Golgi Localization Determinants in ArfGAP1 and in New Tissue-specific ArfGAP1 Isoforms*

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The Arf1-directed GTPase-activating protein ArfGAP1 is a Golgi-localized protein that controls the dynamics of the COP I coat of carriers that mediate transport in the endoplasmic reticulum-Golgi shuttle. Previously the interaction of ArfGAP1 with the Golgi was allocated to a portion of the non-catalytic, carboxyl part of the protein, but the mechanism of this interaction has not been established. In this study we identify a short stretch in the non-catalytic part of ArfGAP1 (residues 204–214) in which several hydrophobic residues contribute to Golgi localization. Even single alanine replacement of two of these residues (Leu-207 and Trp-211) strongly diminished Golgi localization. Mutations in the hydrophobic residues also diminished the in vitro activity of ArfGAP1 on Arf1 bound to Golgi membranes. The stretch containing the hydrophobic residues was recently shown to mediate the binding of ArfGAP1 to loosely packed lipids of highly curved liposomes (Bigy, J., Casella, J. F., Drin, G., Mesmin, B., and Antonny, B. (2005) EMBO J. 24, 2244–2253). Whereas short fragments containing the hydrophobic stretch were not Golgi-localized, a proximal 10-residue in-frame insertion that is present in new ArfGAP1 isoforms that we identified in brain and heart tissues could confer Golgi localization on these fragments. This localization was abrogated by alanine replacement of residues Phe-240 or Trp-241 of the insertion sequence but not by their replacement with leucines. Our findings indicate that ArfGAP1 interacts with the Golgi through multiple hydrophobic motifs and that alternative modes of interaction may exist in tissue-specific ArfGAP1 isoforms.

Membrane traffic in the endoplasmic reticulum-Golgi shuttle is mediated by the COP I and COP II trafficking systems. The COP II system mediates the initial exit from the endoplasmic reticulum, whereas subsequent transport to the Golgi apparatus and retrograde Golgi to endoplasmic reticulum traffic involves COP I carriers. The COP I and COP II coats are composed of evolutionarily distinct sets of proteins yet follow similar pathways of coat assembly and disassembly (for recent reviews see Refs. 1–3). In both systems a small GTPase (Arf1 and Sar1) respectively plays a key regulatory role. Following activation by a guanine nucleotide exchange protein, the GTPase translocates from cytosol to the organelle membrane, where it initiates the process of coat formation by the direct binding of coat subunits (4–6). The coat in turn recruits cargo and polymerizes causing membrane deformation to form a bud.

In the second phase of the GTPase cycle of Arf1 and Sar1, bound GTP is hydrolyzed with the aid of a GTP-activating protein (GAP). The hydrolysis of GTP is a prerequisite for coat dissociation, and its inhibition leads to the accumulation of coated vesicles that are prevented from fusing with the target membrane (4, 7). The GAP for Sar1 is a subunit of the first layer of the COP II coat, the Sec23/24 complex (8). ArfGAPs constitute a large family of proteins containing a highly conserved catalytic domain of ~130 residues with a characteristic zinc finger structure, whereas the non-catalytic parts are highly diverse (9). At least two types of ArfGAPs appear to function at the Golgi complex, ArfGAP1 (10, 11) and ArfGAP2/3 (12–14) (Gcs1 and Glo3 in Saccharomyces cerevisiae, respectively, Refs. 15 and 16). Evidence for the Golgi function of the mammalian proteins includes their localization at the Golgi complex (11, 13, 14), their functional (17, 18) and physical (14, 19, 20) interaction with the COP I coat, and the perturbation of Golgi integrity upon overexpression of the ArfGAP1 protein (21).

A unique feature of ArfGAP1 is its avid interaction with loosely packed lipids presented by liposomes containing diacylglycerols (22) or by small liposomes where the high curvature of the bilayer creates open spaces between the phospholipid acyl chains (23, 24). The response of ArfGAP1 to membrane curvature was proposed to serve as a mechanism for the regulation of GTP hydrolysis during bud formation. Thus the positive curvature in the central region of the bud would cause ArfGAP1 recruitment and hence facilitate GTP hydrolysis on Arf1, whose presence may not be required following the polymerization of the coat, whereas the negative curvature in the bud rims would exclude ArfGAP1 from this region, allowing Arf1-GTP-dependent recruitment of new coat subunits (23, 25, 26).

