Identification of Arfophilin, a Target Protein for GTP-bound Class II ADP-ribosylation Factors*

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Yeast two-hybrid screening of a human kidney cDNA library using the GTP-bound form of a class II ADP-ribosylation factor (ARF5) identified a novel ARF5-binding protein with a calculated molecular mass of 82.4 kDa, which was named arfophilin. Northern hybridization analysis showed high level arfophilin mRNA expression in human heart and skeletal muscle. Arfophilin bound only to the active, GTP-bound form of ARF5 and did not bind to GTP-ARF3, which is a class I ARF. The N terminus of ARF5 (1–17 amino acids) was essential for binding to arfophilin. The GTP-bound form of ARF5 containing the N terminus of ARF3 also bound to arfophilin, suggesting it is a target protein for GTP-bound forms of class II ARFs. The binding site for ARF on arfophilin was localized to the C terminus (residues 612–756), which contains putative coiled-coil structures. Recombinant arfophilin overexpressed in CHO-K1 cells was localized in the cytosol and translocated to a membrane fraction in association with GTP-bound ARF5. ARF5 containing the N terminus of ARF5 did not promote translocation indicating that class II ARFs are specific carriers for arfophilin.

The ADP-ribosylation factors (ARFs)† were originally identified as cofactors for cholera toxin-catalyzed ADP-ribosylation of Goα, the α-subunit of the G protein that stimulates adenylate cyclase. ARFs have now been associated with intracellular membrane trafficking events such as recruitment of clathrin-coated vesicle adaptors or coatomer proteins to Golgi membranes, and receptor-mediated endocytosis (1, 2). As a subfamily of the Ras-related small GTP-binding proteins, ARF proteins transmit signals to downstream effectors in a guanine nucleotide-dependent manner. Guanine nucleotide exchange factors (GEFs) act on ARFs to catalyze replacement of bound GDP with GTP, converting them to the active GTP-bound form, while GTPase-activating proteins hydrolyze the bound GTP, converting them to the inactive GDP-bound form.

Six mammalian ARF genes have been cloned. Based on phylogenetic analysis, deduced amino acid sequence, protein size, and gene structure, ARFs can be divided into three classes:

- class I (ARF1, ARF2, and ARF3), class II (ARF4 and ARF5), and class III (ARF6) (3). ARF6 is found in plasma membranes or endosomes, and is involved in peripheral vesicle trafficking such as endocytosis and exocytosis (4–6). GDP-bound forms of class I and class II ARFs are mainly found in the cytosol (7). However, GTP-bound forms of class I and II ARFs can be associated with Golgi, endoplasmic reticulum, and endosomes (7, 8). Class I and class II ARFs appear to be similar in both their cellular localization and functions. Both ARF1 and ARF5 are equally effective in promoting the recruitment of the AP-1 adaptor complex in Golgi (9). Peptides corresponding to the N-terminal 17 amino acids of ARF1 or ARF4 inhibit endoplasmic reticulum to Golgi vesicle transport (10), and expression of either ARF1 or ARF4 genes corrects the impairment of secretion in yeast induced by ARF deletion (11). However, different GEFs seem to be involved in the activation of class I and class II ARFs. Several class I ARF-specific GEFs have been cloned and purified, such as cytohesin-1, a brefeldin A (BFA)-insensitive 55-kDa protein, and a BFA-sensitive 200-kDa protein (2).

Recently, a BFA-insensitive GEF (GBF1) was identified in CHO-K1 cells (12). The BFA-sensitive 200-kDa protein acts on class I ARFs (13), and the BFA-insensitive GBF1 is specific for ARF5 (12). Both ARF1 and ARF5 specifically translocate to the Golgi (8, 9), possibly due to the GEFs located in this organelle.

Regardless of the fact that different GEFs act on class I and class II ARFs, both classes of ARFs share many overlapping cellular functions (9–11). Due to the 3–10-fold lower cellular concentrations of class II ARFs compared with class I ARFs, it is possible that class II ARFs have only supplementary role to class I ARFs. However, it is also possible that class II ARFs have unique cellular functions that cannot be replaced by class I ARFs.

To define the cellular actions of class II ARFs we used the yeast two-hybrid system to identify proteins that bind to ARF5. We employed a constitutively active form of ARF5 in which Glnt has been mutated to Leu to inhibit GTP hydrolysis (14). Our studies revealed a protein named arfophilin that selectively binds to active class II ARFs.

