculation that Rabphilin binds to other, more C-terminal residues that extend beyond this helix and were not deciphered in the three-dimensional structure. It is noteworthy that the last α helix varies in length between Rab proteins because this feature is known to be important for certain effector interactions (18). Thus, differences in helix lengths contribute to the generation of distinct Rab protein surfaces.

The first glimpses at the structure of hypervariable domain sequences come from the work of Goody and co-workers (13) who determined the structure of a monoprenylated Rab protein (yeast Ypt1) in complex with a binding partner, GDI. GDI binds to prenylated Rab proteins in their GDP-bound conformations and can remove Rab proteins from membranes for delivery to their membranes of origin (see Refs. 25 and 26 for review). As shown in Fig. 2, the Rab GTPase (shown in *yellow*) sits at the top of the GDI protein and interacts with the so-called Rab-binding platform (26). The hypervariable domain extends down the side of GDI in an extended conformation; two key hydrophobic residues in the Rab make important contacts with GDI and appear to mount the hypervariable domain onto the face of GDI. Rab hypervariable domains contain conserved hydrophobic residues that can serve this interaction role (13, 16) (Fig. 2*B*). Finally, the monoprenyl group lies in a pocket at the bottom of GDI (13).

Rak *et al.* (16) also reported the structure of monoprenyl Rab7 bound to the Rab escort protein 1 (REP) that presents Rab proteins to Rab geranylgeranyl transferase. Again, the Rab C terminus is extended over the surface of REP, and the same two hydrophobic residues in the Rab hypervariable domain seem key to this interaction. Indeed, mutation of the corresponding leucine residue in Rab7 decreased the prenylation of this Rab by >90% (16). Thus, a key feature of hyper-

variable domains is to provide a polypeptide extension between the prenylation site and the globular GTPase domain. This feature suggests further that Rabs may extend some distance from a membrane by virtue of a long, hypervariable domain tether.

A side view of the Ypt1-GDI complex (Fig. 2*A*, *right*) shows that the relatively short, unstructured Ypt1 C terminus displays a significant bulge between the end of the last α helix of the Rab and the GDI surface. This gap may be more extreme for other Rabs; for example, Rab5 contains eight additional residues between the end of the last α helix and the two conserved residues that are proposed to anchor this Rab to GDI (Fig. 2*B*). This distinction is likely to influence the relative affinities of the interaction between different prenlyated Rabs and GDI and also their relative recognition by cellular proteins that recognize hypervariable domain sequences.

A final noteworthy feature of hypervariable domain sequences is their high content of proline and glycine residues, which likely contribute to helix breakage and generation of the extended structure that is important for GDI binding (13), Rab geranylgeranylation (16), and likely also, other protein interactions.

Interactions of Rab Proteins with their Effectors

Early endosome antigen 1 (EEA1) is a Rab5 effector that is important for endosome-endosome fusion (27, 28). It interacts with Rab5A, -5B, and -5C and Rab22, and these interactions seem not to require hypervariable domain sequences (29). Using BIAcore, Merithew *et al.* (29) showed that glutathione *S*-transferase-Rab5 bound to the EEA1 N terminus (residues 1–218) with a K_d comparable with that obtained using glutathione *S*-transferase-Rab5 residues 18–185 (2.3 *versus* 3.3 μM). Indeed, comparison of the hypervariable domain sequences of this group of Rab GTPases shows as little as 19% identity of Rab22A with Rab5C. Thus, for Rab effectors that interact with multiple Rabs, it is highly unlikely that hypervariable domain sequences will be critical for Rab-effector interaction.

Zhu *et al.* (10) have reported the structure of Rab5 bound to Rabaptin 5, another Rab5 effector involved in membrane fusion (30). As expected, Rab5 binding to Rabaptin 5 relies on extensive contact between Rab5 Switch domain constituents that define the GTP-bound state and the Rabaptin 5 protein dimer. All residues involved in Rab5 binding are absolutely conserved among Rab5 isoforms (A, B, C), and these include some residues conserved beyond Rab5 including the invariant hydrophobic triad residues discussed earlier. Importantly, these conserved residues present their side chains at different angles in the structures of other Rabs and can therefore contribute specificity in effector interaction (19).

Although the structures of only two Rab protein-effector complexes have been reported to date, it already appears that effectors (Rabphilin and Rabaptin 5) interact with distinct faces of Rab GTPases (10, 18). Whereas Rabphilin interacts significantly with Rab3A, N- and C-terminal features, Rab5 interaction with Rabaptin 5 relies predominantly on Switch domain interactions. Because truncated Rabs were used in both of these structural studies, it is possible that additional interactions are important. However, the lack of conservation of Rab5 hypervariable domain sequences between Rab5 A, B, and C strongly suggests that these regions will be less important for effectors that interact with multiple relatives of a single Rab class; such related Rabs have divergent hypervariable sequences.

Other examples of Rab effectors that interact with multiple Rabs include the Rab3A effectors Rim1, Rim2, Rabphilin, Noc2, and Slp4; all interact with multiple yet related Rabs (3) (Fig. 3). It is likely that the modes of interaction with these Rabs will involve Rab3-related features analogous to the Rab5 subfamily effector interactions described above. Determination of the structures of these Rab protein-effector pairs will be required to learn whether an overlapping set of Rab determinants are used for these effector interactions.

Summary and Future Perspectives

The next 5 years are sure to provide structural information for many more Rab proteins and their effectors. Hopefully, we will continue to learn more about how effector specificity is generated, how GDI interacts with doubly prenylated Rabs, and how Rabs and their effectors catalyze so many important processes in the eukaryotic cell cytoplasm. However, several conclusions are already clear. First, Rabs share a very similar fold but very distinctive surfaces that permit selective recognition by a diverse array of effector proteins. For effectors that interact with multiple related Rabs, these interactions are unlikely to involve Rab hypervariable domain recognition. Finally, even highly conserved amino acid residues can contribute to structural distinctions between members of this important protein superfamily.

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