Arfaptin 1, a Putative Cytosolic Target Protein of ADP-ribosylation Factor, Is Recruited to Golgi Membranes*  

(Received for publication, August 7, 1996, and in revised December 19, 1996)

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ADP-ribosylation factors (ARFs) have been implicated in vesicle transport in the Golgi complex. Employing yeast two-hybrid screening of an HL60 cDNA library using a constitutively active mutant of ARF3 (ARF3-Q71L), as a probe, we have identified a cDNA encoding a novel protein with a calculated molecular mass of 38.6 kDa, which we have named arfaptin 1. The mRNA of arfaptin 1 was ubiquitously expressed, and recombinant arfaptin 1 bound preferentially to class I ARFs, especially ARF1, but only in the GTP-bound form. The interactions were independent of myristoylation of ARF. Arfaptin 1 in cytosol was recruited to Golgi membranes by ARF in a guanosine 5′-O-(3-thiotriphosphate)-dependent and brefeldin A-sensitive manner. When expressed in COS cells, arfaptin 1 was localized to the Golgi complex. The yeast two-hybrid system yielded another clone, which encoded a putative protein, which we have named arfaptin 2. This consisted of the same number of amino acids as arfaptin 1 and was 60% identical to it. Arfaptin 2 was also ubiquitously expressed and bound to the GTP-bound but not GDP-ligated form of class I ARFs, especially ARF1. These results suggest that arfaptins 1 and 2 may be direct target proteins of class I ARFs. Arfaptin 1 may be involved in Golgi function along with ARF1.

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‡ The abbreviations used are: ARF, ADP-ribosylation factor; ARF1–6, ARF isoforms 1–6; GAP, GTPase-activating protein; GEP, guanine nucleotide exchange protein (GEP); GST, glutathione S-transferase; GTP·S, guanosine 5′-O-(3-thiotriphosphate); PAGE, polyacrylamide gel electrophoresis; kb, kilobase pairs.

Yeast Strain and Cell Lines—The yeast strain used in the two-hybrid screening and interaction assay was HFP7c (MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, lys2::GAL1-HIS3, URA3::(GAL4 17-mers)-CYC1-lacZ). HL60 cells were purchased from ATCC and maintained as described (17). COS7 cells were purchased from ATCC and grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere of 10% CO2 and 90% air.

Plasmids and cDNA Library—The ARF cDNAs used in the two-hybrid screening and interaction assays were polymerase chain reaction-amplified from human ARF3 cDNA as a template (kindly provided by J. Moss, National Institutes of Health). Full-length wild type ARF3 cDNA was generated using 5′ (sense) oligonucleotide primer A (5′-CG GAA TTC ATG GCC AAT ATC TTC GGA AAT ATC TTC GGT TTT GAG CGC ATG ATT GGC C). Substitution of Gln71 with Leu (Q71L) was introduced by synthesizing the N-terminal half (corresponding to amino acids 1–71) and C-terminal half (corresponding to amino acids 71–181). The N-terminal half was generated using 5′

2 Apto (απητο) is Greek for “I bind to.”
primer A and mutant 3′ primer C (3′-G CTC TAG ACC ACC CAC ATC CCA CAC TGT AAA GCT; Leu71 is underlined). The C-terminal half was generated using mutagenic 5′ primer D (5′-GCT GTA AAC AAG ATT CGA GCC CCC TCT TGG AGA CA; Leu71 is underlined) and 3′ primer B. The fragments were digested with XhoI and ligated to produce pKK233-2-ARF3. Double mutant ARF3-G2A-Q71L was synthesized using N-terminal (amino acids 1–71) and C-terminal (amino acids 71–181) fragments. The N-terminal half was synthesized using mutagenic 5′ primer E (5′-CG GAA TTC AGC GCC AAT TTC GGA AAC CTT CTC; Ala8 is underlined) and 3′ primer C. The C-terminal half was synthesized as described above. The fragments were digested with XhoI and ligated to produce pKK233-2-ARF3. E.coli strain JM109 was transformed with pKK233-2-ARF3, and protein expression was induced with 2 µM isopropyl-1-thio-β-D-galactoside in 50 °C at 37 °C. Recombinant ARF3 was purified by successive column chromatography on DEAE-Sephalose and then Sephadex 75 as described by Weiss et al. (20) with slight modifications.

