The interaction of the Arf1-directed GTPase-activating protein ArfGAP1 with the Golgi apparatus depends on motifs in its noncatalytic part that are unstructured in solution but are capable of folding into amphipathic helices in vitro upon interaction with poorly packed lipids. In previous studies a few hydrophobic residues that are critical for lipid binding and Golgi localization were identified, but the precise topology of the amphipathic motifs has not been determined. Here we present a detailed analysis of the Golgi targeting and in vitro folding features of the region encompassing the amphipathic motifs (residues 199–294). Point mutation analysis revealed that most hydrophobic residues within this region contribute to Golgi localization, whereas analysis by proline replacements and alanine insertions revealed that Golgi interaction depends on folding into two amphipathic helices with a short interrupting sequence. Analysis of splice isoforms containing 10-residue in-frame insertions within their first amphipathic motifs revealed that the insertion causes a truncation of the amphipathic helix that does not extend beyond the insertion sequence. Lastly, a lysine replacement mutant recently reported to bind to negatively charged liposomes in a curvature-independent manner showed normal cellular distribution, suggesting that Golgi targeting of ArfGAP1 may involve factors other than sensing lipid packing.

Arf (ADP-ribosylation factor) GTPases serve as regulators of membrane traffic at multiple cellular compartments (reviewed in Refs. 1 and 2). At the Golgi, the Arf1 GTPase regulates the recruitment of the COPI coat to cisternal membranes (3, 4). Arf1 is converted to the GTP-bound form by the action of guanine nucleotide exchange proteins (5), whereupon it translocates from cytosol to the Golgi membrane. The COPI coat is then recruited to the membrane through its interaction with the activated Arf1 (6–8). The subsequent hydrolysis of GTP on Arf1, driven by a GTPase-activating protein, is a prerequisite for coat protein dissociation (9, 10).

ArfGAPs3 constitute a large family of proteins sharing a small catalytic domain with a characteristic zinc finger signature (11–13). Among several ArfGAPs that may function at the Golgi, the best characterized is ArfGAP1, a 415-residue protein with a conserved 130-residue catalytic domain at its amino terminus and a unique noncatalytic part (14, 15). ArfGAP1 rapidly cycles on and off the Golgi apparatus (16), and its interaction with the Golgi is determined primarily by the noncatalytic part (16–19). In addition to its GAP function, ArfGAP1 was proposed to play a role in processes such as cargo sorting (20, 21) and vesicle budding and scission (22, 23). An important indication of how ArfGAP1 might regulate the sequence of GTP binding and hydrolysis during COPI vesicle formation came from studies by Antonny and co-workers (24). These investigators observed that GAP activity on liposome-bound Arf1 is dramatically increased with decreasing liposome size, apparently because of increased avidity of ArfGAP1 to these liposomes and the resulting proximity to its substrate. This has led to a model whereby GTP hydrolysis is restricted to curved regions of the bud where the coat has already been stabilized due to lateral interaction, whereas the negative curvature at the base of the bud prevents ArfGAP1 binding, allowing efficient recruitment of new coat by binding to Arf1-GTP. A subsequent study (25) showed that ArfGAP1 interacts with the loosely packed lipids found in highly curved liposomes through a motif in the noncatalytic part that was termed ArfGAP1 lipid packing sensor (ALPS). This motif is unstructured in solution, but upon binding to liposomes it folds into an amphipathic helix that is stabilized by the insertion of bulky hydrophobic residues into open spaces in the outer lipid bilayer. A role of the ALPS motif in vivo was demonstrated by our finding that Golgi localization of ArfGAP1 is abrogated by point mutations of hydrophobic residues near the amino side of ALPS (19). More recently, a second amphipathic motif (ALPS2) with similar physicochemical characteristics was identified in ArfGAP1. The ALPS2 motif was shown to function in cooperation with the originally identified motif (ALPS1) in conferring liposome interaction, and a point mutation in an ALPS2 hydrophobic residue (V279D) was found to abrogate Golgi localization (26). However, the precise extent and topology of the ALPS motifs and the role of the multiple hydrophobic residues along their sequence in Golgi localization have not been defined.

