Structural Basis for Arl1-Dependent Targeting of Homodimeric GRIP Domains to the Golgi Apparatus

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Summary

Golgins are large coiled-coil proteins that play a role in Golgi structure and vesicle traffic. The Arf-like GTPase Arf1 regulates the translocation of GRIP domain-containing golgins to Golgi membranes. We report here the 1.7 Å resolution structure of human Arf1-GTP in a complex with the GRIP domain of golgin-245. The structure reveals that the GRIP domain consists of an S-shaped arrangement of three helices. The domain forms a homodimer that binds two Arf1-GTPs using two helices from each monomer. The structure is consistent with golgin-245 forming parallel coiled-coils and suggests how Arf1-GTP/GRIP complexes interact with Golgi membranes via the N termini of Arf1-GTP and the C-terminal tails of the GRIP domains. In cells, bivalent association with Arf1-GTP would increase residence time of the golgins on Golgi membranes. Despite no conservation of sequence, topology, or even helical direction, several other effectors form similar interactions with small GTPases via a pair of α helices, suggesting a common structural basis for effector recognition.

Introduction

Small GTPases of the Ras superfamily control a wide range of cellular events and are divided into the Ras, Rho, Ran, Rab, and Arf classes (Takai et al., 2001). For all classes, a cycle of GTP binding and hydrolysis is linked to a conformational change which alters interaction with effectors (Vetter and Wittinghofer, 2001). The Arf class of GTPases is distinguished from the others by an N-terminal amphipathic helix, typically myristoylated, that mediates interaction with membranes (Chavrier and Goud, 1999). In addition, the Arf GTPases undergo a “front-back” change so that in the GTP-bound form the amphipathic helix is displaced from a hydrophobic pocket in the GTPase and instead acts to stabilize interaction with lipid bilayers (Antony et al., 1997; Pasqualato et al., 2002). Genetic and biochemical evidence has shown that Arf GTPases play central roles in both membrane traffic and cytoskeletal organization. Activation of Arf GTPases is usually controlled by exchange factors resident on specific organelles, with the GTP-bound GTPase then recruiting specific effectors to that organelle (Donaldson and Jackson, 2000). For example, Arf1 recruits COP I vesicle coats to Golgi membranes, and the distantly related Sar1 recruits COP II coats to the endoplasmic reticulum (ER). Arf1 also binds directly to the Golgi-localized protein GGA1, an adaptor that recruits cargo into Golgi-derived clathrin-coated vesicles. Arf1 has a number of close relatives, including the plasma membrane-localized Arf6 that modulates the actin cytoskeleton and endocytosis via generation of PtdIns(4,5)P2 (Krauss et al., 2003; Niedergang et al., 2003). Beyond this, there is a large number of more distantly related proteins known as Arf-like (Arl) GTPases that share structural features with Arf1 and Sar1, but whose functions are less well understood (Clark et al., 1993; Pasqualato et al., 2002). There are at least ten Arls in humans which all share with the Arfs an N-terminal amphipathic helix and, in most cases, a consensus sequence for N-myristoylation (Antony et al., 1997; Pasqualato et al., 2002). The function of most Arl proteins is unclear, but their importance is suggested by the observations that mutation of Arf1 in Drosophila is zygotically lethal (Tamkun et al., 1991) and that deletion in mice of Arf-related protein (ARFRP1 or ARP) causes embryonic lethality (Mueller et al., 2002). Arf2 has been implicated in tubulin assembly and microtubule polymerization, but its effector in this process is not known (Bhamidipati et al., 2000). In addition, Arf2-GTP binds to the phosphodiesterase (PDE) δ subunit, which has been proposed to act as a transport factor for the PDE catalytic subunits, and other prenylated proteins including GTPases (Hanzal-Bayer et al., 2002). Of the other Arls, perhaps the best understood is Arf11, which localized to the Golgi apparatus of both mammalian cells and yeast (Lowe et al., 1996; Gangi Setty et al., 2003). Arf1 is myristoylated, and mutation of the N-terminal myristoylation site abrogates Golgi targeting (Lee et al., 1997; Lu et al., 2001). Yeast two-hybrid screens for potential effectors of human Arfl identified two Golgi-localized proteins, golgin-97 and golgin-245 (also termed p230) (Fritzler et al., 1995; Erlich et al., 1996; Lu et al., 2001; Van Valkenburgh et al., 2001). These are two members of a set of large coiled-coil proteins that have been found on the Golgi apparatus and which are often referred to as “golgins.” The golgins have been implicated in Golgi formation and maintenance as well as in vesicular transport. Golgin-97 and golgin-245, along with GCC88 and GCC185, are peripheral membrane proteins that share a C-terminal GRIP domain. This ~50 residue domain is both necessary and sufficient for targeting to the Golgi apparatus and is conserved in animals, fungi, plants, and protozoa (Barr, 1999; Kjer-Nielsen et al., 1999a; Munro and Nichols, 1999; McConville et al., 2002). Arf1 is required for targeting GRIP domain proteins to the Golgi apparatus in both yeast and mammalian cells and has been shown to bind directly to the GRIP domain in a GTP-dependent manner (Gangi Setty et al., 2003; Panic et al., 2003). Arf1 appears to be predominantly localized to Golgi membranes, and relocation of Arf1-GTP to a different organ-
elle causes relocation of a GRIP golgin (Lu and Hong, 2003).