EXPERIMENTAL PROCEDURES

Cell Lines—The CHO-K1 (ATCC CCL-61) cell line was purchased from ATCC, and the cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and proline (0.034 g/liter) at 37 °C in a humidified atmosphere of 5% CO2 and 95% air.

Yeast Two-hybrid Screening—ARF5-Q71L cDNA containing EcoRI site at 5’ and BamHI site 3’ was amplified by polymerase chain reaction (PCR) from human ARF5 cDNA as a template (kindly provided by Dr. J. Moss, National Institutes of Health, Bethesda, MD) using Pfu polymerase (Stratagene). ARF5-Q71L cDNA was subcloned into the EcoRI/BamHI sites of pGBT9 vector, a GAL4 DNA binding domain vector (CLONTECH), and used as a bait for human kidney cDNA library screening. For screening, the H7F yeast reporter strain was transformed sequentially with pGBT9-ARF5-Q71L and then with a human kidney cDNA library fused to GAL4 activation domain vector, pACT2 (CLONTECH) using a lithium acetate-based method.

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† The abbreviations used are: ARF, ADP-ribosylation factor; GEF, guanine nucleotide exchange factor; PCR, polymerase chain reaction; GST, glutathione S-transferase; BFA, brefeldin A; CHO, Chinese hamster ovary; GTPyS, guanosine 5’-3-O-thiotriphosphate; GDPβS, guanylyl-5’-y-thiophosphate.
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transformation efficiency, $4 \times 10^6$ yeast cells containing both pGBo9-ARF5-Q71L and pACT2-human cDNA were screened for β-galactosidase-positive clones. β-galactosidase activity was determined by a liquid culture assay using O-nitrophenyl β-D-galactopyranoside (Sigma) as substrate.

Construction of ARF Mutant and Yeast Two-hybrid Binding Assay—For construction of ARF5-Q71L containing the N-terminal 1–17 amino acids of ARF3 (N3-ARF5-Q71L), a Fsp1 restriction enzyme site was introduced at 56 site of ARF5 cDNA by a PCR mutation method. The primer with the mutation point underlined and the Fsp1 site indicated by italic letters was 5'-GGGAAGAAGCGATGGCAGTTCTCAGTTGTT-3'. P. Xerri terms exchange between ARF5 and ARF3, the 5' end of ARF5 cDNA was cut out after EcoRI/Fsp1 digestion, and the remaining part of ARF5 cDNA was ligated with the EcoRI/Fsp1-digested 5' end of ARF3 cDNA. For construction of ARF3-Q71L containing the N-terminal 1–17 amino acids of ARF5 (N5-ARF3-Q71L), an Eco57I restriction enzyme site was introduced at 109 site of ARF3 cDNA. For construction of ARF3-Q71L containing the N-terminal 1–17 amino acids of ARF5 (N5-ARF3-Q71L), an Eco57I restriction enzyme site was introduced at 109 site of ARF3 cDNA by a PCR method. The primer with the mutation point underlined and the Eco57I site indicated by italic letters was 5'-ATCCCTATAAAGCT- GAGCTCAGGGAGACTC-3'. The EcoRI/Eco57I-digested 5' ends were then exchanged. For construction of ARF5-Q71L containing the C-terminal 174–181 amino acids of ARF3 (ARF5-C3-Q71L), an Msc1 restriction enzyme site was introduced at 521 site of ARF5 cDNA by a PCR mutation method. The primer with the mutation points underlined and the Msc1 site indicated by italic letters was 5'-TGACAGCTCCTT- GCGGCAGCCAGTCCAGACC-3'. The Msc1/HindIII-digested 3' ends were then exchanged. All ARF mutants were confirmed by DNA sequencing.

For yeast two-hybrid binding assay, SFY526 yeast were co-transformed by both DNA binding and activation domain vectors and β-galactosidase activity was measured. For the colony-lift filter β-galactosidase assay, yeast colonies were transferred onto 75-mm VWR grade 410 paper filters and permeabilized in liquid nitrogen. Each filter was placed on another filter paper that had been presoaked in X-gal buffer (0.01% NaHPO$_4$, 40 mM β-mercaptoethanol, and 0.33 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside, pH 7.0) and incubated at 30 °C until color developed.