ArfGAP1 is expressed in several isoforms that present variations in the ALPS motifs. In addition to a ubiquitous form expressed in all rat tissues examined so far, there are tissue-specific isoforms (19). One isoform expressed in heart tissue contains an in-frame 10-residue insertion in proximity to the ALPS1 motif, whereas a second isoform that constitutes the major form of ArfGAP1 in the rat brain contained the same insertion together with a 22-residue deletion within the ALPS2 motif. Even though this deletion diminishes the ability of the modi-
fied ALPS2 motif to support Golgi localization, the brain isoform is Golgi localized, apparently because of an enhanced Golgi-localizing propensity conferred on the ALPS1 motif by the insertion sequence.

In this study, we carried out a detailed investigation of the structure-function relationship of the region containing the ALPS motifs in ArfGAP1 and its splice isoforms. The combined results of Golgi localization and in vitro experiments indicate that the first amphipathic helix is considerably longer than previously thought and that a short nonhelical segment separates the two amphipatic motifs. We also show that the insertion peptide in the splice isoform creates a break that truncates the first amphipathic helix. Based on these and additional data, we discuss the mechanism of ArfGAP1 interaction with the Golgi.

EXPERIMENTAL PROCEDURES

DNA Constructs, Transfection, and Western Blot Analysis—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; full-length ArfGAP1 cloned into the pmCherry-C1 vector was provided by J. Bigay. Mutations were introduced by PCR and were confirmed by DNA sequencing. Plasmids (0.2 μg) were transfected into HeLa cells grown on 13-mm glass coverslips in 24-well plates using 1 l of FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. GFP fused to nuclear export signal peptides in the splice isoform creates a break that truncates the first amphipathic helix. Based on these and additional data, we discuss the mechanism of ArfGAP1 interaction with the Golgi.

To determine the localization of ArfGAP1 and its splice isoforms, cells were grown on 13-mm glass coverslips in 24-well plates using 1 l of FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. GFP fused to nuclear export signal peptides in the splice isoform creates a break that truncates the first amphipathic helix. Based on these and additional data, we discuss the mechanism of ArfGAP1 interaction with the Golgi.

Protein expression was monitored by Western blot analysis using anti-GFP antibodies, confirming that all constructs gave rise to a fusion protein with the expected size.

Labeling with Antibodies and Fluorescence Microscopy—The cells were fixed with 4% paraformaldehyde 20–22 h following transfection. For immunostaining, the 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, 1:400). The coverslips were mounted on glass slides using Mowiol as glue and were visualized under a Leica DMIRE2 inverted fluorescent microscope at a 40× or 63× magnification.

Quantitation of ArfGAP1 Cellular Distribution—Digital images of cells that have been transfected with GFP-ArfGAP1 constructs and counter-stained with anti-GM130 were analyzed by marking the GM130-stained area and determining the average pixel intensity of the GFP fluorescence in this area (defined as the Golgi GFP fluorescence). A thin area of cytoplasm near the Golgi was manually marked, and the average GFP fluorescence was determined (cytosolic fluorescence; see Fig. 1). Cells in which the Golgi image overlapped with the nuclear area were excluded from the analysis. Following subtraction of background from cell-free areas of the image, the ratio of Golgi to cytoplasmic fluorescence was calculated. For each mutant the entire experiment from transfection to counting was repeated at least twice, and the combined data from a total of 30–50 cells were averaged; the results are presented as the means with error bars indicating the standard deviation.

Statistical Analysis—The statistical significance of data sets was tested and established by the Kruskal-Wallis one-way analysis of variance by ranks (28). The significance of differences between wild type and mutants was estimated by a one-sided multiple comparison test implemented in function kruskalmc of the R-package pgirmess. Statistical analysis was performed using the R statistical system.