Whereas the catalytic domain of ArfGAP1 contains the machinery required for the catalysis of GTP hydrolysis (17, 27), the non-catalytic part contains information for the targeting of ArfGAP1 to the Golgi, as indicated by the correct targeting of GFP fusions of non-catalytic fragments (28). The non-catalytic part mediates the interaction of ArfGAP1 with loosely packed lipids (24) and is also involved in protein-protein interactions, as demonstrated for the interaction of ArfGAP1 with the COP I cargo, the KDEL receptor (29, 30). However, a role of any of these interactions in the Golgi localization of ArfGAP1 has not been established.

Here we report the identification of a short stretch within the non-catalytic part of ArfGAP1 containing hydrophobic residues that are necessary for Golgi localization. These residues closely match those recently reported to constitute a lipid-binding motif in ArfGAP1 (24), suggesting a role of lipid binding in ArfGAP1 targeting. We also describe new tissue-specific ArfGAP1 isoforms that contain a small in-frame insertion and deletion in proximity to the lipid-binding motif and provide evidence that these insertion and deletion regions function as Golgi localization determinants.
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MATERIALS AND METHODS

DNA Constructs and Transfection—GFP fusion proteins were prepared by cloning PCR-amplified fragments of ArfGAP1 cDNA into the pEGFP-C2 vector (Clontech) using the EcoRI and BamHI sites. To facilitate the introduction of point mutations, silent mutations were introduced in the codons for amino acids 209–210 (from TCGGGC to TCCGGGA) creating a BspEI site. Primers that included point mutations and a BspEI extension were used in PCR amplification of either amino- or carboxyl-terminal fragments, and the products were used to replace the corresponding fragment in the non-mutated GFP fusion plasmids. Plasmids encoding myc-tagged ArfGAP1 and its mutants were prepared by PCR amplification of the ArfGAP1 coding region in the corresponding GFP fusion constructs and cloning the products into the pCDNA3.1/myc-His(-)B vector (from Invitrogen) using the EcoRI and BamHI sites.

Plasmids (0.2 μg) were transfected into cells grown on 13-mm glass coverslips in 24-well plates using 0.3 μl of FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instructions. GFP-fused constructs were transfected into HeLa cells, whereas myc-tagged constructs were transfected into HeLa cells stably expressing N-acetylgalactosaminy transferase-2 (GalNAc-T2) fused to GFP (31). Cells were fixed with 4% paraformaldehyde 20–24 h following transfection.

Labeling with Antibodies and Fluorescence Microscopy—For immunostaining, coverslips were transferred into PSS (phosphate-buffered saline containing 0.05% saponin and 0.2% bovine serum albumin), and incubated with a monoclonal antibody against GM130 (from Transduction Laboratories, 1:500) or against the myc epitope (clone 9E10, from Santa Cruz Biotechnology, 1:100), followed by CY3-conjugated donkey anti-mouse IgG (ImmunoResearch Laboratories, 1:400). Coverslips were mounted on glass slides using Mowiol as glue and were visualized under a Leica DMIRE2 inverted fluorescent microscope at a 40× magnification.

Preparation of Recombinant ArfGAP1 and Its Mutants—ArfGAP1 cDNA containing silent mutations avoiding rare codon usage (19) was subjected to PCR amplification with primers that included a 5′-Xbal site and a 3′-BamHI site, generating PCR products encoding residues 6–415. These were cloned into the T7 polymerase-driven pKM260 vector between the NheI and the BamHI sites. Mutations were introduced by two-stage PCR. Proteins were expressed in the BL21/DE3 Escherichia coli strain harboring pLysS by induction for 3 h at 30°C in the presence of 0.1 mM isopropyl-β-d-thiogalactopyranoside. Bacterial pellets were extracted with 6 M guanidine hydrochloride, and proteins were purified to near homogeneity by nickel-nitrilotriacetic acid chromatography followed by dialysis and Resource Q chromatography as previously described (27).

GAP Activity Assays—Measurement of GAP activity on Golgi membranes was carried out as previously described (18). Briefly, recombinant myristoylated Arf1 (32) was loaded with [γ-32P]GTP onto rat liver Golgi membranes (33) using the guanine nucleotide exchange activity that is present in the membrane. The exchange activity was stopped by the addition of 0.3 mM brefeldin-A and was followed 1 min later (zero time) by the addition of GAP. Following different incubation times at 30°C, samples were filtered through a nitrocellulose filter, and the amount of radioactivity remaining on the filter (representing Arf1-bound GTP) was determined.

