Arfophilin Is a Common Target of both Class II and Class III ADP-Ribosylation Factors

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ABSTRACT: Arfophilin was first identified as a target protein for GTP-ARF5. The N-terminus of ARF5 (amino acids 2−17), which is distinct from that of class I or class III ARFs, is essential for binding to the C-terminus of arfophilin (amino acids 612−756). This study using GST fusion proteins in pulldown experiments in CHO-K1 cell lysates showed that, unexpectedly, ARF6 also bound to full-length arfophilin or the C-terminus of arfophilin (amino acids 612−756) in a GTP-dependent manner. Studies with ARF1/ARF6 chimeras further showed that the amino acid sequence of residues 37−80 of ARF6, which is different from the corresponding sequences in class I and class II ARFs, was essential for binding to arfophilin. Both GTP-ARF5 and GTP-ARF6 bound to arfophilin in CHO-K1 cell lysates, while GTP-ARF1 did not bind. In contrast, all three forms of ARF bound to arfaptin 2, with ARF1 showing the strongest binding. Yeast two-hybrid studies with wild-type, dominant negative, and constitutively active forms of ARF1, -5, and -6 and with ARF1/ARF6 chimeras confirmed these results, except that constitutively active ARF6 was autoactivating. Our findings suggest that both class II and III ARFs may influence the same cellular pathways through arfophilin as a common downstream effector.

ADP-ribosylation factors (ARFs)[1] regulate membrane trafficking and organelle integrity in eukaryotic cells (*1*). ARFs are divided into three classes: class I (ARF1−3), class II (ARF4 and -5), and class III (ARF6) (*2*). ARF1 and -3 are involved in trafficking in the endoplasmic reticulum, Golgi, and endosomal systems (*3*−*5*). GTP-bound ARF5 specifically translocates to Golgi membrane in rat brain homogenate (*6*), but its cellular function is not fully appreciated. ARF6 is involved in endosomal recycling to the plasma membrane in regulated secretion (*7*). ARF6 is also involved in phagocytosis (*8*), adipsin secretion (*9*), clathrin endocytosis (*10*), and Glut4 translocation (*11*).

GDP-bound class I and II ARFs are localized in the cytosol and are recruited to the membrane in a GTP-dependent manner (*12*). Several studies have suggested that ARF6 is always in the membrane fraction (*12*, *13*), but recent studies indicate that the distribution of ARF6 between membranes and cytosol is regulated by its GTPase cycle like class I and II ARFs (*14*). According to a structural analysis of GDP-ARF6, its conformation is similar to that of GDP-ARF1, which cannot bind to membranes with high affinity (*15*).

ARFs transmit signals to downstream effectors in a guanine nucleotide-dependent manner. The myristoylated N-terminus (amino acids 2−17) of ARF1 was identified as an effector domain for interaction with ARF GTPase-activating protein and Gαs (*16*, *17*). Analysis of the GTP-dependent conformational changes of ARF1 has indicated that amino acids 38−83 represent a possible effector domain which encompasses switch I, strand β2, strand β3, intervening loop λ3, and switch II (*18*). The constitutively active Q71L mutant of ARF1 interacts with the PDZ domain of a protein interacting with C kinase 1 (PICK1) as shown by a yeast two-hybrid interaction assay (*19*). ARF1-Q71L without the three C-terminal residues fails to interact with the PDZ domain, suggesting that the C-terminus (amino acids 179−181) of ARF1 may also be an effector domain (*19*).

Utilizing both protein purification and biochemical analysis approaches, phospholipase D (*20*), phosphatidylinositol 4-phosphate 5-kinase (*21*), cholera toxin (*22*), and clathrin adaptor protein (*23*) have been identified as direct downstream effectors of ARFs. Since our laboratory first identified arfaptin 1 and arfaptin 2 as direct binders for GTP-ARFs using yeast two-hybrid screening (*24*), POR1 (arfaptin 2 missing the first 38 amino acids) (*25*, *26*), mitotic kinesin-like protein 1 (*27*), arfophilin (*28*), and Vear/GGAs (*29*−*32*) have also been cloned as possible ARF effectors.

Arfophilin is the only known ARF effector which does not interact with class I ARFs (*28*). Much work has been focused on class I ARFs, especially ARF1, but virtually nothing is known about the function of class II ARFs (*33*). In human pancreas, arfophilin message has been reported to be increased during the development of cancer (*34*), suggesting that the arfophilin−ARF5 pathway may be involved in secretion because cancer cells usually upregulate secretion for metastatic spreading.