In Vitro Binding Assay—Recombinant ARFs used for in vitro binding assays were produced in CHO-K1 cells after transformation of eukaryotic expression vectors (pALTER-MAX, Promega) containing each ARF mutant cDNA. Briefly, $9 \times 10^6$ CHO-K1 cells were plated in 100-mm plates and incubated for 24 h. Then 6 ml of Opti-MEM medium (Life Technologies, Inc.) containing 5 µg of DNA and 30 µl of LipofectAMINE reagent (Life Technologies, Inc.) was added to each washed plate with Opti-MEM medium. Six ml of Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and proline (0.034 g/liter) was supplemented after 5 h of incubation, and then the cells were incubated another 19 h. CHO-K1 cells transfected with each ARF mutant cDNA were washed twice with 0.8% g/ml M protease and cultured in 10% serum-free DMEM with 100,000 g for 1 h. ARF5-V5L7A5S112L was made by a PCR-based mutation method. The primer with the mutation points underlined was 5'-ATGGGCTCT- CACAGTCCTCGCTCGCTTGCCTGCTCTTC-3'. All mutation points were confirmed by DNA sequencing.

Escherichia coli or yeast does not make full-length arfophilin recombinant protein (amino acids 1–756) due to the 5' GC-rich region of arfophilin cDNA. To make arfophilin protein in E. coli, we excised the 5' GC-rich region of arfophilin cDNA. Recombinant arfophilin (amino acids 321–756) was produced as a glutathione S-transferase (GST) fusion protein. For GST-arfophilin-(321–756) protein, EcoRI/Xhol-digested arfophilin-(321–756) cDNA was ligated into a pGEX4T2 vector (Amersham Pharmacia Biotech) to generate pGEX4T2-arfophilin-(321–756). E. coli DH5α was transformed with pGEX4T2 or pGEX4T2-arfophilin-(321–756). Transformed cells were washed three times with cold phosphate-buffered saline. The cells were then washed in buffer (60 mM Na$_2$HPO$_4$, 40 mM NaCl, 5 mM MgCl$_2$, 0.5 mM EGTA, 1 mM EDTA, 2.1 mM MgCl$_2$, 0.25 µl/µl leupeptin, 0.1% Triton X-100, 1 mM dithiothreitol, and 2 µM phenylmethylsulfonyl fluoride, pH 7.2) and then washed with 10 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM EDTA and kept at 80 °C until used. GST or GST-arfophilin-(321–756) protein was extracted using 1.5% sodium N-lauroylsarcosine (Sigma) (15) and then affinity-purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech).

GST or GST-arfophilin-(321–756) protein (10–25 µg) attached to the glutathione-Sepharose was washed twice with 1 M GTP and incubated with CHO-K1 cytosol containing each recombinant ARF mutant and 10 µM GTP-5P. The protein concentration of CHO-K1 cytosol containing the overexpressed ARF mutants was about 0.5 mg/ml, and 0.5 µl of cytosol was used for binding assays. For binding, each mixture was incubated for 30 min at 4 °C with rocking and then the 50 µl of Sepharose was washed five times with 1 ml of ARF binding buffer. ARF proteins bound to the arfophilin-Sepharose were analyzed by GST or GST-arfophilin-(321–756) protein was extracted with glutathione-Sepharose 4B (Amersham Pharmacia Biotech).

Yeast two-hybrid binding assay was performed on CHO-K1 cells after transformation of eukaryotic expression vectors (pALTER-MAX, Promega) containing each ARF mutant cDNA. Briefly, $9 \times 10^6$ CHO-K1 cells were plated in 100-mm plates and incubated for 24 h. Then 0.5 µl of ARF binding buffer (50 mM HEPE, 100 mM KCN, 5 mM NaCl, 1 mM MgCl$_2$, 0.5 mM EGTA, 1 mM EDTA, 2.1 µM aprotinin, 2.5 µl/µl leupeptin, 0.1% Triton X-100, 1 mM dithiothreitol, and 2 µM phenylmethylsulfonyl fluoride, pH 7.2) was added per 100-mm plate. After scraping, cell suspensions were passed five times through a 27-gauge needle to break cells and the cytosol fractions containing the recombinant ARF mutants were collected after ultracentrifugation (100,000 g for 1 h).

### Table I

Yeast two-hybrid interaction assay between arfophilin-(321–756) and various ARF constructs

| GAL4 DNA binding | β-Galactosidase filter assay (GAL4-activating) |
|------------------|---------------------------------------------|
|                  | pGAD424-mouse α-actin | pGAD424-arfophilin | pGAD10-arfaptin-1* |
| pGBo9-ARF5-Q71L  | Blue<sup>a</sup> | Blue<sup>a</sup> | Blue<sup>a</sup> |
| pGBo9-ARF5      | Blue<sup>a</sup> | Blue<sup>a</sup> | Blue<sup>a</sup> |
| pGBo9-ARF5-T31N | Blue<sup>a</sup> | Blue<sup>a</sup> | Blue<sup>a</sup> |
| pALTER-MAX-ARF5-Q71L | Blue<sup>a</sup> | Blue<sup>a</sup> | Blue<sup>a</sup> |
| pALTER-MAX-ARF5-C3-Q71L | Blue<sup>a</sup> | Blue<sup>a</sup> | Blue<sup>a</sup> |

<sup>a</sup> pGBo9, pGAD424, and pGAD10 vectors use a truncated form of the alcohol dehydrogenase promoter inducing very low protein expression.