GAP activity on the Arf1 mutant lacking the first 17 residues (Δ17-Arf1, ref. 17) loaded with [γ-32P]GTP was measured in the absence of membranes as described (27) except that the assay buffer contained 50 mM NaCl, 1 mM MgCl2, 1 mM dithiothreitol, and 25 mM MOPS, pH 7.5. Following incubation with GAP for different times at 30°C, samples were transferred to a charcoal suspension, which was subjected to centrifugation, and the amount of radioactivity in the supernatant (representing 32Pi) was determined.

Screening for ArfGAP1 Isoforms—cDNAs of various rat tissues were obtained from BD Biosciences (Rat MTC™ Panel I, catalog number K1429-1). These were subjected to PCR amplification using primers encompassing either the entire coding sequence (sense, GCCAGG-CCAGAACCAGAAA, antisense, GGCTCTTACAGTTCTGGTTGTC) or a carboxyl-terminal fragment (sense, ATGACTTCTCAA-CAGCGCCAT, antisense primer as above), and PCR products were cloned into the pDrive vector (Qiagen) and sequenced.

Preparation of Antipeptide Antibodies That Specifically Recognize the ArfGAP1 Isoforms—Antibodies against the insertion peptide that is present in the brain/heart isoforms was prepared as follows. A peptide representing the insertion peptide with three residues on each side from the flanking ArfGAP1 sequence and amino-terminal cysteine residue, CSQKFWGYYKQQSEPASE, was synthesized, conjugated to keyhole limpet hemocyanin, and injected into rabbits; the entire procedure was carried out by Sigma (Rehovot, Israel). Serum was prepared from the fourth and fifth boosts.

Western Blot Analysis—Proteins were separated by SDS-PAGE (10% gels) and transferred to nitrocellulose sheets, which were blocked in phosphate-buffered saline containing 5% defatted milk and 0.1% Tween 20. Blots were incubated with primary antibodies diluted in blocking buffer using the following antibodies/dilutions: polyclonal anti-ArfGAP1 (11), 1:10,000; anti-ArfGAP1 isoform insertion peptide, 1:3000; anti-GFP (from Santa Cruz Biotechnology), 1:500. Blots were then incubated with horseradish peroxidase-coupled secondary antibodies and were developed by the enhanced chemiluminescence system.

RESULTS

Identification of a Golgi Localization Determinant in the Non-catalytic Part of ArfGAP1—Previously, Yu and Roth (28) have demonstrated by the expression of GFP fusion constructs that the non-catalytic part of ArfGAP1 contains information for Golgi targeting, whereas the catalytic part alone is not Golgi localized. They further demonstrated that a fragment encompassing residues 203–334 still shows Golgi localization, whereas fragments starting at residue 252 or ending at residue 280 are not localized. We sought to further define the minimal Golgi localization domain by introducing small truncations at either side of the non-catalytic fragment. Determination of the carboxyl border of the localization domain was not straightforward, as we observed a gradual decrease in the percentage of cells with clear Golgi localization in a series of truncated mutants ending between residues 280 and 359.4 By contrast, a sharp border could be defined at the amino side (Fig. 1A).

In these experiments, we tested the localization of GFP fusions of carboxyl-terminal fragments that contained a series of small truncations at the amino side of ArfGAP1 and extended to the last residue of the protein. In agreement with the findings of Yu and Roth (28), the 203–415 fragment showed good Golgi localization that coincided with the cis-Golgi marker GM130 and resembled that of the entire non-catalytic domain (residues 143–415). However, fragments with further truncations at the amino side (210–415 and 219–415) showed strongly diminished Golgi localization. Western blot analysis (Fig. 1B) showed that the different constructs gave rise to proteins of the expected size, indicating that the abrogated Golgi localization in the shorter constructs was not because of degradation.