In the study presented here, we have found that ARF6 can also bind to arfophilin in a guanine nucleotide-dependent manner. We identified the ARF6 domain involved in binding to arfophilin, and re-evaluated arfophilin as a common target for both class II and III ARFs.

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[1] Abbreviations: ARF, ADP-ribosylation factor; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; GDPβS, guanosine 5′-2-*O*-(thio)diphosphate.

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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% heat-inactivated (57 °C for 45 min) fetal bovine serum and proline (0.034 g/L) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Preparation of Mutated cDNA Constructs. ARF mutations introduced in this study were generated by a PCR mutation method using pfu polymerase (Stratagene). Mutation points as well as the full-length cDNA sequence amplifed by a PCR method were confirmed by DNA sequencing. The 5′-end *Eco*RI and 3′-end *Bam*HI sites were added between both ARF1 and ARF5 cDNAs for in-frame fusion proteins for the yeast two-hybrid interaction assay. Because ARF6 has a *Bam*HI site in the open reading frame, we added an *Eco*RI site at the 5′-end and a *Bgl*II site at the 3′-end.

Yeast Two-Hybrid Binding Assay. SFY 526 yeast was cotransformed by both GAL4 DNA binding and activation domain vectors, and β-galactosidase activity was measured. For the colony-lift filter β-galactosidase assay, 50−300 yeast colonies grown on a tryptophan- and leucine-deficient agar plate 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 [60 mM Na_2HPO_4, 40 mM β-mercaptoethanol, and 0.33 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (pH 7.0)] and incubated at 30 °C until color development.

In Vitro Binding Assay. Recombinant ARFs used for in vitro binding assays were produced in CHO-K1 cells after transfection of eukaryotic expression vectors (either pcDNA3.1 from Invitrogen or pALTER-MAX from Promega) ligated with each cDNA between *Eco*RI and *Bam*HI sites. Briefly, 1×10^6 cells were plated in a 100 mm plate and cultured for 24 h. Then 6 mL of Opti-MEM medium (Life Technology, Inc.) containing 6 μg of DNA and 30 μL of LipofectAMINE reagent (Life Technology, Inc.) was added to each washed plate with Opti-MEM medium. Six milliliters of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and proline (0.034 g/L) was supplemented after incubation for 5 h, and then the cells were cultivated for an additional 19 h. CHO-K1 cells transfected with each cDNA were washed three times using cold phosphate-buffered saline. Then 0.5 mL of binding buffer [50 mM HEPES, 100 mM KCl, 5 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EGTA, 1 mM EDTA, 2.1 μg/mL aprotinin, 2.5 μg/mL leupeptin, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride (pH 7.2)] containing 0.5% Triton X-100 was added per 100 mm plate. After scraping, cell suspensions were incubated at 4 °C for 1 h with rocking, and the clear supernatants containing each ARF were collected after centrifugation (14 000 rpm for 10 min using an Eppendorf microcentrifuge) and stored at −80 °C until they were used.

Escherichia coli DH5α was transformed with pGEX4T2, pGEX4T-arfaptin 2 (*24*), and pGEX4T2-arfophilin (amino acids 321−756) (*28*). Transformed cells were grown at 37 °C to an A_{600} of 0.8, and protein expression was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 37 °C. The cells were washed with buffer [10 mM Tris (pH 8.0), 150 mM NaCl, and 1 mM EDTA] and kept at −80 °C until they were used. GST or GST fusion protein was extracted using 1.5% sodium *N*-lauroylsarcosine (Sigma) (*35*) and then affinity-purified using glutathione−Sepharose 4B beads (Amersham Pharmacia Biotech). GST, GST-arfaptin 2, or GST-arfophilin protein (7−17 μg) attached to the glutathione−Sepharose beads was washed twice with binding buffer containing 0.1% Triton X-100 and incubated with CHO-K1 cell lysates overexpressing each recombinant ARF protein. The protein concentration of CHO-K1 cell lysates containing each recombinant ARF was between 1.3 and 2.0 mg/mL. Recombinant ARF protein represented not more than 0.3% of total protein of cell lysate because overexpression of ARF protein was extremely toxic to the CHO-K1 cells. CHO-K1 lysate (0.5 mL) containing 0.5% Triton X-100 was diluted with 0.5 mL of binding buffer to decrease the Triton X-100 concentration to 0.25%, and the lysates were incubated with GTPγS or GDPβS before the binding assay. For binding, each mixture was incubated for 30 min at 4 °C with rocking, and then 25 μL of Sepharose was washed five times with 1 mL of binding buffer containing 0.1% Triton X-100. ARF protein bound to the arfaptin 2− or arfophilin−Sepharose was analyzed by Western blotting after 14% Tris-glycine SDS−polyacrylamide gel electrophoresis. For ARF protein detection, we employed the amplified alkaline phosphatase method (Bio-Rad).

