Structure of the GTPase-binding Domain of Sec5 and Elucidation of its Ral Binding Site

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Helen R. Mott‡§, Daniel Nietlispach‡, Louise J. Hopkins‡, Gladys Mirey¶, Jacques H. Camonis¶, and Darerca Owen‡‡‡

From the Department of Biochemistry, University of Cambridge, 80, Tennis Court Road, Cambridge CB2 1GA, United Kingdom and the Institute Curie, 26, Rue d’Ulm, 75248 Paris Cedex 05, France

The exocyst complex is involved in the final stages of exocytosis, when vesicles are targeted to the plasma membrane and dock. The regulation of exocytosis is vital for a number of processes, for example, cell polarity, embryogenesis, and neuronal growth formation. Regulation of the exocyst complex in mammals was recently shown to be dependent upon binding of the small G protein, Ral, to Sec5, a central component of the exocyst. This interaction is thought to be necessary for anchoring the exocyst to secretory vesicles. We have determined the structure of the Ral-binding domain of Sec5 and shown that it adopts a fold that has not been observed in a G protein effector before. This fold belongs to the immunoglobulin superfamily in a subclass known as IPT domains. We have mapped the Ral binding site on this domain and found that it overlaps with protein-protein interaction sites on other IPT domains but that it is completely different from the G protein-geranylgeranyl interaction face of the Ig-like domain of the Rho guanine nucleotide dissociation inhibitor. This mapping, along with available site-directed mutagenesis data, allows us to predict how Ral and Sec5 may interact.

Spatial regulation of exocytosis is crucial for a variety of cellular processes, including embryogenesis, establishment and maintenance of cell polarity, and neuronal growth cone formation (reviewed in Ref. 1). The exocyst complex is involved in the final stage of exocytosis, when post-Golgi vesicles are targeted and dock to the plasma membrane. The exocyst consists of an assembly of eight proteins: Sec3, -5, -6, -8, -10, and 15, and Exo70 and -84, which form a complex localized to sites of vesicle docking to the plasma membrane during exocytosis.

In Saccharomyces cerevisiae the exocyst directs vectorial targeting of secretory vesicles to sites of membrane expansion, such as bud sites. It appears that Sec9p, which is always localized to the plasma membrane, forms a targeting patch and is the spatial landmark for polarized exocytosis (reviewed in Ref. 1). A subcomplex of Sec15p and Sec10p is localized to secretory vesicles (2) and a network of protein-protein interactions among the exocyst components bridges Sec15p-Sec10p to the targeting patch made by Sec3p (3).

In mammals the exocyst is involved in the targeting of Golgi-derived vesicles to the basolateral membrane of polarized epithelia. The regulation of the exocyst seems to be somewhat different in higher eukaryotes, because Sec3 does not have the same role. Rather, in polarized epithelial cells, Exo70 is localized to the plasma membrane (4), whereas the other components of the exocyst remain cytosolic, implying that exocyst assembly at the membrane is dependent upon Exo70, rather than Sec3 as in yeast.

Small GTPases of the Ras superfamily are involved in the regulation of a variety of cellular processes, including growth, differentiation, actin cytoskeleton, nuclear transport, and vesicle transport. Because many of these processes involve exocytosis, it was likely that small GTPases could play a role in exocyst regulation (5). In yeast, Sec4p, a homologue of Rab3A GTPase, anchors Sec15p to secretory vesicles (2). The exocyst is anchored to the plasma membrane via the interaction of Sec3p with another GTPase, Rho1p (6). Sec3p has also been shown to bind to Cdc42, another member of the Rho family (7). Finally, it was shown that Exo70p interacts with Rho3p at the plasma membrane (8). The role of the Rho family GTPases, which regulate the actin cytoskeleton, in exocyst regulation implies a coordination of cytoskeletal changes with exocytosis.

In mammalian cells, the exocyst components do not seem to interact with Rab3A or Rho family members. Rather, it was recently shown that the exocyst is regulated by yet another GTPase, RaL (9–12). RaL is a Ras family small G protein that is not present in S. cerevisiae. Both activated Ral and Ral inhibition disrupt polarized exocytosis in epithelial cells, suggesting it is necessary for the GTPase to cycle between the GDP- and GTP-bound forms to direct vesicle movement. RaL, like Rab3A, is localized to secretory vesicles and the plasma membrane, but the exocyst component responsible for interacting with RaLA was found to be Sec5. In yeast, Sec5p is at the center of the exocyst complex, linking the Sec15p-Sec10p subcomplex to the rest of the exocyst via its interactions with Exo70p, Sec3p, and Sec6p (3). Thus, Sec5 may have a different role in the mammalian exocyst.