4 A. Parnis, M. Rawet, L. Regev, B. Barkan, M. Rotman, M. Gaitner, and D. Cassel, unpublished results.
We next tested the role of individual residues within the region whose deletion abrogated Golgi localization (residues 204–215). To avoid any effects of the GFP linker that might be manifested in fusion constructs that start close to the mutated region, the mutations were introduced into the full non-catalytic fragment (residues 143–415). As shown in Fig. 2, single alanine replacements of several hydrophobic residues diminished Golgi localization of the ArfGAP1 non-catalytic fragment. This effect was most pronounced with residue Leu-207, where alanine replacement essentially abolished Golgi localization. A W211A mutation also strongly diminished Golgi localization, although weak Golgi fluorescence was still apparent in some cells (Fig. 2, arrowheads). In M204A and F214A mutants, Golgi localization was diminished but to a lesser extent, whereas replacement of M204 with aspartate abrogated Golgi localization, as did a double M204A/W211A mutation. In contrast to the effect of the W211A mutation, replacement of this residue with other hydrophobic residues (Leu or Phe) did not abrogate Golgi localization. Unlike the hydrophobic residues, alanine replacement of serine/threonine residues that are abundant in this region (residues 205/206, 212, and 215) did not diminish the Golgi localization of the non-catalytic fragment. Western blot analysis demonstrated that all mutants were expressed at the expected size (data not shown).

FIGURE 1. Truncation analysis of localization determinants at the amino side of the ArfGAP1 non-catalytic domain. A, GFP alone or GFP fusions of ArfGAP1 fragments encompassing the residues indicated by the scheme on the left side were transiently expressed in HeLa cells. After 24 h the cells were fixed and were counterstained for the Golgi marker GM130. B, Western blot analysis of the transfected cells.

To test whether the point mutations that abrogate Golgi localization affect the GAP function of ArfGAP1 in cells, we tested the effect of overexpression of full-length ArfGAP1 and its mutants on the Golgi apparatus. This assay is based on our previous finding (21) that inactivation of Arf1 by GAP overexpression leads to a brefeldin A-like phenotype, where the Golgi apparatus fuses with the endoplasmic reticulum and ceases to exist as a discrete organelle (34). As shown in Fig. 3A, the full-length GFP-ArfGAP1 was Golgi-localized in cells expressing low to moderate levels of the fusion protein, but not in mutants containing double alanine replacements of hydrophobic residues (M204A/L207A, M204A/W211A) or a single alanine replacement of Trp-211. Staining with antibodies directed against the Golgi marker GM130 revealed that the Golgi apparatus was dispersed in cells expressing high levels of wild-type GFP-ArfGAP1, but remained intact in cells expressing similar levels of the mutants. These findings suggest that the interaction of ArfGAP1 with the Golgi through the stretch of hydrophobic residues is necessary for the in vivo activity of the protein.

The results reported thus far were obtained with GFP fusions of ArfGAP1. Although GFP fusion proteins are commonly used to study the dynamics of proteins in cells, we sought to verify the results using a smaller tag. To this end, we expressed full-length ArfGAP1 constructs in the pCDNA3.1/myc-His vector encoding carboxyl-terminal additions of a myc epitope and a hexahistidine stretch. In these experiments the status of the Golgi apparatus was tracked by using HeLa cells stably expressing a GFP-fused Golgi enzyme, GalNAc-T2 (31). As shown in Fig. 3B, the results obtained with myc-tagged ArfGAP1 constructs were similar to those obtained with the corresponding GFP-fused constructs. Thus, at relatively low expression levels the wild-type protein was Golgi-localized, whereas mutants containing double alanine replacement of the hydrophobic residues showed cytosolic distribution. At high expression levels, the wild-type ArfGAP1 caused a dispersal of the Golgi, whereas the mutants had little effect.
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Effect of Mutations on GAP Activity at the Golgi Membrane—Bigay et al. (24) have recently demonstrated that mutations in the hydrophilic residues that were found in this study to be important for Golgi localization diminish the ability of ArfGAP1 to interact with tiny liposomes and to induce GTP hydrolysis on Arf1 bound to such liposomes. We sought to determine whether these mutations also affect ArfGAP1-induced GTP hydrolysis on Golgi membrane-bound Arf1. This was tested by using our previously described assay (18), where Arf1 is loaded with [γ-32P]GTP at the Golgi membrane by membrane-bound guanine nucleotide exchange proteins, and the effect of GAP is subsequently assayed as a decrease in the amount of membrane-bound radioactivity following the release of the radiolabeled phosphate from GTP. Full-length ArfGAP1 and its mutants were employed in these assays (Fig. 4A). Whereas wild-type ArfGAP1 caused a rapid decrease in membrane-bound radioactivity, double replacement of Met-204 and Leu-207 with alanines resulted in a strongly diminished activity. A mutant with triple alanine replacement of Met-204, Leu-207 and Trp-211 did not increase GTP hydrolysis above the basal level observed in the absence of added GAP. By contrast, as shown in Fig. 4B, ArfGAP1 and its mutants showed similar activity when assayed in the absence of membranes using a lipid-independent Arf1 mutant lacking the first 17 residues (17, 35), indicating that the mutants were not defective in catalytic GAP activity.