RESULTS

Binding of Different Classes of ARFs to Arfophilin and Arfaptin 2. Arfophilin was first identified as a binding partner for ARF5 (*28*). It also bound ARF4, another class II ARF, but not ARF3, which is a class I ARF. The binding site for arfophilin on ARF5 was shown to be its N-terminal myristoylated switch (amino acids 2−17) which is different in class I ARFs (*28*). We determined the association between different classes of ARFs and arfophilin using GTP-bound mutant forms of ARF1, -5, and -6.[2] The Q71L mutation for ARF1 and -5 and the Q67L mutation for ARF6 were used to eliminate the possible effect of different GTP−GDP exchange rates in CHO-K1 cell lysates. The GTP-bound form of ARF1, another class I ARF, exhibited strong binding to arfaptin 2 but did not bind to arfophilin (Figure 1). The GTP-bound form of ARF5 associated with both arfaptin 2 and arfophilin, but the binding to arfophilin was stronger (Figure 1). The GTP-bound form of ARF6 associated with arfaptin 2, but also bound to arfophilin. This latter finding was surprising because of the great difference between the N-terminal sequences of ARF5 and ARF6 (see Figure 3). Like GTP-ARF5, GTP-ARF6 bound more strongly to arfophilin than to arfaptin 2 (Figure 1). Since similar amounts of the ARF mutants were used in these studies, the results also indicate that ARF1 binds more strongly to arfaptin 2 than does ARF5 or ARF6.

ARF6 overexpressed in CHO-K1 cells (Figure 1) appeared as two bands on Western blotting, but ARF6 produced in *E. coli* migrated with the upper band of CHO-K1 ARF6 (data not shown). This suggests that the upper band is the nonmyristoylated form of ARF6 and that the lower band is the myristoylated form. The mobility difference on SDS−

[2] *E. coli* or yeast does not make full-length arfophilin recombinant protein due to the 5′-GC-rich region of arfophilin cDNA (*28*). Therefore, the GC-rich region was excised to make GST-arfophilin (residues 321−756).

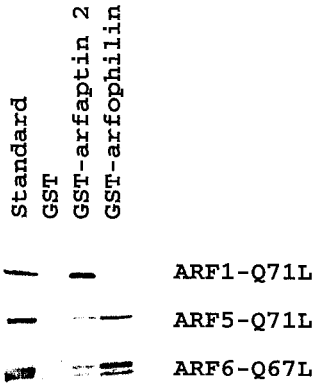

FIGURE 1: In vitro interaction of GST-arfophilin (residues 321−756) with constitutively active mutants of different classes of ARFs. Each ARF mutant was overexpressed in CHO-K1 cells, and 500 μL of cell lysate was used for GST pulldown experiments in the presence of 10 μM GTPγS. Each mutant ARF was incubated with 7 μg of GST (29 kDa), 15 μg of GST-arfaptin 2 (63 kDa), or 17 μg of GST-arfophilin (residues 321−756) (73 kDa) immobilized on 25 μL of glutathione−Sepharose beads at 4 °C for 30 min in the binding buffer described in Experimental Procedures. After the sample had been washed five times with 1 mL of binding buffer, 50 μL of SDS sample buffer was added to each sample, and the samples were boiled for 10 min. Twenty microliters of each sample was used for 14% SDS−polyacrylamide gel electrophoresis, and the level of ARF association was determined by Western blotting using class-specific ARF antibodies (a generous gift from J. Moss). Ten microliters of CHO-K1 cell lysate overexpressing each ARF mutant was used for a standard. The amounts of the standards were as follows: 40 ng of ARF1-Q71L, 50 ng of ARF5-Q71L, and 45 ng of ARF6-Q67L, amounts based on densitometric analysis and Western blotting using ID9 antibody recognizing ARF1−3, -5, and -6 (Affinity BioReagents, Inc.). Three independent experiments were performed with similar results.