<sup>b</sup> Previously our laboratory reported that arfaptin-1 binds to GTP-bound form of either ARF3 or ARF5 (27).

<sup>c</sup> Color development was not observed after 24 h incubation at 30 °C.

<sup>d</sup> Blue color started to develop within 30 min of incubation in all positive samples.

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FIG. 1. Deduced amino acid sequence of arfophilin. The amino acid sequence recovered by yeast two-hybrid screening is indicated by the dotted line (amino acids 321–756). The possible coiled-coil domains are boxed, and the ARF binding domain (amino acids 612–756) is indicated by italic letters.
A preblotted membrane containing 2 ng of poly(A) RNA from various human tissues was hybridized using an arfophilin cDNA probe (A), and then after stripping, the membrane was hybridized using a β-actin cDNA probe (B).

Western blotting after 14% Tris-glycine SDS-polyacrylamide gel electrophoresis.

Northern Blotting—A Northern blot filter with 2 μg of poly(A) RNA from different human tissues (CLONTECH) was hybridized with a 32P-labeled probe according to the manufacturer’s recommendations. A 1.2-kilobase fragment of arfophilin cDNA (corresponding to amino acids 326–756) was 32P-labeled using a random primer DNA labeling kit (Amersham Pharmacia Biotech) and used as a probe. As a loading control, β-actin Northern blotting was performed after stripping.

Determination of Binding between Arfophilin and GTP-bound Form of ARF5 after Overexpression in CHO-K1 Cells—CHO-K1 cells do not express N- or C-terminal epitope-tagged full-length arfophilin. N-terminal GST-tagging of the truncated form of arfophilin (amino acids 321-756) was the most stable form in CHO-K1 cells. To express GST-arfophilin-(321–756) in CHO-K1 cells, we constructed pcDNA3-GST-arfophilin-(321–756). GST structural cDNA was PCR-amplified from pGEX4T2 vector using Pfu polymerase and subcloned between the HindIII and EcoRI sites of pcDNA3 to make pcDNA3-GST vector. GST cDNA nucleotide sequence amplified by PCR in pcDNA3-GST vector was confirmed by DNA sequencing. To construct pcDNA3-GST-arfophilin-(321–756), arfophilin cDNA-(321–756) was inserted between EcoRI and XhoI sites of pcDNA3-GST.

GST-arfophilin-(321–756) and either ARF5-Q71L or N3-ARF5-Q71L were overexpressed in CHO-K1 cells after transfection of eukaryotic expression vectors containing each cDNA using LipofectAMINE as described above. ARF-binding buffer containing 0.5% Triton X-100 was added to plates washed three times with cold phosphate-buffered saline, and the cells were scraped. The cell homogenate was passed five times through a 27-gauge needle to break the cells, rocked for 30 min at 4 °C, spun at 14,000 rpm for 10 min using a microcentrifuge, and the supernatant was taken for the binding assays. 25 μl of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) was added to 1.5 mg (1 ml) of clear cell supernatant containing 20 μg GTPγS, and then rocked for 2 h at 4 °C. The Sepharose was washed five times each using 1 ml of ARF binding buffer, and arfophilin and ARF5 association was determined by Western blotting.

Production of Arfophilin Antibody—Rabbit polyclonal anti-arfophilin antibody was raised against recombinant GST-arfophilin-(321–756) protein. For Western blotting, whole serum was used at a 1:2000 dilution.

Western Blotting—Western blotting was carried out using the enhanced chemiluminescence method using horseradish peroxidase-conjugated second antibody. Since ARF5 antibody (a generous gift from Dr. J. Moesl) was not sensitive enough to detect nanogram levels of ARF5 in cell homogenates, we employed the amplified alkaline phosphatase method (Bio-Rad) for this protein. All immunoblots were scanned by an Apple OneScanner.