Preparation of Recombinant ArfGAP1 Fragments—ArfGAP1 cDNA containing silent mutations avoiding rare codons (20) was subjected to PCR amplification with primers that included a 5’ XbaI site and a 3’ BamHI site, and the PCR products were cloned into the T7 polymerase-driven pKM260 vector between the Nhel and the BamHI sites. Proteins were expressed in the BL21/DE3 Escherichia coli harboring pLysS by induction for 3 h at 30 °C in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside. For GAP activity assay, bacterial pellets were extracted with 6 mM guanidine hydrochloride, 20 mM Tris, pH 8, 5 mM 2-mercaptoethanol, and proteins were purified by nickel-nitritolriatic acid chromatography, eluted with 200 mM imidazole, dialyzed, and further purified by Resource Q chromatography as previously described (29). Proteins to be used for CD measurements were prepared by lysing a pellet of a 400-ml induced bacterial culture by freezing and thawing three times. The pellets were resuspended at room temperature in 12 ml of 100 mM NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA, 10 mM MgCl2 containing 0.5 mg of DNase I and left at room temperature for a few minutes, followed by the addition of 1% Triton X-100. Inclusion bodies were collected by centrifugation (9,000 rpm for 15 min), washed, and resuspended in 100 mM NaCl, 20 mM Tris, pH 8, and solubilized in 5 mM guanidine hydrochloride, 20 mM Tris, pH 8. Proteins were purified by nickel-nitritolriatic acid chromatography in the presence of 5 mM guanidine followed by elution with 0.2 M imidazole in the guanidine solution, overnight dialysis against 10 mM Tris, pH 8, 150 mM KCl, and centrifugation to remove any insoluble material. Purity of the recombinant proteins was analyzed by SDS-PAGE and Coo massie Blue staining.

GAP Activity Assay—Measurement of GAP activity on Golgi membranes was carried out as previously described (30). Briefly, recombinant myristoylated Arf1 (31) was loaded with [γ-32P]GTP onto rat liver Golgi membranes using the guanine nucleotide exchange activity that is present in the membrane. Exchange was stopped by the addition of 0.3 mM brefeldin A, followed by the addition of GAP. After different incubation times at 30 °C, the samples were filtered through a nitrocellulose filter, and the amount of radioactivity remaining on the filter (representing Arf1-bound GTP) was determined. The data were fitted to an exponential decay curve (y = ae−bx) using the algorithm provided by SigmaPlot 9.0; the b value of the control without GAP was subtracted from b values of the samples, and the percentage of activity of mutants was calculated by comparing their corrected b values with that of wild type GAP. For each mutant assays were repeated two to three times, and the variation of the b values ranged between 13 and 19% of the mean.
Topology of ArfGAP1 Localization Motifs

Liposome Preparation and Circular Dichroism Measurements—Liposomes used in circular dichroism measurements were produced by sonication. The composition of the liposomes was 70 mol % dioleoyl-PC, 30 mol % dioleoyl-PS. A dried film was prepared by evaporation of a mixture of the lipids in chloroform and was resuspended in 150 mM KCl, 10 mM Tris, pH 8, to a final lipid concentration of 15 mM. The mixture was sonicated for 5 min using a Microson sonicator set to low intensity and centrifuged at 100,000 × g for 20 min to remove debris. The liposomes were stored at room temperature under nitrogen and used within 2 days. Average liposome size, determined by dynamic light scattering, ranged between 21 and 30 nm in different experiments.

CD spectroscopy was performed on a Chirascan spectropolarimeter (Applied Photophysics). The experiments were performed at room temperature in a Hellma quartz cell with an optical path length of 0.05 cm in the presence or absence of 2 mM liposomes. Each spectrum was obtained by averaging several scans recorded from 200 to 260 nm with a bandwidth of 1 nm, a step of 1 nm, and a scan speed of 50 nm/min. Control spectra of liposomes in buffer were subtracted from the protein spectra. The data were analyzed by the CDPro software (32).