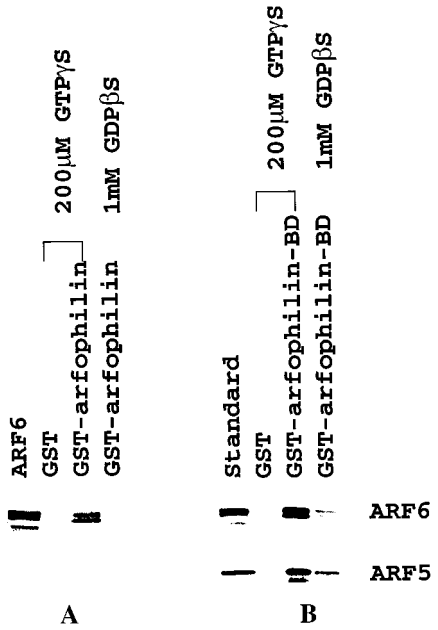

FIGURE 2: Effects of GTPγS or GDPβS on ARF6 binding to GST-arfophilin (residues 321−756). (A) Wild-type ARF6 was overexpressed in CHO-K1 cells, and the total cell lysate was used for GST pulldown experiments. Five hundred microliters of cell lysate overexpressing ARF6 was incubated with 200 μM GTPγS or 1 mM GDPβS for 30 min at 30 °C and used for a GST pulldown experiment. For this specific experiment, 17 μg of GST or GST-arfophilin (residues 321−756) protein immobilized on glutathione−Sepharose beads was incubated with GTPγS- or GDPβS-treated cell lysate for 30 min at 4 °C. After the sample had been washed five times with 1 mL of buffer, 50 μL of SDS sample buffer was added to each sample, and the samples were and boiled for 10 min. Twenty microliters of sample preparation was used for 14% SDS−polyacrylamide gel electrophoresis, and the level of ARF6 association was determined by Western blotting using anti-ARF6 antibody. Ten microliters of CHO-K1 cell lysate overexpressing ARF6 (approximately 70 ng) was used for a standard. Three independent experiments were performed with similar results. (B) CHO-K1 cell lysates overexpressing either ARF6 or ARF5 were incubated with GTPγS or GDPβS as described for panel A. For this specific pulldown experiment, 17 μg of GST or GST-arfophilin-BD (ARF5 binding domain of arfophilin, amino acids 612−756) immobilized on glutathione−Sepharose beads was incubated with the cell lysates for 30 min at 4 °C. Washing and Western blotting were performed as described for panel A. Another independent experiment was performed with similar results.

polyacrylamide gels after N-terminal myristoylation of ARF6 has been well-documented (*40*), and this is consistent with our observation. Thus, N-terminal myristoylation of ARF6 appears not to be essential for binding to arfophilin because both the upper and lower bands of ARF6 overexpressed in CHO-K1 cells were associated with arfophilin (Figure 1).

When wild-type ARF6 was overexpressed in CHO-K1 cells, it associated with GST-arfophilin (Figure 2A). The association was much stronger in the presence of GTPγS than GDPβS, and was not seen with GST alone. These data indicate that the binding of ARF6 to arfophilin in CHO-K1 cell lysates is specific and guanine nucleotide-dependent. Figure 2B shows that ARF6 also bound to the C-terminal ARF5 binding domain of arfophilin (amino acids 612−756) in a guanine nucleotide-dependent manner.

Definition of the ARF6 Binding Domain for Arfophilin. As stated above, the finding that both GTP-ARF5 and GTP-ARF6 bound to the same domain of arfophilin, even though their N-termini are quite different (Figure 3), was unexpected. For this reason, we identified the arfophilin binding domain of ARF6 using ARF1/ARF6 chimeras in a "gain of function" approach (Figures 3 and 4A). Amino acids between ARF1 and ARF6 are well-conserved in only the switch I (amino acids 45−54 of ARF1) and switch II (amino acids 70−80 of ARF1) regions, among possible effector domains such as the N-terminus, strands β2 and β3, intervening loop λ3, and the C-terminus (Figure 3). Comparison of the N-terminal amino acids among the different classes of ARFs indicated that S and R/K residues (residues 10 and 11 for ARF4 and -5 and residues 6 and 7 for ARF6) are well-conserved in class II and III ARFs, but not in class I ARFs (Figure 3). Figure 4B shows that the GTP-bound mutant form of ARF1 with K10S and G11R mutations did not bind to arfophilin, while it did bind to arfaptin 2, indicating that the S and R/K residues in the N-termini of ARFs are not critical for binding to arfophilin.