In Vitro Translocation of GTP-bound ARF5 and Arfophilin Complex to the Membrane Fraction—Recombinant arfophilin was overexpressed in CHO-K1 cells, and the cytosol fraction was used for translocation experiment after discarding proteins smaller than 50 kDa using Centricon-50 tubes (Millipore Corp.). Recombinant ARF5-Q71L and N3-ARF5-Q71L were overexpressed in CHO-K1 cells as described, and the cytosol fractions were used without further purification. For translocation experiments, CHO-K1 cell membranes (23 μg of protein) were mixed with 16 μg of CHO-K1 cytosol expressing arfophilin and 51 μg of CHO-K1 cytosol expressing either ARF5-Q71L or N3-ARF5-Q71L in a...
membrane fraction was determined by Western blotting. Experiments min. After washing, 50 μl of cytosol fraction was used for GST pull-down experiments. A, native form of ARF5 was incubated with various concentrations of GTPγS or GDPβS at 30 °C for 30 min. Subsequently, these mixtures were incubated with 14 μg of GST or GST-arfophilin-(321–756) immobilized on glutathione-Sepharose beads at 4 °C for 30 min. After washing, 50 μl of SDS sample buffer was added to each sample and boiled for 10 min. Each 20 μl of sample preparation was used for 14% SDS-polyacrylamide gel electrophoresis, and ARF association was determined by Western blotting. 

RESULTS

Yeast two-hybrid screening using pGBT9-ARF5-Q71L identified a novel ARF5-binding protein among 4 × 10^6 HF7c yeast cells containing both pGBT9-ARF5-Q71L and an activation domain vector containing a human kidney cDNA library (Fig. 1). The cDNA sequence of the protein, termed arfophilin, is identical to a previously undefined 4080-base pair human mRNA (KIAA0665 gene EMBL/GenBank accession no. AB014565). Arfophilin cDNA contained an open reading frame encoding a protein of 756 amino acids with a calculated molecular mass of 82.4 kDa. Primary structure analysis indicated that arfophilin has four possible coiled-coil domains, located at amino acids 531–561, 564–601, 602–648, and 655–694.

Because both yeast and E. coli did not make full-length arfophilin, we used a truncated form containing amino acids 321–756 for both yeast two-hybrid binding assays and GST pull-down experiments. Since yeast does not express human ARF5 protein from a high copy number plasmid construct (16), we employed a pGBT9 vector inducing very low levels of protein expression. The growth rate of SFY526 yeast transformed by pGBT9-ARFs was similar to that of untransformed yeast. Conserved amino acid substitutions are grouped as follows: C; S, T, P, G; R, K; M, I, L, V, F, Y, W, B. Arfophilin-(321–756) binding to ARF5-V5I,A7S,I12L. 500 μl of CHO-K1 cytosol containing both pGBT9-ARF5-Q71L and an activation domain vector containing a human kidney cDNA library (Fig. 1). The cDNA sequence of the protein, termed arfophilin, is identical to a previously undefined 4080-base pair human mRNA (KIAA0665 gene EMBL/GenBank accession no. AB014565). Arfophilin cDNA contained an open reading frame encoding a protein of 756 amino acids with a calculated molecular mass of 82.4 kDa. Primary structure analysis indicated that arfophilin has four possible coiled-coil domains, located at amino acids 531–561, 564–601, 602–648, and 655–694.

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200-μl reaction volume. Based on densitometric analysis after separation of each protein on SDS-polyacrylamide gels, 0.1 μg of arfophilin, 0.38 μg of ARF5-Q71L, and 0.43 μg of N-ARF5-Q71L protein were used for this experiment. Each mixture was incubated for 30 min at 4 °C without GTPγS to prevent activation of endogenous class II ARF5 in CHO-K1 cytosol, and then the membrane fraction was isolated (100,000 × g for 1 h). Association of arfophilin and ARFs with the membrane fraction was determined by Western blotting. Experiments were carried out three times with similar results.

FIG. 5. In vitro interaction of GST-arfophilin-(321–756) with various ARF constructs. Each ARF mutant was overexpressed in CHO-K1 cells, and 500 μl of cytosol fraction was used for GST pull-down experiments. A, native form of ARF5 was incubated with various concentrations of GTPγS or GDPβS at 30 °C for 30 min. Subsequently, these mixtures were incubated with 14 μg of GST or GST-arfophilin-(321–756) immobilized on glutathione-Sepharose beads at 4 °C for 30 min. After washing, 50 μl of SDS sample buffer was added to each sample and boiled for 10 min. Each 20 μl of sample preparation was used for 14% SDS-polyacrylamide gel electrophoresis, and ARF association was determined by Western blotting. 10 μl of CHO-K1 cytosol overexpressing ARF was used for standard. Another independent experiment was performed with similar results. B, the arfophilin binding domain in ARF5 was determined in vitro using the N5-ARF3 mutant. Each ARF3 mutant was incubated with 21 μg of GST or GST-arfophilin-(321–756) immobilized on glutathione-Sepharose beads at 4 °C for 30 min. After washing, 50 μl of SDS sample buffer was added to each sample and boiled for 10 min. Each 20 μl of sample preparation was used for 14% SDS-polyacrylamide gel electrophoresis, and ARF association was determined by Western blotting. 10 μl of CHO-K1 cytosol overexpressing ARF was used for standard. Another independent experiment was performed with similar results.