RESULTS

Definition of Amphipathic Helices Mediating ArfGAP1 Golgi Localization—Previously, the role of the ALPS motifs in targeting ArfGAP1 to the Golgi was inferred from the effect of a few mutations in selective regions of these motifs (19, 25, 26). For a mutagenesis study aimed at defining the extent of the motifs mediating Golgi localization, it was necessary to design a quantitative analysis of ArfGAP1 interaction with the Golgi. Among several approaches, we obtained the best results by the method described in Fig. 1 and under “Experimental Procedures.” The cellular distribution of GFP fusions of ArfGAP1 and mutants thereof was determined as the ratio of the average pixel intensity at the Golgi to that of a thin section of cytosol in the close proximity of the Golgi. Because Golgi staining was faint in many ArfGAP1 mutants, the Golgi area was defined in all experiments by counter-staining with GM130, a cis-Golgi marker that shows a good colocalization with ArfGAP1 (19). As shown in Fig. 1, the data for the noncatalytic fragment (residues 143–415) of the wild type protein showed a good fit to a linear curve, indicating that the amount of Golgi-bound GFP-ArfGAP1 is proportional to that of the cytosolic protein. This was also true for a localization-defective mutant (V268A; Fig. 1), as well as for all other mutants tested, as revealed by $R^2$ values above 0.75 (average = 0.85 ± 0.07). We therefore used the Golgi/cytosol ratio as a measure of the extent of Golgi localization of the mutants, averaging ratios measured in multiple cells.

To analyze the amphipathic motifs, we first tested the effect of alanine replacements of hydrophobic residues all along the region where the presence of two amphipathic motifs (ALPS1 and ALPS2) was previously indicated (19, 25, 26) (Fig. 2A). As shown in Fig. 2B, single or double alanine replacements of most of the hydrophobic residues in the region encompassing residues Met204–Phe294 caused a decrease in ArfGAP1 Golgi localization. GFP with a nuclear export signal that served as a negative control gave a ratio of 1.18, apparently reflecting a greater proximity of the Golgi. Because Golgi staining was faint in many cells expressing wild type GFP-ArfGAP1(143–415) or a localization-defective mutant thereof (V268A) double-labeled with antibodies to the Golgi marker GM130. On the right are shown the same GFP-ArfGAP1-expressing cells with the Golgi area encircled according to the GM130 labeling and with a thin section of cytosol in the immediate vicinity of the Golgi selected for evaluation of cytoplasmic GFP-ArfGAP1 fluorescence. The plots show the average intensity of Golgi GFP fluorescence versus that in the selected cytoplasmic areas in multiple cells, fitted to a linear plot (see under “Experimental Procedures”). WT, wild type.

The finding that the region between the previously defined ALPS motifs contains hydrophobic residues that are required...
Topology of ArfGAP1 Localization Motifs

A

DFLNASAMSSLYSGWSSPTGASKFAASAKEGATKFGSGQASQKASELGH

L

Figure 2. Mutation analysis of ArfGAP1 amphipathic motifs. Mutations were introduced into the GFP-fused noncatalytic fragment of ArfGAP1 (residues 143–415), and their effect on Golgi localization was assessed as described in the legend to Fig. 1 and under “Experimental Procedures.” A, sequence of the ArfGAP1 region under study. Hydrophobic residues that have been subjected to alanine replacement are highlighted in bold type; replacements introduced in the experiment shown in C are indicated on top of the sequence, and points of dialanine insertions (D) are marked by arrowheads. Shading indicates the region between the previously identified amphipathic ALPS motifs. B, effect of alanine replacement of hydrophobic residues on Golgi localization. C, effect of replacement of hydrophilic residues with helix-disrupting residues. Residue 255 was replaced with Gly to generate a Pro-Gly sequence with endogenous Pro254. D, effect of insertion of two alanine residues between the indicated residues. E, effect of mutations on Golgi localization of GFP-fused full-length ArfGAP1. WT, wild type.