Although the precise function of the GRIP golgins is still unclear, golgins such as p115 and GM130 have been shown to participate in both tethering of transport vesicles to Golgi membranes prior to fusion and in stacking cisternae to create the higher order structure of the Golgi apparatus. Golgin-245 is found on the trans face of the Golgi apparatus (Koo et al., 1992), and the GRIP domains of all four human GRIP domain golgins are sufficient to confer this compartment-specific targeting (Luke et al., 2003).

In this paper, we report a 1.7 Å resolution X-ray structure of a complex between a human GRIP domain and GTP-bound Arf1. The structure reveals that the GRIP domain is a dimer, allowing two Arf1 proteins to interact simultaneously with a golgin. To our knowledge, such a dyad-symmetrical interaction of a small GTPase with an effector has not been described previously. The interaction between Arf1 and the GRIP domain shares features with those formed between several Ras-like GTPases and their effectors, despite a range of effector topologies. The specificity of the interactions is dictated by packing restraints at the interface with the GTPase.

Results and Discussion

Preparation and Crystallization of an Arf1-GTP/GRIP Domain Complex

The GRIP domain was initially defined on the basis of related sequences present at the C termini of several Golgi coiled-coil proteins (Barr, 1999; Kjer-Nielsen et al., 1999a; Munro and Nichols, 1999). There are four GRIP domain-containing proteins in humans and Drosophila, and one in fungi and plants (Figure 1A). Direct binding has previously been reported between recombinant forms of the GRIP domain of S. cerevisiae Imh1p and Arf1p-GTP. It has also been reported that human Arf1p interacts with golgin-245 and golgin-97 in a yeast two-hybrid assay (Li et al., 2001; Van Valkenburgh et al., 2001). E. coli–expressed forms of all four human GRIP domains bind directly to human Arf1 in a GTP-dependent fashion (Figure 1B), suggesting that GRIP domains defined by sequence may all share the capacity to bind to Arf1p-GTP.

The C-terminal 59 residues of human golgin-245 encompassing the GRIP domain was coexpressed in E. coli with human Arf1 lacking the N-terminal 14 residues. This N-terminal region is very likely to form an amphipathic helix, as such a helix has been observed in the structures of the GDP-bound forms of all Arf family members so far examined, including that of yeast Arf1p (Amor et al., 2001). This helix is displaced in the GTP-bound forms of other Arf family GTPases to expose the hydrophobic face for association with membranes, and its removal was necessary for crystallization of GTP-bound forms of Arf1p and Sar1p (Bi et al., 2002; Shibata et al., 2003). The GTPase active site mutant Q71L was incorporated into Arf1 to ensure that the protein accumulated in the GTP-bound form. Complexes were purified by affinity chromatography and gel filtration in the presence of Mg2+ and GTP.

Overall Structure of the Arf1-GTP/GRIP Domain Complex

The complex of the human golgin-245 GRIP domain with human Arf1p(Q71L) crystallized in space group P21, with unit cell dimensions a = 72.5, b = 89.7, c = 72.5, β = 110.7. There are four Arf1p(Q71L)-GTP/GRIP domain complexes in the asymmetric unit. The structure was solved using Se-Met multiple anomalous dispersion (MAD) data (Table 1). There are only minor differences in conformation among the four Arf1p/GRIP complexes in the asymmetric unit.