Ral small GTPases are members of the Ras superfamily of small G proteins implicated in oncogenesis, endocytosis, actin dynamics, and membrane trafficking. Downstream effector
proteins identified for Ral include Ral BP1\(^1\) (or RLIP-76), which mediates the effects of Ral on endocytosis (13, 14) and interactions with the Rho family GTPases (15), filament, an actin filament cross-linking protein, and phospholipase D1, which is involved in vesicle trafficking (16). The discovery that Ral is involved in regulation of exocytosis provides a link between secretory and cytoskeletal pathways.

The exocyst was found to bind specifically to GTP-bound RalA (11). One region of small G proteins that is sensitive to the state of the bound nucleotide is the effector loop, which interacts with downstream effectors. The effector loop mutant D49E does not bind to Sec5 and has been shown to disrupt transport of proteins to the basolateral surface in polarized epithelial cells (9).

The region of Sec5 responsible for Ral binding was identified and comprises the first 80 amino acids (10). The first 95 residues of Sec5 contain a putative domain, the IPT (17), which is found in some cell-surface receptors such as Met and Ron and in intracellular transcription factors, e.g. NF-κB, where it is responsible for DNA binding. A domain of this type has not been found in a G protein effector so far, and it is not possible to predict how it will interact with Ral. In contrast, the topology of the Ras-binding domain of the Ras effectors is structurally conserved and forms a ubiquitin-like fold (18, 48, 49). It is thus of great interest to study how the Ral GTPases (the closest homologues of Ras) interact with their own effectors. There is some precedent for Ig domain-G protein interactions. A guanine nucleotide dissociation inhibitor (GDI) for Rho family proteins contains an Ig domain, whose structure in complex with Cdc42 and Rac has been solved (19–21).

We have solved the structure of the Ral-binding domain of Sec5 using solution NMR techniques and find that it forms an IPT fold. We have mapped the binding of Ral-GMPPNP to this domain and found that the surface of Sec5 that interacts with Ral is similar to that used in other IPT domains for protein-protein interactions. Furthermore, this surface is different from that used in the Rho GDI Ig domain for contacting the Rho family proteins and their geranyl-geranyl moiety. How Ral may bind to the IPT domain of Sec5 will be discussed and compared with other G protein-effector interactions.

**EXPERIMENTAL PROCEDURES**

**Protein Expression**—The IPT domain of murine Sec5 (residues 5–97) was expressed as a His-tagged fusion protein. Labeled protein was produced by growing Escherichia coli BL21 in a medium based on MOPS buffer, containing 5% Celpone (Spectra Stable Isotopes), and \(^{15}\)NH,Cl and \(^{13}\)C-glucose. The fusion protein was affinity purified on a Ni\(^2+\) column and cleaved from its His-tag with Factor Xa (Roche) followed by gel filtration. The NMR buffer was 20 mM sodium phosphate, pH 6.0, 50 mM NaCl, 10 mM dithiothreitol, 0.05% NaN\(_3\). NMR experiments were run with a protein concentration of \(\sim 1 \text{ mM}\).

Ral was expressed as a His-tagged fusion protein and affinity purified in the same manner as Sec5. The purified protein was concentrated in a GTPase reaction mixture for 70 ps. The GTPase reaction mixture was then adjusted to 100 ps, followed by gel filtration. The NMR buffer was 20 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1 mM MgCl\(_2\), 0.05% NaN\(_3\). A 0.4 mM sample was prepared and used to record the first \(^{13}\)N HSQC in the Ral titration.

GMPPNP was then added into the Sec5 sample to give titration points at the following ratios: 1:0.1, 1:0.25, 1:0.5, 1:0.8, 1:1, and 1:1.5 (Sec5:Ral-GMPPNP). \(^{13}\)N HSQC spectra were recorded for each titration point.

**RESULTS**

**Description of the Structure**— Backbone resonances of the Sec5 IPT domain were assigned using intra-HNCA (23), HN(CO)CA, HN(CACB), and CBCA(CO)NH experiments (reviewed in Ref. 24). Side chain resonances were assigned using (H)CC(CO)NH, H(CC)(CO)NH, and HCCH-TOCSY experiments. NOEs were measured from \(^{15}\)N-separated NOESY (961 NOEs) and \(^{13}\)C-separated NOESY (2,662 NOEs) experiments. Initial structures were calculated using a total of 2,941 unique (non-degenerate) NOE restraints, (1,350 unambiguous and 1,591 ambiguous) and 42 pairs of dihedral restraints from TOFALOS. After 8 iterations, there were 2,052 unambiguous NOEs and 828 ambiguous NOEs. In the final iteration, 100 structures were calculated; the 25 with the lowest energy were selected for analysis.