For the preparation of the HL60 cDNA library (custom made by Clontech), total RNA was isolated from undifferentiated HL60 cells by the acid guanidium thiocyanate-phenol-chloroform extraction method (18), and poly(A)+ RNA was purified through an oligo(dT)-cellulose column (19). Double-stranded cDNA was made using both oligo(dT) and random primers and introduced into pGAD10 yeast/ E. coli shuttle vector using EcoRI-NolI-SalI adaptor to generate fusions with GAL4 activation domain.

**Northern Blot Assay**—Two-hybrid screening was performed using a Matchmaker Two-Hybrid System (Clontech) according to the instructions provided by the manufacturer. The yeast reporter strain H76c was transformed sequentially with pGBT9-ARF3, pGBT9-ARF3-Q71L, and pGBT9-ARF3-G2A-Q71L. All constructs were confirmed by DNA sequencing.

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**Vero** budding studies—Nucleotide-free ARF was prepared by dialysis of recombinant ARF against 7 M urea as described (20) and incubated at 0.5 µM with or without guanosine 5′-O-(3-thiotriphosphate) (GTP·S) (5 µM) in a reaction mixture consisting of 25 mM Hepes (pH 7.4), 100 mM NaCl, 25 mM KCl, 2.5 mM MgCl2, 1 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, and 0.5 µM bovine serum albumin at 37 °C for 30 min. Triton X-100 was included to increase GTP·S loading and to stabilize the GTP·S:ARF complex (25). GDP-bound ARF was prepared by incubating ARF (not treated with urea) with GDP (5 µM) in the same reaction mixture. GTP·S, ARF-aptin 1, and GST-aptin 2-immobilized glutathione-Sepharose beads prepared as described above were equilibrated with washing buffer A (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol). Fifty µl of nucleotide-preloaded ARF mixture was then incubated with 25 µl of GST, ARF-aptin 1, or GST-aptin 2-immobilized beads for 20 min at 4 °C with gentle rocking. The beads were then collected by pulse centrifugation in a microcentrifuge, washed four times with 0.5 ml of washing buffer A, and resuspended in SDS-sample buffer. ARF associated with the beads was detected by 14% SDS-PAGE followed by immunoblotting using anti-ARF1 antibody.

To determine the affinity of ARF1 for ARF-GTP·S, ARF3 was loaded with 10 µM [35S]GTP·S (4 µCi/nmol) as described above. The concentration of ARF3-[35S]GTP·S was determined by a nuclease filter binding assay, based on the specific activity of [35S]GTP·S. GST-aptin 1 was then incubated with ARF-[35S]GTP·S at different concentrations at 30 °C for 30 min. Anti-ARF1 antiserum and protein A-Sepharose beads were added to the reaction and the mixture was incubated at 4 °C for 60 min. The beads were then washed three times in washing buffer A and counted to determine ARF-[35S]GTP·S binding. The data were analyzed by Eadie-Hofstee plot to yield Kd values.

When the relative affinity of each ARF isoform to arfaptin 1 or arfaptin 2 was examined, nuclease-free ARF was preloaded with 5 µM [35S]GTP·S (4 µCi/nmol) as described above. Thereafter, aliquots were
determined for [35S]GTP-S binding by the nitrocellulose filter assay. GTP-S-bound ARF was stable on ice for at least 5 h. Each mixture was adjusted to contain the same amount of [35S]GTP-S-ARF and used for the interaction experiment described above. After washing, the beads were mixed with 10 ml of Ready Safe liquid scintillation mixture (Beckman), and the radioactivity was counted. The stoichiometry of [35S]GTP-S binding for ARF1, ARF3, ARF5, and ARF6 was 0.26, 0.28, 0.08, and 0.21, respectively (means of the three determinations).