for Golgi localization raised the possibility that this region also folds into an amphipathic helix, possibly by extending one of the ALPS motifs. To probe for helical structures mediating Golgi interaction, we tested the effect of the replacement of nonhydrophobic residues along the region encompassing the amphipathic motifs by helix-disrupting residues (Pro or Pro-Gly). As shown in Fig. 2C, proline (or Pro-Gly) replacements introduced at different positions between residues 216 and 285 of the noncatalytic ArfGAP1 fragment diminished Golgi localization. A much smaller inhibition was observed with proline replacement of residue Glu261, suggesting that this residue may not take part in a helical conformation. We also tested the requirement for helix amphipathicity by inserting at different points a pair of alanine residues, mutations that should alter the register of the helix and thus prevent the formation of a continuous hydrophobic face. As shown in Fig. 2D, dialanine insertion at different points between residues 217–276 of the noncatalytic fragment caused a decrease in Golgi localization. Quantitatively similar inhibitory effects of dialanine insertion between positions 229 and 230, as well as diproline replacement of residues 222 and 223 and residues 236 and 237, were observed when the mutations were introduced in the full-length ArfGAP1 (Fig. 2E). The effect of dialanine insertions (Fig. 2D) was smaller at several positions between residues 259 and 267, with a minimal effect between positions 263 and 264. Together with the proline replacement analysis, these findings indicate that a region including residues 259–267 may not adopt a helical conformation; however, because alanine replacement of residues Ile256/Phe257 strongly decreased localization, we assume that these residues may be at the beginning of an amphipathic helix, such that the nonhelical segment includes residues 260–263. Accordingly, the region required for Golgi localization appears to be made up of two amphipathic motifs separated by a short break (see Fig. 7). Somewhat unexpectedly, our findings suggest that the proline residue that is present at position 254 of ArfGAP1 does not constitute a helix break, because mutations introduced immediately following this residue (A255G, A255P, and a dialanine insertion between residues 254 and 255; Fig. 2, C and D) strongly diminished Golgi localization. Residue Pro254 is conserved in mammalian ArfGAP1 proteins, but an equivalent residue is not found in orthologues from organisms such as Drosophila and Caenorhabditis elegans. Indeed, this residue does not seem to be essential, because a P254A mutation had only a small effect on Golgi localization (Fig. 2C, rightmost bar).

Remodeling of the First Amphipathic Helix in Splice Isoforms—We have previously described two splice isoforms of ArfGAP1 that are expressed in rat heart and brain. Both isoforms contain a 10-residue insertion within the first amphipathic motif, whereas the brain isoform has also a 22-residue deletion within the second motif (19) (Fig. 3A). Here we investigated the topological relationship between the 10-residue insertion and the surrounding helical motif by testing the effect of helix-modifying mutations introduced before and after the insertion. We employed the brain isoform in these experiments as Golgi locali-
The structure-function relationship of ArfGAP1 and its isoform was further investigated by in vitro assays. First, we investigated the effect of mutations on GAP activity on purified Golgi membranes (19). The assay is based on loading of myristoylated Arf1 with [γ-32P]GTP at the Golgi membrane by the action of endogenous guanine nucleotide exchange proteins. Subsequently exchange is stopped by the addition of brefeldin A, and the decrease in membrane-bound radioactivity is followed in the presence or absence of GAP. Previously we found that GAP activity on Golgi membranes is decreased by alanine replacement of hydrophobic residues at the amino side of the first amphipathic motif (Met204, Leu207, and Trp211) (19). To investigate the extent of the amphipathic motif required for GAP activity, we tested the effect of helix-modifying mutations introduced at different points. As shown in Fig. 4A, the activity of ArfGAP1(5–257) was almost completely inhibited in an A222P/S223P mutant (87% inhibition by exponential decay curve fitting), whereas a dialanine insertion between positions 229 and 230 and an A236P/S237P mutation caused inhibition by 77 and 71%, respectively. These findings are in agreement with the Golgi localization data, indicating that an extension of the amphipathic motif beyond residue 237 is required for its efficient interaction with the Golgi membrane.

Next we tested GAP activity at the Golgi membrane of the isoform containing the 10-residue insertion (Fig. 4B). In comparison with the ubiquitous form, the isoform fragment ending at the same position but containing the insertion (isoform residues 5–267) showed a 2-fold higher activity (note the different GAP concentrations used in Fig. 4, A and B). Replacement of residues shortly before the insertion (at positions 236 and 237) with prolines resulted in strong inhibition of GAP activity, lending support to the suggestion from the Golgi localization studies that Golgi interaction requires a continuity of the amphipathic helix with the amino side of the isoform insertion.