The structure reveals that the GRIP domain forms a homodimer, with each GRIP domain bound to an Arf1-GTP (Figure 2). The resulting dyad symmetric arrangement of the Arf1 proteins leaves their N termini facing the same direction so that the two N-terminal amphipathic helices absent from the structure would be able to interact simultaneously with a lipid bilayer. The overall extent of the residues in the GRIP domain that participate in dimerization or Arf1 binding corresponds well to the 40–50 residue region conserved among different GRIP domains, and specifically to the 45 residue portion of golgin-245 that is sufficient for some Golgi targeting (gray bar in Figure 1C; Kjer-Nielsen et al., 1999b). Previous mutagenesis studies have also identified a number of residues in the GRIP domain that are required for golgin-245 and Arf1 binding in a GTP-dependent manner (see below), which has prevented such artifactual disulfide bond formation.

Structure of Arf1p(Q71L)-GTP

Like other Arf family GTPases, the GTP-bound Arf1p (Q71L) overall fold consists of six β strands surrounded by five α helices (Figure 2). Comparison of the human Arf1p(Q71L)-GTP with the previously reported yeast Arf1p-GDP (Amor et al., 2001), shows that the region from 40 to 82 changes conformation upon GTP binding. This is consistent with other members of the Arf family GTPases (Goldberg, 1998; Pasqualato et al., 2001). The region from 51 to 67 (strands β2 and β3) has been referred to as the “interswitch” region (Pasqualato et al., 2002) and is located between switch 1 (residues 40–50) and switch 2 (residues 68–82). In the GTP-bound form of Arf1, the interswitch region has moved two residues with respect
**Figure 1. Domain Structure and Sequence Alignment of GRIP Domain-Containing Proteins**

(A) Schematic representation of the four human GRIP domain proteins, and their homologs in *Drosophila melanogaster* (Dm) and *Caenorhabditis elegans* (Ce). Also shown is Imh1p, the single GRIP domain protein of *Saccharomyces cerevisiae* (Sc). Sequence homology suggests that the gene encoding the *C. elegans* homolog of golgin-245 probably comprises two adjacent predicted genes in the genome (F59A2.2 and F59A2.6).

There is a fifth protein in the human genome that ends with a GRIP domain, Ran binding protein 2 (RanBP2), but it appears to be the product of a recent fusion of duplications of the genes for RanBP2 and GCC185, and its significance is unclear.

(B) Binding of the GRIP domains from the indicated human golgins to Arl1. Extracts of *E. coli* expressing the His 6-tagged GRIP domains of golgin-245, golgin-97, GCC88, and GCC185 (C-terminal 58, 78, 62, and 110 residues, respectively) were applied to glutathione beads loaded with either human GST-Arl1 with mutations to lock it in the GDP (T31N) or GTP (Q71L) bound forms (upper panels), or GST-Arl1 loaded with GDP or the nonhydrolysable analog GMPPNP (GTP*) as described previously (lower panels) (Panic et al., 2003). After washing, bound proteins were eluted with SDS sample buffer and analyzed by gel electrophoresis and Coomassie blue staining.

(C) Alignment of the C termini of the indicated human GRIP domain proteins and their homologs in other species. Sequences were aligned with Clustal W, and residues identical, or related, in seven or more of the sequences are indicated by black or gray, respectively (BoxShade), and tryptophans are marked in orange. A schematic of the structure of the golgin-245 GRIP domain is shown above the alignment with the interacting residues marked as indicated. The residue numbering for golgin-245 is based on that of Swissprot accession Q13439 (isoform 4) (Erlich et al., 1996). Residues in the GRIP domains of golgin-245 or golgin-97 that have been mutated in previous studies are indicated with circles (filled, loss of Golgi targeting; open, normal targeting; half filled, reduced targeting) (Barr, 1999; Kjer-Nielsen et al., 1999a; Munro and Nichols, 1999; Lu and Hong, 2003). The gray bar indicates the minimal portion of golgin-245 that shows some Golgi targeting (Kjer-Nielsen et al., 1999b).

Species as in (A), or *Candida albicans* (Ca), *Oryza sativa* (Os), and *Trypanosoma brucei* (Tb).