Arfaptin 1 Translocation Studies—Highly concentrated cytosol was prepared from HL60 cells as described by Malhotra et al. (26) except that the cells were homogenized by sonication. Prior to use, the cytosol was stored at −80 °C and thawed and centrifuged at 100,000 × g for 1 h at 4 °C to remove any aggregated proteins. Golgi-enriched membrane fractions were prepared from rat liver by sucrose gradient centrifugation according to Malhotra et al. (26). Membranes were collected at the 0.5 M sucrose/1 M sucrose interface and stored at −80 °C. Prior to use, membranes were washed with 10 mM Tris-HCl (pH 7.4).

Golgi membranes were incubated with HL60 cytosol or comparable amounts of gel-filtered cytosol under conditions previously defined (27) with slight modifications. Briefly, Golgi membranes (7.5 μg of protein) and a saturating concentration of cytosol protein (300 μg) were incubated with or without GTP-S (25 μg) at 37 °C for 10 min in the reaction mixture (100 μl) consisting of 25 mM Hepes-KOH (pH 7.0), 125 mM KCl, 2.5 mM MgCl2, 1 mM dithiothreitol, 0.2 mM sucrose, 1 mM ATP, 5 mM creatine phosphate, and 10 units/ml creatine kinase. When the effect of brefeldin A was examined, Golgi membranes were incubated with brefeldin A for 10 min at 37 °C prior to the addition of cytosol and GTP-S. After incubation, 90 μl of the mixture was layered on 330 μl of 25% (w/v) sucrose in washing buffer B, consisting of 5 mM MgCl2, 1 mM EGTA, 10 mM KCl, 10 mM Hepes, 1 mM dithiothreitol, and 0.2 M sucrose/1 M sucrose interface and stored at −80 °C. Prior to use, membranes were washed with 10 mM Tris-HCl (pH 7.4).

Results

Identification of an ARF3-interacting Protein by Yeast Two-hybrid Screening—To identify molecules that act as downstream effectors of ARF, we employed the yeast two-hybrid system and screened an HL60 cDNA library using a constitutively active mutant of ARF3 (ARF3-Q71L) (16, 29) as a target. When approximately 2 × 106 double transformants were screened and selected for histidine prototrophy, three positive colonies were obtained. These colonies were assayed for activation of second reporter gene lacZ by β-galactosidase filter assay, and all were revealed to be positive. Partial sequencing and restriction mapping of the cDNA inserts of these library clones revealed that two clones (clones 1 and 3) had the same cDNA insert (1.2 kb), while another clone (number 2) had an insert (1.6 kb) distinct from clones 1 and 3. To eliminate false positives, further genetic assays were conducted (Table I). Neither clone 1 nor clone 2 by itself was capable of activating β-galactosidase activity, indicating that they do not contain a latent transcriptional activator. The GAL4-binding domain alone or human lamin C, which is a protein unrelated to ARF, did not interact with either clone 1 or 2. These observations rule out the possibility that clone 1 and clone 2 nonspecifically interact with other proteins.

ARFs are modified by myristoylation at Gly2 through the action of an N-myristoyltransferase in vivo (30). However, ARF3 is expressed as a fusion protein with a GAL4-binding domain cannot be modified, because N-myristoyltransferase can only act at the N terminus (21). Therefore, it is presumed that the interaction of ARF3-Q71L with clone 1 or clone 2 in yeast is independent of myristoylation. This was confirmed by studies with the double mutant ARF3-G2A-Q71L, which has an additional mutation at Gly2 to abolish myristoylation, which showed that this also interacted with clones 1 and 2 with apparently the same affinity as ARF3-Q71L (Table I). Interestingly, the two-hybrid assay indicated that neither clone 1 nor 2 interacted with wild-type ARF3, suggesting that both clones interact only with the active, GTP-bound form of ARF (Table I).