The structure of the Sec5 IPT is well defined by the NMR data and has good covalent geometry (Table I). The family of structures and the closest structure to the mean are shown in Fig. 1. The domain forms an Ig-like \(\beta\)-sandwich, consisting of eight \(\beta\)-strands that pack together into two \(\beta\)-sheets. The topology of the Sec5 domain is shown in Fig. 2, along with the topology of an Ig V-type domain and that of the IPT domain of the transcription factor NF-κB. The first \(\beta\)-strand is split into two, a and a’, connected by three residues that form a bulge centered around Pro-15. This is followed by strand b, which packs against strand a in an anti-parallel fashion and then strands c and c’, which form the edge of the second \(\beta\)-sheet. The b-c loop is interrupted by a single turn of 3\(\alpha\) helix. The c’ strand is followed by strand d, which forms the edge of the first \(\beta\)-sheet, packing against strand e. This is followed by strands f and g, which complete the second \(\beta\)-sheet. The last strand is also split into two: g, which forms an anti-parallel connection with strand f and g’, which forms a parallel connection with strand a’.

**Comparison to Other IPT Domains**—The first structures of IPT domains determined were from the DNA-binding domains of transcription factors such as NF-κB (28, 29) and revealed a 7-stranded \(\beta\)-sandwich, which differs from that of the Ig V-type domains in that it is missing strand c’ from the first \(\beta\)-sheet and strand d is much shorter (Figs. 2, b and c, and 3). A sequence alignment of Sec5 IPT with other IPT domains (Fig. 4) was constructed after the structure of Sec5 IPT was solved, on the basis of tertiary structure alignment of Sec5 with NF-κB (28) and Bacillus stearotherophilus α-amylase (30) (PDB codes 1fs and 1qho), using TOP (31). Alignment on the basis of sequence alone was not accurate, even when the secondary structures of IPT domains such as NF-κB were used to guide the alignment. The sequence alignment available from data bases such as Pfam is also incompatible with this structure-based alignment, probably because the sequence identity between Sec5 and the other IPT domains is extremely low.
binds to Ral, and the binding contacts on Sec5 were mapped by

They include the highlighted regions of the IPT domain that may generally be not likely to be conserved between different proteins, they domains within the same molecule. Although the residues are the IPT domains are also involved in contacting other protein program domains, or other protein domains were analyzed using the x-ray crystallography in complex with either DNA, another IPT dimerization. The IPT domains whose structures have been solved by ribbon representation of the structure that is closest to the mean. The backbone amides changes, and this usually causes a change in any amide depends on the relationship between the chemical shift difference and the rate of exchange between the free and bound states. If the exchange rate were higher than the chemical shift difference, a single peak would appear at a position between the chemical shift of the free and bound forms. If the exchange rate is lower than the chemical shift difference, two peaks would be observed, one for the free form and one for the bound. In the intermediate case (the exchange rate is comparable with the chemical shift difference), the peaks become broadened and may be unobservable. In the Sec5-Ral complex spectra, all the resonances that change significantly are in slow or intermediate exchange. None of the resonances appear to be in fast exchange, because they do not shift gradually as more G protein is added (Fig. 5A). In addition, new resonances appear as the ratio of Sec5:Ral approaches 1:1, for example that of Leu-92 (Fig. 5A). There is a trend for the signal intensities to decrease because of the increase in correlation time between 10 kDa Sec5 and the 30 kDa Sec5-Ral complex. Some resonances in Sec5 disappear completely in the 1:1 complex, e.g. Arg-37 (Fig. 4 and 5A). It should be noted that NMR mapping implicates a larger surface than the actual contact site, because secondary effects will be observed. Thus, changes in backbone amides that are not exposed to the solvent have been excluded from Figs. 3 and 5B. In Sec5, the changes are concentrated on one face of the domain, comprising strands a, b, e, and g (Figs. 2, 3, and 5B). There are no changes in strands c, c', and f.

### DISCUSSION

The structural and sequence alignments reveal that, although the overall folds are similar, the Sec5 domain is more similar to the bacterial a-amylase than to NF-κB (Figs. 3 and 4). One of the major differences between these domains is the region between strands c and e. In NF-κB there is a loop containing a single turn of a 3_10 helix, then the c' strand, then another turn of 3_10 helix and strand d, which is only 2 residues; in Sec5 and a-amylase the loop between c and c' is much shorter and does not contain any helix and strand d is longer (Fig. 4). The other main difference between Sec5/α-amylase and the transcription factors is that there is a 6-residue insertion in the e–f loop in the DNA-binding proteins. This insertion is visible in the structures, because the loop protrudes from the surface in NF-κB (Fig. 3).