Effect of Mutations on Liposome-dependent Transition to Helical Structure—To directly assess the folding of the first amphipathic motif, we studied its interaction with small liposomes by circular dichroism spectroscopy, based on the liposome-induced transition of this motif from random to helical structure (26, 33). CD measurements were carried out using a fragment of ArfGAP1 encompassing residues 137–257. As shown in Fig. 5, the addition of small liposomes (21–30 nm) to

4 S. Levi, M. Rawet, L. Kliouchnikov, A. Parnis, and D. Cassel, unpublished observations.
bic interactions, amphipathic helices frequently interact with membranes (Fig. 3). This probably reflects a helix break introduced by the insertion (residues 137–257) in comparison with that of the ubiquitous (ub) form (residues 137–267). Dashed and solid lines indicate the absence and presence of liposomes, respectively. B, Coomassie Blue-stained SDS gel of the recombinant proteins employed (3 μg). WT, wild type; MRE, mean residue ellipticity.

nonmutated ArfGAP1(137–257) resulted in a strong transition to helical structure, as revealed by the minima around 210 and 225 nm, recapitulating previous observations with the same fragment (25). This transition was much smaller in a mutant with a diproline replacement mutation at positions 222 and 223 and was also significantly decreased in mutants with dialanine insertion and diproline replacement at positions 229 and 230 and positions 236 and 237, respectively. Analysis of the spectra using the CDPro program gave 31.2% helical conformation for the wild type protein in the presence of liposomes, and 7.8% for the nonmutated ArfGAP1(137–257) and 10 μM of the insertion isoform in the absence of liposomes. Interestingly, the CD spectrum of the corresponding fragment containing the 10-residue insertion residues 137–267) decreased to 17.3, 19.8, and 21.8% for the above-mentioned mutants (labeled (2), (3), and (4) in Fig. 5A, respectively) with little change in the helical content in the absence of liposomes. Interestingly, the CD spectrum of the corresponding fragment containing the 10-residue insertion residues 137–267) showed a lower degree of transition to helical conformation in the presence of liposomes (estimated at 20.8%) in comparison with that of the ubiquitous form. This probably reflects a helix break introduced by the insertion sequence, already anticipated from the localization experiments (Fig. 3B). A diproline replacement just before the insertion sequence (positions 236 and 237) had only a small effect on the CD spectrum in the presence of liposomes, indicating that the helix does not extend much beyond this point in the isoform.

Role of Electrostatic Interactions—In addition to hydrophobic interactions, amphipathic helices frequently interact with membranes through positively charged residues (34, 35). We tested the possible role of electrostatic interactions by alanine replacement of lysine residues that are present in the amphipathic motifs. This analysis included three lysine residues of the first amphipathic motif, two of which (Lys226 and Lys231) are predicted to point to a direction favoring membrane interaction (36), as well as the only two lysine residues in the second motif. As shown in Fig. 6A, good Golgi localization was observed in mutants containing double or triple alanine replacements on the localization of a short ArfGAP1 construct (residues 198–240). The cells were counter-stained with antibodies against the Golgi marker GM130. GM130.

FIGURE 5. Effect of liposomes on the CD spectrum of ArfGAP1 mutants. A, CD measurements were carried out as described under “Experimental Procedures” using 20 μM of the ubiquitous (ub) form (residues 137–257) and 10 μM of the insertion isoform (iso, residues 137–267). Dashed and solid lines indicate the absence and presence of liposomes, respectively. B, Coomassie Blue-stained SDS gel of the recombinant proteins employed (3 μg). WT, wild type; MRE, mean residue ellipticity.