Clone 1 was revealed to contain a full-length open reading frame encoding a protein of 341 amino acids with a calculated molecular mass of 38,596 Da (Fig. 1). The translation initiation codon (nucleotides 131–133) was surrounded by a consensus Kozak sequence (31). Furthermore, an antibody raised against a recombinant protein encoded by this open reading frame...
immunoprecipitated a protein from HL60 cell lysate whose migration on SDS-PAGE was similar to that of the recombinant protein (44 kDa) (Fig. 2). Because the recombinant protein was obtained by thrombin cleavage of the GST fusion protein, it had four additional amino acids at the N-terminus. This could partly explain the slight difference in migration. We have named the protein encoded by clone 1 arfaptin 1.2 When cytosol and crude membranes from HL60 cells were subjected to immunoblotting, arfaptin 1 was detected predominantly in cytosol (Fig. 2).

Interestingly, sequence analysis of clone 2 revealed the presence of a potential open reading frame whose size was exactly the same as that of arfaptin 1.2 When cytosol and crude membranes from HL60 cells were subjected to immunoblotting, arfaptin 1 was detected predominantly in cytosol (Fig. 2).

The predicted amino acid sequences of arfaptins 1 and 2 were used to search a protein data base (the BLAST network service at the National Center for Biotechnology Information (NCBI)). However, no homologous proteins of known function were found.3 The highest identity score was found with a Caenorhabditis elegans hypothetical 35-kDa protein of unknown function (CEF54C8.7, EMBL/GenBank™ accession number Z22178). The primary structure analysis indicated that arfaptin 1 and arfaptin 2 were hydrophilic proteins with a few minor hydrophobic regions. Both proteins had several potential phosphorylation sites for protein kinase C,4 protein kinase A, and casein kinase II. Interestingly, arfaptin 2 had a periodic repeat.

3 After submission of this paper, a re-search revealed POR1, a Rac-binding protein that is identical to arfaptin 2, but lacks the first 38 amino acids. It has been implicated in Rac-induced membrane ruffling in fibroblasts (54, 55).

4 Preliminary experiments indicated that both GST-arfaptin 1 and GST-arfaptin 2 but not GST alone were phosphorylated by purified protein kinase C (Promega) but only with poor stoichiometry (25–35% for GST-arfaptin 1, 15–25% for GST-arfaptin 2).
of five leucine residues at every seventh position (leucine zipper) (residues 150, 157, 164, 171, and 178 in Fig. 3). Such a motif, with a highly positively charged region immediately adjacent to its N-terminal side, forms a DNA-binding domain (33). Arfaptin 2, however, lacks such a positively charged region. Arfaptin 1 also has a leucine zipper motif, but this is interrupted at residue 157 by replacement with methionine (Fig. 3).

Northern blot analysis indicated that arfaptins 1 and 2 were ubiquitously expressed as mRNAs of approximately 3.4 and 2.1 kb, respectively, in various human tissues as well as HL60 cells, ruling out the possibility that they are cancer cell-specific products. Both arfaptins 1 and 2 were expressed at a relatively high level in liver, pancreas, and placenta. In addition, arfaptin 1 was expressed at a relatively high level in skeletal muscle and heart (Fig. 4).