- **GRIP Domain Dimerization**

The GRIP domain consists of three antiparallel α-helices arranged in an S-shaped configuration (Figures 2 and 3). The surface of the S-shaped domain is slightly curved, and the convex face interacts with the Arl1 while the concave face pairs with another GRIP domain in a parallel α-helical “handshake.” The interaction between GRIP domains in the crystal is extensive (2282 Å² of solvent-accessible area is buried) and overwhelmingly hydrophobic, with only a single intermolecular hydrogen bond (Arg 2179 to carbonyl oxygen of Leu 2202; Figure 3A). The extensive interface between the GRIP domains suggests that this homodimeric interaction is probably
Table 1. Data Collection, Structure Determination, and Refinement Statistics

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<th>Remote c</th>
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Data sets were collected at ESRF beamline ID14-4 at 12.6612, 12.6590, and 13.2 keV for the peak, inflection, and remote data sets, respectively.

Phasing statistics

| Phasing power (iso) b    | —      | 0.78        | 1.1      |
| Phasing power (anom) b   | 1.48   | 1.42        | 1.2      |
| Se sites found/expected  | 39/48  |             |          |
| FOM after SHARP          | 0.43   |             |          |
| FOM after SOLOMON        | 0.79   |             |          |

Refinement statistics

| Resolution                | 67–1.7 Å |
| Protein atoms             | 7148     |
| Waters                    | 266      |
| Rmerge                    | 0.22     |
| Rmerge (%) (data used)    | 0.25 (3.4) |
| Rmsd from ideality b      | 0.014 Å  |
| Bonds                     | 1.5°     |
| Angles                    |          |
| Dihedrals                 | 6.0°     |

The phasing power is defined as the ratio of the r.m.s. value of the heavy atom structure factor amplitudes to the r.m.s. value of the lack-of-closure error.

Data sets were collected at ESRF beamline ID14-4 at 12.6612, 12.6590, and 13.2 keV for the peak, inflection, and remote data sets, respectively.

Interaction between Arl1-GTP and the GRIP Domain

All of the contacts that the GRIP domain makes with Arl1 are via GRIP helices \( \alpha_1 \) and \( \alpha_2 \), with \( \alpha_1 \) forming most of the switch 1 interactions and \( \alpha_2 \) forming most of the switch 2 interactions (Figure 4). Interaction with the Arl1 switch/interswitch regions in the crystal structure provides a rational explanation for the GTP-dependent binding of the GRIP domain seen in vitro and in vivo. The only residue in the GRIP domain that interacts with both switch 1 and switch 2 is the very well conserved Tyr 2177. Mutation of this residue to alanine in golgin-245 and several other GRIP domains has been shown to result in loss of Golgi targeting in mammals, yeast, and protozoa (Kjer-Nielsen et al., 1999a; Munro and Nichols, 1999; McConville et al., 2002). Moreover, the same residue is required for Arl1 binding by the GRIP domain of the yeast protein Imh1p and for yeast two-hybrid interaction between the GRIP domain of golgin-245 and human Arl1 (Van Valkenburgh et al., 2001; Panic et al., 2003). Tyr 2177 protrudes into a pocket on the surface of the Arl1 that is lined by a cluster of hydrophobic residues from the interswitch and switch 2 (Phe 51, Leu 66, Ile 74, Tyr 77, and Tyr 81; Figure 4). This “selectivity pocket” appears to be one important determinant of the specificity of the Arl1-GTP/GRIP interaction. At the bottom of this selectivity pocket, the OH of Tyr 2177 forms a hydrogen bond with the OH of Tyr 81 in switch 2. Although a tyrosine at the position equivalent to Tyr 2177 is conserved in all GRIP domains, mutagenesis indicates that the hydrogen bond formed with Tyr 81 is present in solution, and this was confirmed in two ways.

First, versions of the GRIP domain were expressed in E. coli with either His6 or MBP tags. The MBP form could be precipitated with Ni\(^2+\)-NTA-agarose only if it was coexpressed with the His6-tagged species, or mixed with it after lysis (Figure 3B). Second, when the GRIP domain was subjected to equilibrium centrifugation, monomers and dimers were found to be in equilibrium in solution, with the sedimentation profile indicating a Kd for dimerization of about 2.5 \( \mu \)M (Figure 3C).