FIGURE 6. Role of electrostatic interactions in ArfGAP1 localization. A, effect of double and triple alanine replacement of lysine residues on the localization of ArfGAP1(143–415). KKK/AAA designates alanine replacement of lysines 226, 231, and 239; KK/AA designates alanine replacement of lysines 278 and 285. B, effect of replacement of residues Ser206 and Thr215 with lysines. The cells were cotransfected with GFP-tagged lysine mutant and wild type Myc-tagged or m-Cherry-fused ArfGAP1 (all as full-length proteins). C, effect of lysine replacements on the localization of a short ArfGAP1 construct (residues 198–240). The cells were counter-stained with antibodies against the Golgi marker GM130. WT, wild type.
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tively, as compared with a value of 2.12 ± 0.26 for the control. These findings indicate that electrostatic interactions do not play a critical role in ArfGAP1 interaction with the Golgi, in agreement with data obtained from studies of GAP interaction with liposomes (26).

We also examined the effect of introducing extra positive charges into the first amphipathic motif at positions recently shown to result in a strong interaction of this motif with negatively charged liposomes regardless of the membrane curvature (37). Mutations of this kind might be expected to alter the cellular distribution of ArfGAP1 as a result of its interaction with all negatively charged cellular membranes. However, as shown in Fig. 6B, such mutant (S206K/T215K) expressed as a full-length GFP fusion showed normal Golgi localization, closely matching that of a Myc-tagged or a mCherry fused wild type protein (except for punctuate structures observed occasionally in the lysine mutant). Similar results were obtained with the noncatalytic part.4 We next tested whether the lysine mutation can affect the localization of a short fragment containing only residues 198–240 of the first amphipathic motif (Fig. 6C). Whereas the wild type 198–240 fragment showed a diffuse cytosolic distribution with only slight Golgi labeling in some cells, the corresponding S206K/T215K fragment displayed significant colocalization with the Golgi marker GM130. In addition, whereas the GFP fusion of the wild type 198–240 fragment appeared to enter into the nucleus (as does GFP itself in HeLa cells), the corresponding fragment of the S206K/T215K mutant was excluded from the nucleus, possibly because of its interaction with endomembranes. Similar cellular distribution was observed with a different lysine replacement mutant (S205K/T216K) where the lysine residues point to the polar face where they were found to have little effect on liposome interaction (37), indicating that the membrane-targeting effect of the lysine residues is not position-specific.

DISCUSSION

Analysis of motifs whose folding depends on lipid interaction is not straightforward, and biophysical approaches may lead to contradictory results, depending on the lipidic environment and the technique employed (e.g. Ref (38, 39). In this study, we have analyzed the structure-function relationship of ArfGAP1 membrane targeting motifs both in vitro and in cells. The combined results of these approaches indicate that Golgi localization of the noncatalytic part of ArfGAP1 requires its folding into two amphipathic helices separated by a short break (Fig. 7). Hydrophobic residues all along these helices contribute to Golgi localization, and helix continuity is required. The first amphipathic motif (ALPS1) is about twice as long as the second motif (ALPS2), which can account for previous findings (26) that ALPS1 contributes more than ALPS2 to liposome interaction. The inhibitory effect of dialanine insertions at multiple points along the two amphipathic motifs (Fig. 2D) demonstrates the role of helix amphipathicity in Golgi localization. In vitro, the ~60-residue-long ALPS1 motif appears to fold onto small liposomes starting at the amino side, because CD experiments suggest that the introduction of helix-interrupting residues allows folding of only the amino side of the motif up to the point of interruption (Fig. 5); this is probably due to the higher density of hydrophobic amino acids near the amino side of this motif.