Arfaptins 1 and 2 Bind Directly to the GTP-bound Form of Class 1 ARFs in Vitro—As described above, the two-hybrid interaction assay suggested that arfaptins 1 and 2 bind ARF3 only in its GTP-bound conformation and that the binding was independent of myristoylation of ARF. To confirm this biochemically, in vitro interactions of nonmyristoylated ARF3 with arfaptins 1 and 2 were examined using recombinant proteins (Fig. 5). Since recombinant ARF binds GTP or GTP\(_7\)S with very low stoichiometry due to its tightly bound GDP, we utilized nucleotide-free ARF to prepare GTP\(_7\)S-bound ARF. Under the conditions employed, the stoichiometry of GTP\(_7\)S binding of ARF3 was 0.28. When GST-arfaptin 1-immobilized glutathione-Sepharose beads were incubated with GTP\(_7\)S-ARF3, GDP-ARF3, or nucleotide-free ARF3, only GTP\(_7\)S-ARF was associated with the beads, while very little nucleotide-free ARF and no GDP-ARF were detected. In addition, GTP\(_7\)S-ARF3 did not bind to GST-immobilized beads, indicating that the binding of GTP\(_7\)S-ARF3 to the beads was through arfaptin 1. The binding, however, was not quantitative (see below). These results indicated that arfaptin 1 was capable of interacting directly with nonmyristoylated GTP-ARF3 but not GDP-ARF3, consistent with the results of the two-hybrid interaction assay. Qualitatively similar results were obtained with arfaptin 2 and ARF3 (Fig. 5). The affinity of arfaptin 1 for GTP\(_7\)S-ARF3 was measured as described under “Materials and Methods.” The mean \(K_d\) from two experiments was \(1.4 \times 10^{-7} \text{ m}\).

The binding of arfaptin 1 and 2 to other ARF isoforms was examined using recombinant nonmyristoylated ARFs preloaded with \(^{[35S]}\text{GTP}\_S\) (Table II). ARF1, which is 96% identical to ARF3 in amino acid sequence, was used as another representative of class I ARF, and ARF5 and ARF6 were used as representatives of classes II and III, respectively. After pre-loading of nucleotide-free ARF with \(^{[35S]}\text{GTP}\_S\), equal amounts of each GTP\(_7\)S-bound ARF were incubated with GST-arfaptin 1- or GST-arfaptin 2-immobilized beads. Both arfaptins 1 and 2 bound ARF1 with the highest affinity. On the other hand, they bound much less ARF5 and very little ARF6. It was confirmed that both arfaptins 1 and 2 bound ARF1 only in its GTP\(_7\)S-bound form by employing the same experiments described for ARF3 in Fig. 5 (not shown). These results indicate that arfaptins 1 and 2 interact preferentially with class I ARFs, especially ARF1.

Arfaptin 1 Is Recruited by ARF from Cytosol to Golgi Membranes in a GTP\(_7\)S-dependent Manner—Among the subfamilies of Ras low molecular weight GTP-binding proteins, ARF is the clearest example of a GTPase whose GTP binding and hydrolysis cycle appears to be strictly coupled to a membrane-cytosol localization cycle. Inactive GDP-bound ARF is cytosolic, whereas active GTP-bound ARF is associated with membranes, especially with the Golgi apparatus (24). As described above,
arfaptin 1 is present in the cytosolic fraction and specifically binds GTP-bound ARF. Therefore, we examined the possibility that arfaptin 1 was translocated from cytosol to Golgi membranes in association with GTP-bound ARF (Fig. 6). Golgi-enriched membranes from rat liver were incubated with HL60 cytosol in the presence or absence of GTP·S and then the membranes were collected by centrifugation. In the presence of GTP·S, arfaptin 1 and ARF were detected in the pellet (lane 3), whereas in the absence of GTP·S very little arfaptin 1 or ARF was detected (lane 1). When Golgi membranes were omitted from the reaction mixture, arfaptin 1 and ARF were barely detectable in the pellet (lane 2), ruling out the possibility that arfaptin 1 and ARF were precipitated due to nonspecific aggregation. Brefeldin A has been shown to disrupt Golgi membranes and inhibit the binding of ARF to Golgi membranes (34). When Golgi membranes were treated with brefeldin A (40 μg/ml) prior to the addition of cytosol and GTP·S, translocation of arfaptin 1 and ARF was inhibited (lane 4), suggesting that the association of arfaptin 1 with Golgi membranes depends on ARF. When the results of three experiments were analyzed by densitometry, the inhibition of arfaptin translocation (32 ± 2%) was similar to that of ARF (38 ± 7%).