Examination of the dimer interface shows that helix \( \alpha_1 \) of the GRIP domain has a pivotal role. This helix has a hydrophobic face consisting of residues Phe 2175, Leu 2178, Leu 2182, and Tyr 2185 that lines up along the dyad axis and primarily interacts with helices \( \alpha_2 \) and \( \alpha_3 \) and connecting loops from the other half of the dimer (Figure 3A). Many of the residues that comprise the interface are well conserved in evolution, with Phe or Tyr equivalent to Tyr 2185 of golgin-245 present in almost all GRIP domains (Figure 1C). This residue, near the C-terminal end of helix \( \alpha_1 \), makes hydrophobic contacts with Tyr 2185 of golgin-245 and Met 2186 from the dyad-related monomer. The importance of these interactions is underscored by mutations in two conserved residues in helix \( \alpha_1 \) having been previously reported to result in loss of Golgi targeting of GRIP domains (Barr, 1999; Kjer-Nielsen et al., 1999a). These mutations are Y2185A in golgin-245 and K699A in golgin-97 (equivalent to Arg 2179 in golgin-245, which forms extensive hydrophobic contacts as well as an intermolecular hydrogen bond).
not essential, as substitution of Tyr 2177 with Phe did not significantly affect Golgi localization (Kjer-Nielsen et al., 1999a). This suggests that the hydrophobic interactions of the Tyr 2177 with the selectivity pocket are paramount for Arf1-GTP/GRIP binding. In the GDP complexes of both Arls and Arfs, the selectivity pocket is occupied by the phenylalanine at residue 51 in Arf1 or its equivalent. This, and other structural changes in the switch regions, presumably prevents binding of the GRIP domain to Arf1-GDP.

Selectivity of the Golgin GRIP Domains for Arf1-GTP
The golgin-245 GRIP domain binds selectively to Arf1-GTP and not to other Arf-family GTPases such as Arfs1-6 and Arfl2 (Van Valkenburgh et al., 2001; Lu and Hong, 2003). Comparison of the Arf1-GTP/GRIP structure with that of Arf1-GTP (Shiba et al., 2003) shows that the switch 2 region involved in effector binding may account for much of the effector selectivity. In particular, a hydrophobic selectivity pocket on Arf1 wide enough to accommodate GRIP Tyr 2177 appears to be a unique feature of Arf1. In Arfl1, the residues lining the selectivity pocket are identical to those of Arf1 except for Leu 77 of Arfl, corresponding to Tyr 77 in Arf1. In Arf1, Leu 77 fills in much of the selectivity pocket because the two methyl groups of the leucine protrude into the pocket due to sp³ hybridization at the γ carbon, whereas the sp² hybridization at the γ carbon of the tyrosine in Arf1 creates more space in the pocket. All of the Arf family GTPases have a Leu at residue 77, whereas only Arf1, Arfl2, and Arf3 have a tyrosine at this position (Figure 4B). Although Arf2 has this tyrosine, the opposite corner of the selectivity pocket in Arf2 is partially filled in by a leucine (residue 73 of Arf2, corresponding to Ile 74 of Arf1), presumably cramping entry of the critical GRIP Tyr 2177. Other residues in the interface are likely to contribute to selectivity for Arf1-GTP. For example, Arf1 Cys 80 makes a hydrogen bond to GRIP Thr 2200 and is well conserved in Arf1 from different species (Figure 4C). In contrast, a histidine is conserved at this position in the Arf proteins, and a C80H mutation in Arf1 eliminates a yeast two-hybrid interaction with the golgin-245 GRIP domain (Lu and Hong, 2003), presumably because of the steric effects of the bulkier histidine residue.

Just as comparison of Arf1-GTP with Arf1-GTP suggests differences that could account for effector selectivity, comparison of the GRIP domain with the Arf1 effector domain N-GAT shows differences that could account for GTPase selectivity. The presence of a GRIP tyrosine (Tyr 2177) that occupies the selectivity pocket of the GTPase. For example, Ile 197 in the N-GAT domain occupies a position in space analogous to Tyr 2177 of golgin-245 GRIP, but the N-GAT isoleucine makes only limited contacts with switch 2 (338 Å² buried in the switch 2/PDEβ interface).
In contrast, the GRIP domain makes a much more extensive interface with switch 2 than with switch 1 (730 Å² buried in the switch 2/GRIP interface versus 308 Å² for the switch 1 interface out of a total of 1329 Å² buried in the Arl1/GRIP interface).