ArfGAP1 Isoforms and Their Interaction with the Golgi—We previously isolated cDNA library clones that appeared to represent ArfGAP1 splice isoforms (11), and the possible existence of additional isoforms is suggested by multiple data bank sequences from several mammalian sources. In the present study we reinvestigated whether ArfGAP1 isoforms exist in different rat tissues and analyzed the interaction of new isoforms that we identified with the Golgi complex.

The expression of ArfGAP1 isoforms was analyzed by subjecting cDNAs from several rat tissues to PCR amplification of the entire or the 3'-part of the ArfGAP1 coding region. Sequencing of multiple clones of PCR products from each tissue revealed the existence of two new isoforms containing small in-frame insertion/deletion (Fig. 5A). In heart tissue, we detected an isoform containing a 10-residue insertion following residue 239, whereas in brain tissue we observed an isoform containing the same insertion together with a proximal in-frame deletion of 22 residues (residues 259–280). Only the form originally identified in the liver (11) was detected in the other rat tissues examined; this form was also detected in PCR clones from heart and brain alongside with the variant forms. To determine the relative abundance of isoforms in heart and brain tissues, products of PCR amplification of a ~500-bp fragment were subjected to restriction analysis using Nael that cuts this fragment uniquely within the region encoding the 10-amino-acid insertion and BsaI that cuts uniquely within the region that is deleted in the brain isoform. The results (Fig. 5B) revealed that most of the brain PCR products are digested by Nael but not by BsaI, indicating that the isoform

5 Genomic data now reveal that some of the variants previously described probably represent misspliced RNA.
containing both insertion and deletion is the major form in the brain. By contrast, this analysis revealed that the isoform containing an insertion is a minor form in heart. To determine whether the isoforms are expressed at the protein level, we raised antibodies against a synthetic peptide representing the 10-residue insertion (Fig. 5C). Western blot analysis of total extracts from rat tissues revealed that the isoform containing the insertion is highly expressed in brain and is weakly expressed in heart, whereas this form is absent in the other tissues, in agreement with the observations at the cDNA level. Blotting with antibodies that recognize both the ubiquitous and variant forms or with antibodies against the insertion peptide found in the brain and heart isoforms (α-Pep).

To study the cellular localization of the new isoforms, we first attempted to identify a cell line that naturally expresses these isoforms by PCR and restriction analysis as described in Fig. 5B. However, the presence of isoforms could not be detected in any of the lines tested, including transformed cells from neuronal or glial origin. Hence, we attempted to identify a cell line that naturally expresses these isoforms. We also generated a form that has a deletion like the one found in the brain form but does not contain the insertion found in this form. Such a form was not identified in rat tissues in the present study, but its possible existence in mice is suggested by data bank mRNA sequences (e.g. BC059817). When fused to GFP and transfected into HeLa cells, the form with a deletion but no insertion showed only a weak Golgi localization, suggesting that the brain isoform deletion region (residues 259–280) contributes to the Golgi localization of the ubiquitous form of ArfGAP1. Western blot analysis showed that the different forms are not subjected to any major degradation in HeLa cells (Fig. 6B).

The difference in Golgi localization between the forms with a deletion only (mostly cytosolic) versus deletion plus insert (Golgi) raised the possibility that the insertion found in the brain and heart isoforms functions as a Golgi localization determinant. This was investigated by testing whether the 10-residue insertion can affect the localization of short ArfGAP1 fragments that terminate before residue 280 and therefore show cytosolic distribution (28). As shown in Fig. 7, a clear Golgi localization was observed in fragments that ended in residue 267 or 249 when these fragments were made from the brain isoform and thus included the 10-residue insertion, whereas, as expected, the equivalent fragments from the ubiquitous isoform were not localized. An isoform fragment that terminated at the insertion peptide and contained just 52 residues (198–249) was still Golgi-localized. These findings suggest that the 10-residue insertion that is present in the isoforms contains a Golgi localization determinant. By contrast, a fragment from the brain isoform that started at residue 210 and extended to the carboxyl terminus of the protein was not localized, indicating that as in the ubiquitous form, the stretch of the hydrophobic residues (204–214) is required for Golgi localization of the brain isoform fragment.