To confirm that translocation of arfaptin 1 is ARF-dependent, we utilized ARF-depleted cytosol (Fig. 7B). To obtain this, HL60 cytosol was subjected to Sephacryl S-200 gel filtration column. Since arfaptin 1 was present in a high molecular weight complex, it could easily be separated from ARF (Fig. 7A). When Golgi membranes were incubated with ARF-depleted cytosol (fractions 22–28) alone, only a trace amount of arfaptin 1 was translocated to membranes even in the presence of GTP·S. This was probably caused by the tiny amount of aggregated ARF that was co-eluted with arfaptin 1. On the other hand, when fractions containing native ARF (fractions 35–40 in Fig. 7A) were included in the above mixture, GTP·S-dependent translocation of arfaptin 1 to the Golgi membranes was restored, and this was well correlated with translocation of ARF. When partially purified recombinant myristoylated ARF3 was used instead of native ARF fraction, the same results were observed. These results indicate that translocation of arfaptin 1 to Golgi membranes requires GTP-bound ARF. It should be noted that although HL60 cytosol contained both ARF1 (lower band of doublet, Fig. 7A) and ARF3 (upper band of doublet, Fig. 7A), translocation of native ARF3 was much less than that of native ARF1. This differential interaction of ARF1 and ARF3 with Golgi membranes is consistent with the previous observations (23). Thus, it appears that translocation of arfaptin 1 to Golgi membranes is mediated mainly by ARF1.

Arfaptin 1 Is Not a Component of Coatomer—One of the well-characterized functions of ARF is the recruitment of cytosolic coat proteins to Golgi membranes, which is thought to play an important role in vesicle trafficking (35). Coat proteins are preassembled in a cytosolic complex, coatomer, which has an apparent molecular mass of ~800 kDa (36). As described above, arfaptin 1 was present in cytosol as a high molecular weight complex that was recruited to Golgi membranes by activated ARF (Fig. 7). These findings raised the possibility that arfaptin 1 was a constituent of coatomer. To examine this, HL60 cytosol was fractionated through a Sephacryl S-400 gel filtration column. Arfaptin 1, however, was clearly separated from β-COP, one of the components of coatomer, since this was eluted at around 800 kDa, whereas arfaptin 1 was eluted at around 450
Discussion

In the present study, we have identified a novel protein arfaptin 1, whose mRNA is ubiquitously expressed in human tissues. Arfaptin 1 is a hydrophilic protein with a few minor hydrophobic regions and is present in cytosol. The calculated mass is 38,596 Da, and it migrates on SDS-PAGE at approximately 44 kDa. Recombinant arfaptin 1 binds preferentially class I ARFs, especially ARF1, in their GTP-bound state and not at all in their GDP-bound form (Fig. 5 and Table II). Thus, arfaptin 1 is likely to be a direct target protein for class I ARFs. We have also obtained another clone encoding a putative protein, arfaptin 2, which consists of the same number of amino acids as arfaptin 1. The mRNA of arfaptin 2 is also ubiquitously expressed, and the deduced amino acid sequence of arfaptin 2 is 60% identical and 81% homologous to that of arfaptin 1. The differences between ARF1 and ARF3 are different in the N-terminal 13 amino acids and 3 amino acids in the C-terminal 8 amino acids (37). These facts suggest that the N and/or C termini may be important domains of ARF1 for interaction with arfaptin 1.

An important feature of arfaptin 1 is the high-affinity interaction with ARF1, which is 10-fold stronger than that with ARF3. Characterization of recombinant ARF revealed that ARF domains that may be important for the interaction with arfaptin 1. Among the isoforms examined, arfaptin 1 binds ARF1 with the highest affinity and ARF3 with the least affinity. It binds much less ARF5 and very little ARF6. The order of affinity for different ARF isoforms is correlated with their structural homology; ARF3, -5, and -6 differ from ARF1 in amino acid sequence by 4, 20, and 32%, respectively (2). It is surprising, however, that the very small difference in the amino acid sequences of ARF1 and ARF3 (7 of 181 amino acids) results in an approximately 3-fold difference in binding affinity for arfaptin 1. The differences between ARF1 and ARF3 are attributed exclusively to their N and C termini; 4 amino acids are different in the N-terminal 13 amino acids and 3 amino acids in the C-terminal 8 amino acids (37). These facts suggest that the N and/or C termini may be important domains of ARF1 for interaction with arfaptin 1.