A Shared Mode of GTPase-Effector Recognition

Several effector domains of Ras superfamily GTPases are helical, including N-GAT domains, Arfaptin2, the protein kinase N (PKN) ACC finger, and from this work, GRIP domains (Maesaki et al., 1999; Tarricone et al., 2001; Collins et al., 2003; Shiba et al., 2003). Structural comparison of the complexes between these effectors and their GTPases shows a shared mode of interaction. For each of the effectors, antiparallel helices contact hydrophobic patches formed by switch/interswitch regions of the GTPase (Figure 5). Because the vast majority of the interactions are with residues in the effector helices and not in the connecting loops, and because the interactions are principally nondirectional hydrophobic interactions between side chains, there are no constraints on the connecting topology or even the direction of the effector helices. Thus, the direction of the equivalent helices is swapped N- to C terminus in N-GAT and Arfaptin2 relative to GRIP, and the connection between the two helices is swapped from one end of the helices to the other in N-GAT relative to Arfaptin2 (Figure 5B). A more distant variant is the complex between Rab3A and rabphilin-3A, where the effector lacks one of the two helices, and instead a short motif in rabphilin-3A makes additional contacts outside of the Rab3A switch regions (Ostermeier and Brunger, 1999). This plastic mode of interaction of these helical effectors with their GTPases leads to a striking structural mimicry that was completely unsuspected from sequence alignments.

The C-Terminal Tail of the GRIP Domain

Although the GRIP domain is located near the C terminus of all proteins in which it has been found, in many cases the domain is followed by an extension of typically 5–30 residues before the end of the protein. Despite being heterogeneous in length and sequence, and indeed not present in all GRIP domain proteins, there are indications that these C-terminal tails may be functionally important. First, some of the tails have shared sequence features, most notably one or two tryptophan residues, which in some cases are interspersed with additional bulky hydrophobic residues (Figure 1C). Second, Golgi targeting of the golgin-97 GRIP domain was reported to be reduced when the tryptophan in the tail (Trp 744) was mutated to alanine (Barr, 1999). Human golgin-245 occurs in two splice isoforms that differ only in these tail residues (Erlich et al., 1996). In the isoform used for these studies, the C-terminal seven residues are SWLRSSS, but inclusion of an extra exon results in these residues being replaced with FTSPRSGIF, with the latter version being represented by 3 of the 20 ESTs for human golgin-245 in the current GenBank database. The significance of this is unclear, as Golgi targeting of the two forms was apparently indistinguishable (Kjer-Nielsen et al., 1999b), but similar alternative splicing is seen in ESTs from mice and rats, suggesting that it has been conserved at least through recent evolution. As with golgin-97, mutation
of Trp 2223 did not prevent the Golgi targeting of the SWLRSSS form of the GRIP domain, but reduced the Golgi-specific signal (Figure 6A). Moreover, photo-bleaching of the cytoplasmic pool of GFP-GRIP revealed that the W2223A mutation reduced the half-time for exchange between the Golgi and cytoplasmic pools from \(\sim 35\) s to \(\sim 13\) s (Figure 6B). This is also consistent with the observation that Golgi targeting of the golgin-245-
GRIP domain was reduced by truncation at residue 2218, or mutation of residues Arg 2219–Leu 2224 to six alanines (Kjer-Nielsen et al., 1999b).

The tail region of the golgin-245 GRIP domain is not well resolved in the crystal structure, with helix α3 ending at residues 2220–2226 for three of the four domains in the asymmetric unit. However, in the case of the fourth GRIP domain, the tail lies against the surface of an adjacent Arl1-GTP/GRIP dimer, and although the side chains, including that of Trp 2223, cannot be resolved, it is clear that the backbone forms an α helix all the way to the C terminus at residue 2228. While this intermolecular interaction is almost certainly a consequence of the crystal environment, it indicates that the apparently unstructured C terminus has a propensity to form an α helix. A possible alternative inducer of a helical conformation is suggested by considering the likely orientation of the Arl1-GTP/GRIP complex on the lipid bilayer (Figure 6C). Insertion of the N-terminal myristoyl group and amphipathic helix of Arl1 into the lipid bilayer would orient the GRIP domain so that the C-terminal tail is adjacent to the bilayer surface. Formation of an α helix might then accompany insertion of the tryptophan and other hydrophobic residues into the lipid interfacial region (Figure 6C). Such an interaction would be analogous to the insertion of hydrophobic residues that accompanies the interaction of FYVE or PX domains with specific lipid head groups (Misra et al., 2001; Karathanassis et al., 2002; Stahelin et al., 2002). The interaction may serve to stabilize membrane attachment or to orient the domain to facilitate interaction with free Arl1-GTP diffusing in the plane of the bilayer.