FIG. 6. Association between arfophilin and the GTP-bound form of ARF5 in CHO-K1 cell homogenates. Association of the GTP-bound form of ARF5 with N-terminal GST-tagged arfophilin was determined in CHO-K1 cell homogenate expressing both proteins as described under “Experimental Procedures.” Lane 1, GST-arfophilin in 10 μl of homogenate; lane 2, GST-arfophilin associated with ARF5-Q71L; lane 3, GST-arfophilin associated with N3-ARF5-Q71L; lane 4, GST-arfophilin in 10 μl of homogenate; lane 5, ARF5-Q71L in 20 μl of homogenate; lane 6, ARF5-Q71L associated with GST-arfophilin; lane 7, N3-ARF5-Q71L associated with GST-arfophilin; lane 8, N3-ARF5-Q71L in 20 μl of homogenate.

FIG. 7. ARF5 with N-terminal ARF4 amino acids binds to arfophilin. A, comparison of N-terminal amino acids among different classes of ARFs. Different amino acids in the same class of ARF are marked by bold letters, and conserved amino acids are underlined. Conserved amino acid substitutions are grouped as follows: C; S, T, P, G; R, K; M, I, L, V, F, Y, W, B. GST-arfophilin-(321–756) binding to ARF5-V5I,A7S,I12L. 500 μl of CHO-K1 cytosol overexpressing the GTP-bound form of ARF5 mutant was incubated with 21 μg of GST or GST-arfophilin-(321–756) immobilized on glutathione-Sepharose beads at 4 °C for 30 min. After washing, 50 μl of SDS-sample buffer was added to each sample and boiled for 10 min. Each 20 μl of sample preparation was used for 14% SDS-polyacrylamide gel electrophoresis, and ARF association was determined by Western blotting. 10 μl of CHO-K1 cytosol overexpressing ARF was used for standard. Another independent experiment was performed with similar results.

200-μl reaction volume. Based on densitometric analysis after separation of each protein on SDS-polyacrylamide gels, 0.1 μg of arfophilin, 0.38 μg of ARF5-Q71L, and 0.43 μg of N-ARF5-Q71L protein were used for this experiment. Each mixture was incubated for 30 min at 4 °C without GTPγS to prevent activation of endogenous class II ARF5 in CHO-K1 cytosol, and then the membrane fraction was isolated (100,000 × g for 1 h). Association of arfophilin and ARFs with the membrane fraction was determined by Western blotting. Experiments were carried out three times with similar results.

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**Determination of ARF5 binding site in arfophilin by a yeast two-hybrid interaction assay**

\[ \beta \text{-Galactosidase activity was determined by a filter assay for the 4-day-old SFY526 yeast transformants containing the indicated plasmids. Two other independent yeast transformations for interaction assays showed the same results.} \]

| DNA binding site | Activating | \( \beta \text{-Galactosidase activity} \) |
|------------------|------------|----------------------------------------|
| pGBT9-ARF5-Q71L  | pACT2-arfophilin-(612–756) | Blue \(^{a}\) |
| pGBT9-ARF5       | pACT2-arfophilin-(612–756) | — \(^{b}\) |
| pGBT9-ARF5-T31N  | pACT2-arfophilin-(612–756) | — |

\(^{a}\) Blue color started to develop within 30 min of incubation at 30 °C.

\(^{b}\) Color development was not observed after 24-h incubation at 30 °C.