A brain-specific ArfGAP1 isoform is also present in humans (GenBank™ BC028233). This isoform has a deletion of only 2 residues (compared with a 22-residue deletion in the rat), whereas the insertion sequence is well conserved (FWGHKQQPEP in humans, FWGKYKQSEP in rats). We focused our attention on the two hydrophobic residues that are conserved between humans and rats (Phe-240 and Trp-241), and tested their role in Golgi interaction. As shown in Fig. 7B, alanine replacement of either the Phe-240 or the Trp-241 residue within the GFP-fused fragment encompassing residues 198–249 of the isoform strongly diminished Golgi localization. By contrast, leucine replacement of both residues (FW/LL) did not
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![Diagram](Image)

FIGURE 7. Effect of the insertion region found in the ArfGAP1 isoforms on the localization of short ArfGAP1 fragments. A, localization of GFP fusions of fragments representing the ubiquitous or the brain isoform defined by the schemes on the left side; the dark box represents the 10-residue insertion sequence, and the striped box represents the region that is deleted in the brain isoform (see Fig. 5). B, effect of replacement of hydrophobic residues within the insertion sequence on Golgi localization. The GFP-fused construct encompassing residues 198–249 in the brain isoform (see Fig. 5) was subjected to alanine or leucine replacements of Phe-240 and Trp-241. C and D, Western blot analysis of the expression of the different constructs shown in A (C) and in B (D).

We found that hydrophobic ALPS residues are also necessary for GAP activity of the full-length ArfGAP1 on Golgi membrane-bound Arf1 in vitro (Fig. 4). Because the catalytic activity of ALPS mutants in a membrane-free assay resembled that of wild-type ArfGAP1, their reduced activity at the Golgi membrane must be because of a role that the hydrophobic residues play in the binding the GAP to the membrane, where it interacts more efficiently with its membrane-bound Arf1-GTP substrate (see Refs. 22, 23, and 27). The results of this in vitro experiment with purified components indicate that the interaction of the hydrophobic ALPS residues with the Golgi is direct and does not require the recruitment of any cytosolic factor.

Together with the findings by Bigay et al. (24), our results thus lend support to the idea that ArfGAP1 interaction with the Golgi is mediated in part by direct binding of the protein to membrane domains containing loosely packed lipids. The presence of such domains may be expected in budding sites, where ArfGAP1 binding to the highly curved bud membrane was proposed to serve as a mechanism for inactivating Arf1 at the bud center but not at the bud rims, where recruitment of new coat subunits takes place (23, 25, 26). Loosely packed lipid domains could also be formed by cone-shaped lipids such as diacylglycerols that are continuously generated at the Golgi from phosphatidylcholine by the phospholipase-D and the sphingomyelin synthase pathways (36–38). Diacylglycerols were found to promote the recruitment of ArfGAP1 to liposomes (22) and to stimulate the activity of both ArfGAP1 and its yeast orthologue Gcs1 (22, 39). Furthermore, genetic evidence in yeast suggests that proper Golgi trafficking depends on diacylglycerol-mediated regulation of the activity of the ArfGAPs Gcs1 and Age2 (39, 40). Because the structural requirements of the acyl chains in diacylglycerols that mediate GAP binding correspond to those generated at the Golgi but not to those generated by the phosphoinositide-specific phospholipase C pathway at the plasma membrane (22), diacylglycerols could mediate specific targeting of ArfGAP1 to the Golgi membrane.

There are some quantitative differences between the results of the
in vivo and in vitro studies. Thus, the relative contribution of individual residues to Golgi interaction in our study is by the order Leu-207 -> Trp-211 > Met-204 > Phe-214, whereas the order observed for binding to small liposomes (24) was Trp-211 > Phe-214 > Leu-207. Additionally, the avid interaction of ArfGAP1 with small liposomes (24) contrasts with the rapid on/off kinetics of its interaction with the Golgi as observed by fluorescence recovery after photobleaching of GFP-fused ArfGAP1 (20). Possible explanations for these apparent discrepancies are that Golgi membrane domains with which the hydrophobic residues in ArfGAP1 interact are not as unstructured as those in small liposomes and that the interaction of ArfGAP1 with the Golgi phospholipid bilayer is hindered by the presence of proteins at the membrane surface. Although in vitro experiments with purified components (41) have demonstrated that ArfGAP1 is incorporated into Golgi-derived COPI vesicles, further studies will be required for determining the membrane domains with which ArfGAP1 interacts in vivo and the role of membrane curvature and/or cone-shaped lipids in this interaction.