Conclusions
The GRIP domain is responsible for targeting four golgins to the membranes of the trans Golgi. This targeting
is mediated by binding of the GRIP domain to the GTPase Arl1, which is itself localized to the Golgi apparatus. In this paper we have shown that the GRIP domain forms a dimer that interacts bivalently with Arl1-GTP. In solution, the isolated domain is in a dimer-monomer equilibrium, but in the context of the golgins the extensive regions of coiled-coil in the rest of the proteins will presumably stabilize the dimer form. The interaction between Arl1-GTP and the GRIP domain is such that the membrane-bound GTPase will orient golgin-245 so that the C-terminal tail faces the lipid bilayer, and the rest of the molecule projects into the cytoplasm, either away from the Golgi membrane or along its surface. The interface formed between Arl1 and helices α1 and α2 of the GRIP domain is similar to that formed by several other Ras-family GTPases with their effectors, suggesting that recognition of paired helices may be a widespread mechanism for effector binding. However, these other GTPases do not recognize their effectors with the dyad symmetric bivalency found in the Arl1-GTP/GRIP complex. Instead, this latter feature is strikingly reminiscent of the bivalent interaction between the endosomal tethering factor EEA1 and its lipid ligand PtdIns(3)P. In the case of EEA1, a C-terminal FYVE domain forms a dimer when stabilized by the parallel coiled-coils in the rest of the protein (Dumas et al., 2001). The resulting bivalent interaction with PtdIns(3)P is critical for ensuring the effective and accurate targeting of EEA1 to endosomes (Gillooly et al., 2000). Although both EEA1 and golgin-245 make dimeric association with membranes, the bivalent interaction of GRIP dimers with Arl1-GTP stands in distinction from the bivalent interaction of the FYVE domains with PtdIns(3)P. In EEA1, the affinity of each FYVE domain for PtdIns(3)P is fairly weak, and the presence of both domains in the dimer is necessary for translocation of EEA1 to membranes in cells. Given that a soluble, GTP-locked Arl1 forms a stable complex with GRIP that can be copurified in vitro, a single Arl1-GTP would probably be sufficient to cause GRIP homodimer translocation in cells. However, binding of a second Arl1-GTP by a GRIP homodimer at the membrane surface would increase the residence time of the complex on the membrane. Accuracy of membrane trafficking processes seems likely to be dependent on the cell being able to accurately specify both the location and the duration of targeting of the proteins that mediate docking and fusion (Munro, 2002). Bivalent interaction with organelle-specific GTPases or lipids may be one means by which this can be achieved.

Experimental Procedures

Protein Expression and Purification

Human Arl1 (residues 15–181, with the Q71L mutation and an N-terminal GST tag) was coexpressed with the nontagged GRIP domain from human golgin-245 (residues 2170–2228) using the poly-cistronic coexpression vector pOPCG. There are a number of splice variants of golgin-245, and all the work in this paper is based on isoform 4, Swissprot accession Q13439. SeMet-substituted protein for the crystallography was expressed in the E. coli methionine auxotroph B834(DE3) in M9 minimal medium supplemented with...
amino acids, seleno-(L)-methionine, and vitamins as described previously (Karathanassis et al., 2002). Cells were grown to OD_{600} = 1 at 37°C and then induced with 0.3 mM IPTG at 17°C for 12 hr. Cells were lysed with a French press in buffer A (50 mM Tris-HCl [pH 7.5, 4°C], 100 mM NaCl, 5 mM MgCl₂, 10 mM β-mercaptoethanol, and 0.2 mM GTP). After ultra centrifugation, the complex was bound to glutathione-Septarose (Amersham Biosciences), washed with buffer A, and cleaved on resin using 0.125 mg of tobacco etch virus protease (TEV) per mg of complex for 12 hr at 4°C. After elution, Arf1 retained an N-terminal GSHM linker sequence. The complex was further purified by gel filtration on Superdex 75 16/60 equilibrated in buffer B (20 mM Tris [pH 7.5, 4°C], 100 mM NaCl, 1 mM MgCl₂, 5 mM DTT, and 10 μM GTP). The complex was concentrated to 11 mg/ml and snap frozen in liquid nitrogen.