ARF1 with its 15 N-terminal amino acids switched to ARF6 ($N6^{1-11}$ARF1-Q71L, Figure 4A) bound to arfaptin 2, as expected from Figure 1, but did not bind to arfophilin (Figure 4B). On the other hand, ARF6 with N-terminal amino acids switched to ARF1 ($N1^{1-23}$ARF6-Q67L, Figure 4A) bound to both arfophilin and arfaptin 2 (Figure 4B), indicating that the N-terminus of ARF6 is not critical for binding to arfophilin (Figure 4B). A GTP-bound mutant form of ARF6 with deletion of N-terminal amino acids (ΔN^{1-13}-ARF6-Q67L, Figure 4A) also bound to GST-arfophilin (data not shown), further indicating that the N-terminus of ARF6 is not essential for binding to arfophilin.

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ARF1    MGNIFANLFKGLFGKKEMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIG  50
ARF3    MGNIFGNLLKSLIGKKEMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIG  50
ARF5    MGLTVSALFSRIFGKKQMRILMVGLDAAGKTTILYKLKLGEIVTTIPTIG  50
ARF6    MG----KVLSKIFGNKEMRILMLGLDAAGKTTILYKLKLGQSVTTIPTVG  46

ARF1    FNVETVEYKNISFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERV  100
ARF3    FNVETVEYKNISFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERV  100
ARF5    FNVETVEYKNICFTVWDVGGQDKIRPLWRHYFQNTQGLIFVVDSNDRERV  100
ARF6    FNVETVTYKNVKFNVWDVGGQDKIRPLWRHYYTGTQGLIFVVDCADRDRI   96

ARF1    NEAREELMRMLAEDELRDAVLLVFANKQDLPNAMNAAEITDKLGLHSLRH  150
ARF3    NEAREELMRMLAEDELRDAVLLVFANKQDLPNAMNAAEITDKLGLHSLRH  150
ARF5    QESADELQKMLQEDELRDAVLLVFANKQDMPNAMPVSELTDKLGLQHLRS  150
ARF6    DEARQELHRIINDREMRDAIILIFANKQDLPDAMKPHEIQEKLGLTRIRD  146

ARF1    RNWYIQATCATSGDGLYEGLDWLSNQLRNQK 181
ARF3    RNWYIQATCATSGDGLYEGLDWLANQLKNKK 181
ARF5    RTWYVQATCATQGTGLYDGLDWLSHELSKR- 181
ARF6    RNWYVQPSCATSGDGLYEGLTWLTSNYKS-- 175
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FIGURE 3: Comparison of the amino acid sequences of ARF1, -3, -5, and -6. Different amino acids are denoted by bold letters, and conserved amino acid substitutions are underlined. Conserved amino acid substitutions are grouped as follows: C; S, T, P, A, and G; N, D, E, and Q; H, R, and K; M, I, L, and V; F, Y, and W. The GTP-myristoylated switch regions (amino acids 2−17) are denoted by the solid line, and the central regions are denoted by the dotted line. S and R/K residues which are well-conserved in both class II and class III, but not in class I, ARFs are boxed.