#### Table I

| Number of experimental restraints | Unambiguous | 2052 |
|----------------------------------|-------------|------|
| Dihedral restraints from Talos   | 828         |      |
| Co-ordinate precision          |             |      |
| r.m.s.d. of backbone atoms (7–94) (Å) | 0.55 ± 0.07 | 0.44 |
| r.m.s.d. of all heavy atoms (7–94) (Å) | 0.94 ± 0.12 | 0.82 |
| From the experimental restraints |             |      |
| NOE distances (Å)               | 0.009 ± 0.002 | 0.008 |
| Tales dihedral angles (°)       | 0.317 ± 0.051 | 0.271 |
| From idealized geometry         |             |      |
| Bonds (Å)                       | 0.0015 ± 0.0009 | 0.0015 |
| Angles (°)                      | 0.321 ± 0.007 | 0.315 |
| Improper (°)                    | 0.392 ± 0.013 | 0.215 |
| Final energy                    | –669.34 ± 7.93 | –671.67 |
| Ramachandran analysis           |             |      |
| Residues in most favored region | 70.4%       | 72.0% |
| Residues in additionally allowed regions | 21.1% | 20.0% |
| Residues in generously allowed regions | 8.5% | 8.0% |
| Residues in disallowed regions  | 0.0%        | 0.0% |

*SA* represents the average r.m.s. deviations for the ensemble.

*SA* represents values for the structure that is closest to the mean.

r.m.s.d., root mean square deviations.

The Lennard-Jones potential was not used at any stage in the refinement.

### Fig. 1. The structure of the Sec5 IPT domain. On the left is a backbone trace of the 25 lowest energy structures. On the right is a ribbon representation of the structure that is closest to the mean. The β-strands are labeled. This figure was generated with Molscript (46) and Raster3D (48).

The transcription factor IPT domains have two major roles; they are involved in DNA binding and homo- and heterodimerization. The IPT domains whose structures have been solved by x-ray crystallography in complex with either DNA, another IPT domain, or other protein domains were analyzed using the program CONTACT (32) with a cutoff distance of 4.0 Å. The residues involved in DNA binding are generally basic or polar (Fig. 4, shaded blue) and are not conserved in IPT domains that do not interact with nucleic acids, such as α-amylase and Sec5. The IPT domains are also involved in contacting other protein domains within the same molecule. Although the residues are not likely to be conserved between different proteins, they highlight regions of the IPT domain that may generally be involved in protein-protein interactions (Fig. 4, shaded green). They include the a’/g’ surface (Figs. 3 and 4) and the b/e surface in both α-amylase and the transcription factors.

**Delineation of the Ral Binding Site**—The Sec5 IPT domain binds to Ral, and the binding contacts on Sec5 were mapped by titrating unlabeled Ral into ^15N-labeled Sec5. The resulting changes in the ^15N HSQC spectrum are shown in Fig. 5A. When two proteins interact, the chemical environment of the backbone amides changes, and this usually causes a change in chemical shift. Such changes can be grouped into three regimes: fast, intermediate, and slow. The regime observed for any amide depends on the relationship between the chemical shift difference and the rate of exchange between the free and bound states.
The residues involved in dimerization in the transcription factors map to one surface of the domain, comprising residues from strands a, b, d, and e (Figs. 4 and 5, shown in red). This is in contrast to Ig-Ig interactions, such as the Cd2-Cd58 complex, where the interaction surface is on the opposite surface of the β-sandwich and is composed of residues in strands g, f, c, and c’ (33). The residues involved in dimerization are generally hydrophobic, although some salt bridges are also found in the dimer interfaces. These hydrophobic residues are not conserved in Sec5 except where they are involved in packing (e.g. Ile-13). There is one notable exception, Val-61 in Sec5, which is not conserved in α-amylase and is equivalent to Val-313 in NF-κB. This side chain is exposed on the surface of the Sec5 even though it is hydrophobic. Despite this, it is not likely that Sec5 dimerizes via the IPT domain in a manner similar to the transcription factors; the correlation time of this domain, determined from the T1/T2 ratios of residues in secondary structure, is 5.9 ns (data not shown), which is consistent with a monomeric protein of this size.