Crystallization
Initial crystals were obtained at 17°C by vapor diffusion in sitting drops made by mixing 100 nl protein and 100 nl reservoir from crystallization screen Wizard I, condition 1 (Emerald BioStructures) in 96 well crystallization plates (Corning). Final conditions were optimized using hair seeding in hanging drops made by mixing 1 μl protein with 1 μl of reservoir solution consisting of 20% PEG 3350 and 0.2 M Tris-HCl (pH 6.5). After the crystals approached their full size, the reservoir solution was exchanged for one containing 31% PEG 3350, and drops were incubated over this for approximately 2 days at 17°C. Crystals were frozen in cryoprotectant (31% PEG 3350, 0.2 M Tris-HCl [pH 8.5]).

Data Collection, Phasing, and Model Refinement
For data collection, crystals were frozen in a nitrogen gas stream, and data were collected at 100 K. Multiple anomalous dispersion (MAD) data sets were collected at ESRF beamline ID14-4 using an ADSC CCD detector. Prior to data collection, a fluorescence spectrum for the crystal was obtained, and three data sets were collected at wavelengths corresponding to the fluorescence peak, inflection, and a high energy remote. Table 1 lists statistics for data collection. Fifty putative Se sites were located using the program SHARP (Turner et al., 1998; Weeks and Miller, 1999), and subsequent refinement with autoSHARP (de La Fortelle and Bricogne, 1997) resulted in 39 Se sites (Table 1). Solvent flattening was carried out with SOLOMON (Abrahams and Leslie, 1996), using a solvent content of 30.4% as optimized by SHARP. An initial model was built using Arp/warp (Perrakis et al., 1999) and refined by alternating rounds of refinement with REFMACS (CCP4, 1994) and manual rebuilding with the program O (Jones et al., 1991). Finally statistics for the 1.7 Å resolution model are given in Table 1. There are no residues in the disallowed regions of the Ramachandran plot, and 95% of residues are in the most favored regions as defined by PROCHECK. Residues 2171–2220 are ordered for each of the GRIP domains in the asymmetric unit. The C-terminal residue ordered differs for each of the GRIP domains in the asymmetric unit: 2220, 2221, 2226, and 2228.

Coprecipitation Assays
His₄-tagged GRIP (golgin-245 residues 2170–2228) and MBP-tagged GRIP were produced in E. coli C41(DE3) either by coexpression of a polycistronic pPOC plasmid, with MBP-GRIP in the first cassette and His₄-GRIP in the second, or by expressing individually MBP-GRIP (from pOPTM plasmid, encoding an N-terminal tag) or His₄-GRIP (from pOPTH plasmid, encoding an N-terminal MetAlaHis₄Met tag). Cells were grown in 2xTY/Amp to OD₆00 1 at 37°C, and then induced with 0.3 mM IPTG at 17°C for 12 hr. Cell pellets were lysed in PBS and bound to Ni-NTA (QIAGEN) resin, washed with PBS, and eluted with buffer containing 0.3 M imidazole, and aliquots were run on 20% Phast SDS gels (Amersham Biosciences).

Analytical Ultracentrifugation
Sedimentation equilibrium was performed on a Beckman Optima XL1 ultracentrifuge using a Ti-60 rotor and interference and absorbance at 280 and 230 nm, at 10°C. His₄-GRIP domain from human golgin-245 expressed in E. coli C41(DE3) was purified by Ni²⁺-affinity chromatography followed by gel filtration on a Superdex 75 16/60 column (Amersham Pharmacia) equilibrated in 20 mM Tris-HCl (pH 7.5, 4°C) and 100 mM NaCl. The protein was loaded into six-sector 12 mm path length cells at three different concentrations: 8 μM, 60 μM, and 220 μM. The samples were centrifuged until they reached equilibrium as judged by the changes in the subsequent scans. Speeds were 32,000, 38,000, and 45,000 rpm. Data were analyzed using UltraSpin software (www.mrc-cpe.cam.ac.uk).

Immunofluorescence
COS cells were transfected using FuGene (Roche), split onto glass slides, and fixed 30 hr post transfection with 4% paraformaldehyde, 0.1% glutaraldehyde. Cells were permeabilized with 0.5% (v/v) Triton X-100 in PBS, blocked with 20% (v/v) fetal calf serum/0.25% (v/v) Tween 20 in PBS, and probed with rabbit TGN46 antibodies (Pescott et al., 1997) and Alexa488 anti-rabbit antibodies (Molecular Probes). Images were obtained on a BioRad Radiation confocal microscope.

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Accession Numbers

The coordinates of the Arl1-GTP/GRIP domain complex have been deposited in the Protein Data Bank under the ID code 1UPT.