Although our findings demonstrate that the hydrophobic residues in the ALPS domain are necessary for Golgi localization, the ALPS domain by itself does not confer Golgi localization on GFP fusion constructs (Ref. 28 and the present study). Golgi localization requires the presence of ~100 residues at the carboxyl side of ALPS (28), suggesting that ArfGAP1 interaction with the Golgi is mediated by multiple low affinity binding sites. Interestingly, we observed that in the new ArfGAP1 isoforms that we have identified, Golgi localization requires much shorter carboxy-terminal sequences. Analysis of these requirements (Fig. 7) indicated that an in-frame insertion of 10 residues that is present in the new isoforms can function along with the ALPS domain to confer Golgi localization even in the absence of ArfGAP1 sequences beyond the insertion point (residue 239). Unlike the ALPS domain, the insertion sequence (239-249) and its neighboring residues cannot form an amphipathic helix. Nevertheless, the finding that alanine replacement of either Phe-240 or Trp-241 abrogates Golgi localization of the carboxyl-truncated ArfGAP1 fragment suggests that the insertion sequence is involved in hydrophobic interaction with the Golgi membrane. Indeed, like ALPS motif residues, the nature of the hydrophobic residues in the insertion sequence did not appear critical, as replacement of both the Phe and Trp residues with leucines did not diminish Golgi localization. This promiscuity of the hydrophobic residues favors a protein-lipid rather than a protein-protein interaction mechanism.

While the isoform in the heart contains just the 10-residue insertion, the one in rat brain contains in addition a proximal deletion of 22 residues (residues 240–249 for the insertion, 259–280 for the deletion). Unlike the brain isoform that is well localized at the Golgi, deletion of residues 259–280 in the ubiquitous form of ArfGAP1 strongly diminished its Golgi localization (Fig. 6A). Interestingly, the deletion sequence contains part of a motif displaying physicochemical properties that are reminiscent of ALPS and hence could also contribute to the recognition of the physical state of the lipid membrane.6

The unexpectedly complex pattern of ArfGAP1 localization motifs that appears to emerge points to regulatory complexity, where different motifs on GAP may respond differentially to local changes in lipid composition and/or membrane curvature. The addition/deletion of membrane-interacting motifs in splice isoforms may thus provide alternative modes of ArfGAP1 targeting in the tissues expressing the isoforms. Finally, ArfGAP1 was reported to interact with a number of Golgi-associated proteins (19, 20, 29, 33, 42), and such interactions, as well as the interaction with the Arf1 substrate (19), are also likely to contribute to ArfGAP1 interaction with the Golgi apparatus.