The Ral binding surface on the Sec5 IPT domain can be compared with the regions of the IPT domains involved in interactions with other molecules (Figs. 4 and 5B, shown in green). The α-amylase IPT domain uses the same face to contact other domains within the same protein (30). NF-κB and the other DNA-binding proteins use the same face both for contacting other IPT domains and for contacting other domains within the same protein (28, 29, 34–40). The DNA-binding residues in these IPT domains are close to the protein-protein interaction surface but only partially overlap. Interestingly, the other Ig-like domain that has been found to contact G proteins, that from Rho GDI, uses the opposite face to contact the Rho family proteins (Fig. 5B) (19–21). It is perhaps not surprising that this divergence in binding interfaces exists between the GDI and Sec5, because the GDI domain is quite different; it has several extra β-strands, one of which is involved in G protein binding (Figs. 3 and 5B). In addition, the GDI domain, although it interacts with the G protein, makes the majority of its contacts with the geranyl-geranyl moiety that is covalently attached to the C terminus of the Rho family proteins.

A number of G protein-effector complex structures have been solved and have shown that the way that proteins can contact G proteins varies significantly. In most G protein-effector complexes, the effector contacts switch I (the effector loop). The Sec5 domain also makes contacts with the effector loop, because mutation of Asp-49 of Ral abrogates its interaction with Sec5 (9). In other G protein-effector complexes, the structural motifs that interact with the effector loop vary widely, so it is not possible to predict which region of Sec5 may be contacting this region of Ral.

In several complexes, for example the Ras-effector complexes (18, 48, 49) and the Cdc42-CRIB effector complexes (41, 50, 51), an intermolecular β-sheet is observed, formed by an interaction...
between the β-strand of the G protein and a strand from the effector. In the case of Sec5, it is tempting to speculate that a similar intermolecular β-sheet will be formed in this effector complex, because many of the residues implicated in binding to the Ral are within strands a, b, e, and g. If an intermolecular β-sheet is formed in this complex, it must involve a β-strand of the Sec5 IPT that is available to make hydrogen bonds. The β-strand that fulfils this criterion and experiences chemical shift changes on Ral binding is strand d. If, however, this were involved in an intermolecular β-sheet interaction, there would have to be some structural rearrangement of the Sec5 because there is not enough space between strand d and the single turn of helix in the b-c loop to insert part of the G protein there.

The other Ral effector that has been studied in some detail is Ral BP1 or RLIP. The Ral-binding domain of this protein has been delineated and comprises residues 403–499 (15). Although there is no structure available for this protein, secondary structure predictions show that RLIP is predominantly α-helix and that the Ral-binding domain partially overlaps a potential coiled-coil region. Thus, the interaction between Ral and RLIP is likely to be significantly different from that between Ral and Sec5. There are other G protein-effector complexes where the effector domain is predominantly α-helix, e.g., Rho-PKN (42), Rac-Arfaptin (43), and Rab-Rabphilin (44), but the structures and interactions are highly variable.

Although the IPT fold exists in most phyla, the IPT domain of Sec5 seems to appear with Ral in evolution. In S. cerevisiae and Arabidopsis thaliana, where Ral does not exist, Sec5 lacks the IPT. In S. cerevisiae the exocyst components that bind to G proteins do not contain any regions with homology to the IPT domains, so it is possible that Ral is the only small GTPase controlling the exocyst that binds an IPT. The other Ral effector proteins, Ral BP1, filamin, and phospholipase D, do not contain an IPT domain. It remains to be seen whether other Ral effectors are isolated that use an IPT to mediate their interaction with Ral.

Directed exocytosis is crucial to the regulation of several cellular processes. Central to the understanding of regulation of exocytosis is the role of small G proteins in recruitment of the components of the exocyst complex. We have solved the structure of the Ral-binding domain of the mammalian exocyst protein, Sec5. This is the first structure of a Ral effector domain and the first G protein effector that utilizes an IPT fold to contact the GTPase. We have shown that the Sec5 Ral-binding domain forms an IPT domain that is topologically closer to α-amylase than to the transcription factors such as NF-κB.
Mapping of the Ral binding site in the Sec5 domain reveals that, within the family of IPT domains, a similar region is used for protein-protein interactions and that this region is not the same as that used for interacting with DNA. Comparison of the Ral-binding surface of Sec5 with the region of GDI that interacts with Rho family G proteins shows that the contacts between Sec5-Ral and GDI-Cdc42 are significantly different. A detailed analysis of the contacts that Sec5 makes with Ral awaits determination of the three-dimensional structure of the complex. The information presented here can be used to help design mutants that disrupt Ral-Sec5 interactions. Such mutants could be used to elucidate the role of the Sec5-Ral interaction in exocyst assembly at the plasma membrane.

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