There is considerable evidence that arfaptin 1 is a direct cytoplasmic target of ARF. Both ARF1 and ARF5 bind arfaptin 1 with the highest affinity. The affinity of class I ARFs for arfaptin 1 is 60% identical and 81% homologous to that of arfaptin 1, which suggests that the N- and/or C-terminal domains of ARF are the major determinants of affinity for arfaptin 1. Arfaptin 1 has been shown to be a direct cytoplasmic target of ARF1, and the affinity of ARF for cholera toxin ADP-ribosyltransferase activity is dependent on the interaction of ARF with arfaptin 1. Arfaptin 2 has also been identified as a target protein of ARF1, and it binds preferentially to ARF1 and ARF3, especially ARF1, in their GTP-bound state and not at all in their GDP-bound form. Arfaptin 2 is 60% identical and 81% homologous to arfaptin 1, and its amino acid sequence is 60% identical and 81% homologous to that of arfaptin 1.

We have also obtained another clone encoding a putative protein, arfaptin 2, which consists of the same number of amino acids as arfaptin 1. The mRNA of arfaptin 2 is also ubiquitously expressed, and the deduced amino acid sequence of arfaptin 2 is 60% identical and 81% homologous to that of arfaptin 1. The differences between ARF1 and ARF3 are different in the N-terminal 13 amino acids and 3 amino acids in the C-terminal 8 amino acids (37). These facts suggest that the N and/or C termini may be important domains of ARF1 for interaction with arfaptin 1.
the interaction with GAP and for the cofactor activity (39). More recently, analysis of the GTP-dependent conformational change of ARF1 has led to the proposal that the N terminus of ARF1 is an effector domain (40). Thus the N terminus rather than C terminus of ARF might be more important for interaction with arfaptin 1. In addition to the N terminus itself, modification of the N terminus by myristoylation is also critical for ARF functions, because myristoylation confers an ability to associate with membranes (41) and is critical for the GTP-dependent conformational change (40). Although the present study indicates that arfaptin 1 interaction with ARF does not require myristoylation (Table I), the possibility that the affinity may be increased by myristoylation remains to be examined.

It is reported that different ARF isoforms have different affinities for Golgi membranes and other cellular membranes. Among the isoforms, it has been shown that ARF1 as well as ARF5 associate with Golgi membranes with high affinity and specificity in vitro, while ARF3 associates with the membranes with lower affinity and is distributed to other cellular membranes (23). Localization of ARF1 to the Golgi complex was confirmed in overexpression experiments, whereas ARF6, to which arfaptin 1 does not bind, was localized to the endosomal/plasma membrane system (42). Another study has shown that ARF6 is uniquely localized to the plasma membrane of Chinese hamster ovary cells (43). Therefore, the preferential binding of arfaptin 1 to ARF1 shown by in vitro interaction experiments is in good agreement with the localization of arfaptin 1 in the Golgi complex shown by overexpression in COS cells in vivo (Fig. 8).