Even though the precise mode of the interaction of the ALPS motifs with the Golgi has not been demonstrated, the correlation between Golgi localization of ArfGAP1 mutants and their ability to interact with liposomes (Refs. 19, 25, and 26 and the present study) suggests that ArfGAP1 may directly interact with the Golgi lipid membrane. In support of this idea are our previous findings of the promiscuous nature of some ALPS1 hydrophobic residues, whose replacement with other hydrophobic residues has no effect on Golgi interaction (19). Originally, the ALPS motifs were proposed to interact with membranes by inserting hydrophobic residues into open spaces between lipid chains caused by the high curvature that prevails in COPI buds and vesicles (25). As previously pointed out (26), a structure consisting of two helices should be more compatible than one long helix for binding to highly curved membranes. However, the ALPS motifs can also bind to membranes containing conical lipids such as diacylglycerols (26, 40), and a functional link between ArfGAPs and diacylglycerols was demonstrated in yeast (41, 42) and recently also in mammalian cells (43). Our findings showing that alanine replacement of almost any hydrophobic residue along the ALPS motifs decreases Golgi localization suggest binding through multiple weak interactions, which would be compatible with only moderate lipid disorder at the target membrane. Such a situation could correspond to the presence of a few percentages of phosphatidylincholine-derived diacylglycerols in the Golgi membrane or to a moderate membrane curvature that may exist at Golgi domains like the rims of the cisternae.

Different splice isoforms of ArfGAP1 display modifications in the ALPS motifs (19). Here we analyzed the modulation of the first amphipathic motif by the 10-residue insertion that is present in splice isoforms from rat heart and brain. Previously, we provided evidence that this insertion can increase the interaction of the first amphipathic motif with the Golgi in cells, making this interaction independent on the second amphipathic motif (19); this effect could be largely attributed to the FW sequence at the amino side of the insertion (Ref (19). The results reported herein show that the insertion forms a continuous helix with the upstream sequence but also introduces a helix break that prevents the extension of the helix beyond the insertion sequence (Fig. 7; this is in accordance with
the presence of proline residue(s) at the insertion carboxyl side (one proline residue in rats, two in humans). Apparently, the segment between the isoform insertion and the beginning of the second amphipathic motif (isofrom residues 249–270) does not independently fold into a helix, because mutations in this segment that abrogate Golgi localization of the ubiquitous form had no effect on the localization of the isoform (Fig. 3). Why different tissues possess ArfGAP1 isoforms with modified ALPS motifs is yet unknown, although a logical explanation would be that the characteristics of the helices needs to be adjusted to differential composition of the Golgi membrane in each tissue.

Drin et al. (37) have recently demonstrated that the curvature-sensitive ALPS motifs in ArfGAP1 belong to a class of amphipathic motifs that are present in different proteins. Unlike “classical” amphipathic motifs where membrane interaction is reinforced by the presence of positively charged residues, the ALPS-like motifs are largely devoid of such residues and therefore are designed to specifically interact with membranes containing loosely packed lipids such as those found in highly curved membranes. In agreement with this idea, we found that the interaction of the ALPS motifs with the Golgi is mostly hydrophobic in nature, whereas the few lysine residues that are present in these motifs do not appear to contribute significantly to Golgi localization (Fig. 6A). As a test for their model, Drin et al. (37) investigated the effect of introducing lysine residues at the interface between the polar and nonpolar faces of the ALPS1 motif of ArfGAP1, a position known to be optimal for lipid interaction (the “snorkeling” effect) (36). Such mutations were found to strongly diminish the curvature dependence of ALPS1 interaction with negatively charged liposomes, whereas lysines introduced at the polar face had little effect (26). In cells, the curvature-insensitive lysine mutants might be expected to loose Golgi-selective binding and interact with multiple negatively charged compartments including the plasma membrane. However, tagged versions of such mutants did not show any significant difference in cellular distribution when compared with the wild type (Fig. 6). Moreover, the introduction of lysine replacement mutations into a GFP-fused fragment containing only the first 40 residues of ALPS1 resulted in significantly enhanced Golgi localization with no evidence for plasma membrane interaction. These findings cannot be readily explained by a mechanism in which specific Golgi targeting of the ALPS motifs is solely based on lipid interactions. Rather, a more specific targeting mechanism that might involve an initial interaction of the ALPS motifs with a Golgi membrane protein should be considered. Potential candidates for such “GAP receptor” could be Golgi membrane proteins previously reported to interact with ArfGAP1 such as SNAREs (44, 45) and the KDEL receptor (46). Such an initial recruitment step would then be followed by the transfer of ArfGAP1 to the membrane surface, similarly to the mechanism proposed for targeting Arf1 to the Golgi (47).

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