**FIG. 8. Determination of ARF5 binding site in arfophilin by in vitro GST pull-down assay.** GST pull-down experiments were performed using recombinant ARF5 and GST-arfophilin C-terminal partial sequence. 500 \( \mu \text{l} \) of CHO-K1 cytosol overexpressing the GTP-bound form of ARF5 was incubated with 25 \( \mu \text{g} \) of GST or GST-arfophilin C terminus immobilized on glutathione-Sepharose beads at 4 °C for 30 min. After washing, 50 \( \mu \text{l} \) of SDS-sample buffer was added to each sample and boiled for 10 min. Each 20 \( \mu \text{l} \) of sample preparation was used for 14% SDS-polyacrylamide gel electrophoresis, and ARF5 association was determined by Western blotting. 10 \( \mu \text{l} \) of CHO-K1 cytosol overexpressing ARF5 was used for standard. Three independent experiments were performed with similar results.

the GDP-bound form of ARF5 (ARF5-T31N) showed \( \beta \)-galactosidase activity when cotransformed with arfophilin-(321–756), indicating that arfophilin interacts only with the GTP-bound form of ARF5 (Table I). To determine whether expression of ARF5 or ARF5-T31N at a higher level resulted in detectable interaction with arfophilin, we expressed these proteins fused to the GAL4 DNA binding domain using a pAS2–1 vector in CHO-K1 cells. Fig. 6 shows that the GTP-bound form of ARF5 containing the ARF3 N terminus did not. The N-terminal 17 amino acids of ARF5 (N5-ARF3-Q71L), confirming that the N terminus of ARF5 is involved in binding to arfophilin in yeast two-hybrid binding assay (Table I and Fig. 4B). ARF5-Q71L, ARF5-C3-Q71L, and N5-ARF3-Q71L fused to the GAL4 DNA binding domain did not show \( \beta \)-galactosidase activity when cotransformed with mouse \( \alpha \)-actin fused to the GAL4 activation domain, suggesting that the binding of these ARF mutants to arfophilin is specific and that they are not autoactivating. N3-ARF5-Q71L and ARF3-Q71L showed \( \beta \)-galactosidase activity when cotransformed with arfaptin-1 (Table I), indicating that they were adequately expressed in a functional form.

**In vitro GST pull-down experiments** were performed using recombinant ARF5s and GST-arfophilin-(321–756). The native form of ARF5 was produced in CHO-K1 cells, and the whole cytosol fraction of the cells was used for binding to GST-arfophilin in vitro. GTPγS treatment resulted in ARF5 binding to GST-arfophilin but not to GST alone, and incubation with GDP/βS caused dissociation of ARF from arfophilin (Fig. 5A). The GTP-bound form of ARF5 did not bind to arfophilin, while the GTP-bound form of ARF3 containing N-terminal ARF5 amino acids did, confirming that N terminus of ARF5 is essential for binding to arfophilin (Fig. 5B). We also determined whether both GST-arfophilin-(321–756) and the GTP-bound form of ARF5 can associate after expression in CHO-K1 cells. Fig. 6 shows that the GTP-bound form of ARF5 associated with arfophilin, while the GTP-bound form of ARF5 containing the ARF3 N terminus did not. The N-terminal sequences of ARF5s are well conserved in each class (Fig. 7A). To determine whether ARF4 can also bind to arfophilin, we constructed ARF5 with its N-terminal amino acids mutated to those of ARF4 (ARF5-V5I,A7S,I12L). Arfophilin-(321–756) bound to the GTP-bound forms of both ARF5 and ARF5-V5I,A7S,I12L (Fig. 7B), suggesting that arfophilin is a specific target protein for the GTP-bound forms of class II ARFs. We did not determine whether ARF6 could bind to arfophilin because the N terminus of ARF6 is quite different from those of class I and II ARFs and is therefore unlikely to bind to arfophilin.
Fig. 9. The GTP-bound form of ARF5 in complex with arfophilin translocates to membranes. A, recombinant arfophilin was overexpressed in CHO-K1 cells and total particulate (P) and cytosol (S) fractions were prepared by centrifugation of a post-nuclear supernatant (H) in the presence or absence of 1% Triton X-100 at 100,000 × g for 1 h. Pellets and supernatants were collected separately and adjusted to equal volumes in buffer. After SDS-polyacrylamide gel electrophoresis, arfophilin was detected by Western blotting. B, effects of GTPγS and GDPβS on localization of arfophilin were determined. Recombinant arfophilin was overexpressed in CHO-K1 cells, and the post-nuclear supernatant was incubated at 30 °C for 30 min with GTPγS or GDPβS. Total particulate (P) and cytosol (S) fractions were prepared by centrifugation at 100,000 × g for 1 h. These were collected separately and adjusted to equal volumes in ARF binding buffer. After SDS-polyacrylamide gel electrophoresis, arfophilin was detected by Western blotting.

The GTP-bound form of ARF5 was found to bind to the C terminus of arfophilin (amino acids 612–756) (Table II and Fig. 8). This ARF5 binding domain has a possible coiled-coil structure (amino acids 612–694), but this coiled-coil structure alone did not bind the GTP-bound form of ARF5 (Fig. 8).