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REFERENCES

1. Bonifacino, J. S., and Glick, B. S. (2004) Cell 116, 153–166
2. McMahon, H. T., and Mills, I. G. (2004) Curr. Opin. Cell Biol. 16, 379–391
3. Lee, M. C., Miller, E. A., Goldberg, J., Orci, L., and Schekman, R. (2004) Annu. Rev. Cell Dev. Biol. 20, 87–123
4. Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M., and Schekman, R. (1994) Cell 77, 895–907
5. Matsuoka, K., Orci, L., Amherdt, M., Bednarz, S. Y., Hamamoto, S., Schekman, R., and Yeung, T. (1998) Cell 93, 263–275
6. Zhao, L., Helms, J. B., Brunner, J., and Wieland, F. T. (1999) J. Biol. Chem. 274, 14198–14203
7. Malhotra, V., Serafini, T., Orci, L., Shepherd, J. C., and Rothman, J. E. (1989) Cell 58, 329–336
8. Yoshihisa, T., Barlowe, C., and Schekman, R. (1993) Science 259, 1466–1468
9. Randazzo, P. A., and Hirsch, D. S. (2004) Cell Signal 16, 401–413
10. Mekler, V., Cukierman, E., Rotman, M., Admon, A., and Cukierman, E. (1999) J. Biol. Chem. 270, 5232–5237
11. Cukierman, E., Huber, I., Rotman, M., and Cukierman, E. (1995) Science 270, 1999–2002
12. Zhang, C., Yu, Y., Zhang, S., Liu, M., Xing, G., Wei, H., Bi, J., Liu, X., Zhou, G., Dong, C., Hu, Z., Zhang, Y., Luo, L., Wu, C., Zhao, S., and He, F. (2000) Genomics 63, 400–408
13. Liu, X., Zhang, C., Xing, G., Chen, Q., and He, F. (2001) FEBS Lett. 490, 79–83
14. Watson, P. J., Frigerio, G., Collins, B. M., Duden, R., and Owen, D. J. (2004) Traffic 5, 79–88
15. Poon, P. P., Wang, X., Rotman, M., Huber, I., Cukierman, E., Cassel, D., Singer, R. A., and Johnston, G. C. (1999) EMBO J. 18, 555–564
16. Goldberg, J. J. (1999) Cell 96, 893–902
17. Szafir, L., Rotman, M., and Cassel, D. (2001) J. Biol. Chem. 276, 47834–47839
18. Lee, S. Y., Yang, J. S., Hong, W., Premont, R. T., and Hsu, V. W. (2005) J. Cell Biol. 168, 281–290
19. Liu, W., Duden, R., Phair, R. D., and Lippincott-Schwartz, J. (2005) J. Cell Biol. 168, 1053–1063
20. Huber, I., Cukierman, E., Rotman, M., Aoe, T., Hsu, V. W., and Cassel, D. (1998) J. Biol. Chem. 273, 24786–24791
21. Antonny, B., Huber, I., Paris, S., Chabre, M., and Cassel, D. (1997) J. Biol. Chem. 272, 30848–30851
22. Bigay, J., Gounon, P., Robinneau, S., and Antony, B. (2003) Nature 426, 563–566
23. Bigay, J., Casella, J. F., Drin, G., Mesmin, B., and Antony, B. (2005) EMBO J. 24, 2244–2253
24. Lippincott-Schwartz, J., and Liu, W. (2003) Nature 426, 507–508
25. Holthuis, J. C., and Burger, K. N. (2003) Dev. Cell 5, 821–822
26. Szafir, L., Pick, E., Rotman, M., Zuck, S., Huber, I., and Cassel, D. (2000) J. Biol. Chem. 275, 23615–23619
27. Yu, S., and Roth, M. G. (2002) Mol. Biol. Cell 13, 2559–2570
28. Aoe, T., Cukierman, E., Lee, A., Cassel, D., Peters, P. J., and Hsu, V. W. (1997) EMBO J. 16, 7305–7316
29. Aoe, T., Huber, I., Vasudevan, C., Watkins, S. C., Romero, G., Cassel, D., and Hsu, V. W. (1999) J. Biol. Chem. 274, 20545–20549
30. Storrie, B., White, J., Rotger, S., Stelzer, E. H., Suganuma, T., and Nilsson, T. (1998) J. Cell Biol. 143, 1505–1521
31. Franco, M., Chardin, P., Chabre, M., and Paris, S. (1993) J. Biol. Chem. 268, 24531–24534
32. Lanoix, J., Ouwendijk, J., Lin, C. C., Stark, A., Love, H. D., Ostermann, J., and Nilsson, T. (1999) EMBO J. 18, 4935–4948
33. Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992) J. Cell Biol. 116, 1071–1080
34. Paris, S., Beraud-Dufour, S., Robineau, S., Bigay, J., Antony, B., Chabre, M., and Chardin, P. (1997) J. Biol. Chem. 272, 22221–22226

6 B. Mesmin, J. Bigay, and B. Antonny, personal communication.
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36. Pagano, R. E. (1988) Trends Biochem. Sci. 13, 202–205
37. Bankaitis, V. A. (2002) Science 295, 290–291
38. Litvak, V., Dahan, N., Ramachandran, S., Sabanay, H., and Lev, S. (2005) Nat. Cell Biol. 7, 225–234
39. Yanagisawa, L. L., Marchena, J., Xie, Z., Li, X., Poon, P. P., Singer, R. A., Johnston, G. C., Randazzo, P. A., and Bankaitis, V. A. (2002) Mol. Biol. Cell 13, 2193–2206
40. Wong, T. A., Fairn, G. D., Poon, P. P., Shmulevitz, M., McMaster, C. R., Singer, R. A., and Johnston, G. C. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 12777–12782
41. Yang, J. S., Lee, S. Y., Gao, M., Bourgoin, S., Randazzo, P. A., Premont, R. T., and Hsu, V. W. (2002) J. Cell Biol. 159, 69–78
42. Hirst, J., Motley, A., Harasaki, K., Peak Chew, S. Y., and Robinson, M. S. (2003) Mol. Biol. Cell 14, 625–641