Accumulating evidence suggests that there is a cyclic localization of ARF between cytosol and membrane fractions. Inactive GDP-bound ARF present in the cytosol is activated by GEP in Golgi membranes or cytosol in a brefeldin A-sensitive manner and is translocated to Golgi membranes. Subsequently, GTP bound to ARF is hydrolyzed in the presence of GAP in Golgi membranes, and the resulting GDP-ARF is released to cytosol (13, 27, 44-47). Our in vitro translocation data and in vivo transient expression experiments suggest that the GTP-bound form of ARF serves as a membrane anchor for arfaptin 1 and that arfaptin 1 cycles between cytosol and Golgi membranes depending on the activity status of ARF. Such a relationship between a GTP-binding protein and its target, i.e., a GTP-binding protein that serves as a regulatable membrane anchor for its target protein, has also been observed for other small GTP-binding proteins and their functional target proteins. Ras, when it is activated, translocates its cytosolic target Raf-1 protein kinase to the plasma membrane, where Raf-1 is activated and initiates a phosphorylation cascade (48). RhoA, which is involved in morphological events involving the actin cytoskeleton, recruits its target serine/threonine kinase ROKα to peripheral membranes (49). Rab3A and Rab3C have been suggested to recruit rabphilin-3A, a Rab3A target protein, to synaptic vesicle membranes (50). Rab5, a potent regulator of endocytic transport, recruits its target protein rabaptin-5 to early endosomes in a GTP-dependent manner (51). Therefore, it is possible that ARF, which belongs to another subfamily of Ras-related proteins, also has a cytosolic target protein (arfaptin) and recruits it to membrane fractions.

Arfaptin 2 has a leucine zipper structure in the middle of the molecule (Fig. 3). Arfaptin 1 also has a similar repetition of leucine residues, although one of them is replaced with methionine (Fig. 3). The leucine zipper structure provides hydrophobic faces through which zipper proteins interact to form dimers, which can be homodimers or heterodimers. The leucine zipper structure is often found in transcription factors, since the dimer can interact with DNA through a domain enriched with positively charged amino acids immediately adjacent to the N terminus of the zipper (33). Since arfaptin 1 and arfaptin 2 lack such a domain, they are unlikely to bind DNA, but they may still be able to form homo- or heterodimers. In fact, when HL60 cytosol was separated by gel filtration chromatography, arfaptin 1 was found as a high molecular weight complex (Fig. 6), raising the possibility that it may exist as an oligomer and/or as a complex with other proteins.

A major question of the present study is the physiological function of arfaptin 1. This is being explored in in vitro studies carried out in collaboration with the group of Dr. J. Moss (National Institutes of Health). These results will be reported elsewhere when complete, but initial findings are that recombinant arfaptin acts as an inhibitor of the in vitro action of ARF on phospholipase D and cholera toxin-catalyzed ADP-ribosyltransferase activity. However, it does not alter the binding of GTPγS or GDP to ARF in the presence of GEP (11) or alter GTPase activity. Irrespective of these observations, the fact that arfaptin 1 only interacts with ARF liganded to GTPγS, but not GDP, suggests that it may be an effector i.e., a physiological target of ARF whose function is presently unknown. An additional possibility is that arfaptin 1 is an adaptor protein that may require a third component before its function can be observed.

The relative affinity of arfaptin 2 for different ARF isoforms is strikingly similar to that of arfaptin 1 (Table II). Tissue
distribution of arfaptin 2 is also similar to that of arfaptin 1 except for muscle (Fig. 4). Furthermore, arfaptin 2 may form a heterodimer with arfaptin 1 as discussed above, raising the possibility that arfaptin 2 might act cooperatively with arfaptin 1. Alternatively, arfaptin 1 may require other protein(s) beside arfaptin 2. As mentioned above, estimation of the size of arfaptin 1 using gel filtration (Fig. 6) suggests that it may associate with other proteins of unknown identity. GFP-bound ARF recruits not only arfaptin 1 to Golgi membranes, but also coat proteins including coatomer (27, 35), p200 protein (52), and AP1 adaptins (53). Recently, ARF1-GAP has also been shown to be recruited to the Golgi complex by ARF (13), suggesting complex interactions among the various molecules. Thus, experimental conditions that are more physiological may be required to explore the function of arfaptin 1, e.g. those involving overexpression or “knockout” of arfaptin 1.

Acknowledgments—We thank S.-C. Tsai, R. Adamik, W. Patton, and J. Moss (National Institutes of Health) for generous gifts of ARF3 cDNA, anti-ARF1 antibody, and recombinant ARF1, -5, and -6 and J. J. Moss (National Institutes of Health) for generous gifts of ARF3.