Recombinant arfophilin overexpressed in CHO-K1 cells was mainly localized in the cytosol, but a small amount was found in the particulate fraction, possibly due to the presence of endogenous class II ARFs. Arfophilin was solubilized from the particulate fraction by 1% Triton X-100, suggesting that it was associated with membranes (Fig. 9A). When a CHO-K1 cell homogenate expressing arfophilin was incubated in 30 °C for 30 min, a significant amount of arfophilin was associated with the membrane fraction. This translocation of arfophilin may be related to activation of ARF5 in CHO-K1 cell cytosol by the ARF5-specific GEF, GBF1 (12). GTPγS clearly promoted arfophilin translocation to the membrane fraction while GDPβS triggered dissociation of arfophilin from this fraction (Fig. 9B). Adding ARF5-Q71L to the cytosol had the same effect as GTPγS (Fig. 9C). Both ARF5-Q71L and N3-ARF5-Q71L translocated to the membrane fraction in vitro, but only ARF5-Q71L promoted translocation of arfophilin to membrane fraction, indicating that only class II ARFs are carriers for arfophilin (Fig. 9C).

DISCUSSION

In the present study, we identified arfophilin a novel protein that binds to the active GTP-ligated form of class II ARFs (ARFs 4 and 5). We also determined the domains in both arfophilin and ARF5 that are involved in the binding.

The N terminus of ARF5 (amino acids 1–17), which is different from the N termini of class I ARFs, was essential for binding to arfophilin. In previous studies, analysis of the GTP-dependent conformational change of ARF1 indicated that the N terminus of ARF1 is the domain at which it interacts with its effectors and that N-terminal myristoylation of ARF1 is critical for this change (17). The consistency between our findings and the proposed model for ARF1 interaction with its effectors suggests that arfophilin is a true target for the GTP-bound form of ARF5.

All classes of ARFs activate phospholipase D and cholera toxin in vitro (1, 2), but the cellular roles of each ARF seem to be diverse. ARF6 (class III) is clearly distinct from other ARFs based on its cellular localization and functions (4–7). In contrast, class I and II ARFs locate to similar cellular organelles and share several cellular functions. For this reason many researchers believe that class II ARFs have only a supplementary role to class I ARFs, and much work is therefore focused on class I ARFs. The cellular function(s) of class II ARFs is not known, and it has been suggested that there is no functional difference between class I and class II ARFs (18). Therefore, the identification of a class II ARF-binding protein suggests for the first time that there may be a unique cellular pathway for class II ARFs, involving arfophilin, that cannot be replaced by other classes of ARFs.

The observation that arfophilin binds the GTP-bound form of ARF5 but not the GDP-bound form suggests that it is a down-
stream effector of ARF5, but its cellular role is unknown. Several lines of evidence suggest that it may be involved in vesicle trafficking especially in Golgi. The GTP-bound form of ARF5 translocates to intracellular membranes such as Golgi, endoplasmic reticulum, and endosomes in CHO-K1 cells (7). In rat brain homogenate, the GTP-bound form of ARF5 seems to localize more specifically in Golgi (8), and our data indicate that arfophilin moves to membranes in association with ARF5. Recently, an ARF5-specific GEF GBF1 was cloned from CHO-K1 cells. Even though GBF1 was located mainly in cytosol, a significant amount was associated with Golgi and Golgi-related tubular/vesicular structures, suggesting that ARF5 activation may be related to the early exocytic pathway (12).

Our results indicate that class II ARFs may have a unique role in determining cellular organization. We do not believe that arfophilin exhibits GEF activity, since it does not have the Sec-7 domain that is essential for ARF-GEF activity (13, 21–23). Furthermore, it does not associate with the T31N mutant of ARF5, which likely represents the transition state of ARF that is devoid of guanine nucleotide (24). The idea that arfophilin may have a function in Golgi is supported by the presence of putative coiled-coil structures, which are commonly found in proteins involved in vesicle trafficking such as a-SNAP and SNARE (19). In human pancreas, an expressed sequence tag (xs176) showing a very similar sequence to that of arfophilin is overexpressed during development of cancer (20). Cancer cells usually up-regulate secretion for metastatic spreading. Our efforts to demonstrate the subcellular localization of arfophilin by immunofluorescence have been complicated by the drastic changes in cell morphology that are induced when arfophilin is overexpressed in eukaryotic cells. These changes imply that arfophilin has a significant role in determining cellular organization.

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