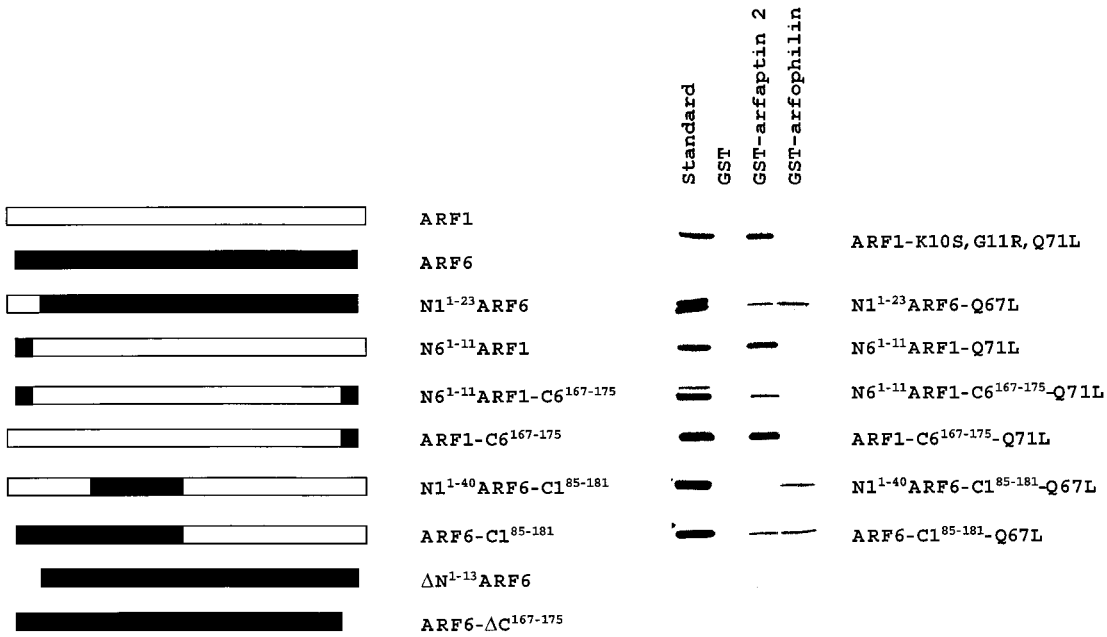

FIGURE 4: Identification of the arfophilin binding domain of ARF6 with the in vitro GST pulldown assay. (A) Depiction of ARF1/ARF6 chimeras and ARF6 modifications. (B) Each ARF mutant was overexpressed in CHO-K1 cells, and 500 μL of cell lysate was used for GST pulldown experiments in the presence of 10 μM GTPγS. Each mutant ARF was incubated with 7 μg of GST (29 kDa), 15 μg of GST-arfaptin 2 (63 kDa), or 17 μg of GST-arfophilin (73 kDa) immobilized on 25 μL of glutathione−Sepharose beads at 4 °C for 30 min. After the sample had been washed five times with 1 mL of buffer, 50 μL of SDS sample buffer was added to each sample, and the samples were boiled for 10 min. Twenty microliters of sample preparation was used for 14% SDS−polyacrylamide gel electrophoresis, and the level of ARF association was determined by Western blotting. Ten microliters of CHO-K1 cell lysate overexpressing each ARF mutant was used for a standard. Two to five independent experiments were performed with similar results.

We also determined whether the C-terminus of ARF6 is involved in binding to arfophilin using other ARF1/ARF6 chimeras. Both N6$^{1-11}$ARF1-C6$^{167-175}$-Q71L and ARF1-C6$^{167-175}$-Q71L bound to arfaptin 2, but not to arfophilin (Figure 4B). This suggests that the C-terminus of ARF6 is not involved in binding to arfophilin. The binding of both N6$^{1-11}$ARF1-C6$^{167-175}$-Q71L and ARF1-C6$^{167-175}$-Q71L to arfaptin 2 indicates that both chimeras were produced as functional proteins in these experiments. We also tried to determine whether ARF6 lacking the C-terminus (ARF6-ΔC$^{167-175}$-Q67L, Figure 4A) could bind to arfophilin, but our results were inconclusive since the mutant ARF6 bound to GST alone (data not shown).

Since the preceding studies indicated that both the N- and C-termini of ARF6 were not critical for binding to arfophilin, we exchanged amino acids 41−84 of ARF1 for amino acids 37−80 of ARF6 (N1$^{1-40}$ARF6-C1$^{85-181}$-Q67L, Figure 4A). This chimera bound to arfophilin, indicating that amino acids

Table 1: Yeast Two-Hybrid Interaction Assay between Various Mutant Forms of ARFs and Arfophilin[a]

GAL4 DNA binding domain (pGBT9)	pGAD424-mouse α-actin	pGAD10-arfaptin 2	PGAD424-arfophilin
ARF1-Q71L	–[b]	blue[c]	–
ARF1	–	–	–
ARF1-T31N	–	–	–
ARF5-Q71L	–	blue	blue
ARF5	–	–	–
ARF5-T31N	–	–	–
ARF6-Q67L	blue[d]	blue	blue
ARF6	–	–	–
ARF6-T27N	–	–	–
ARF1-K10S/G11R/Q71L	–	blue	–
$N1^{1-23}$ARF6-Q67L	blue[d]	blue	blue
$N6^{1-11}$ARF1-Q71L	–	blue	–
$N6^{1-11}$ARF1-$C6^{167-175}$-Q71L	–	blue	–
ARF1-$C6^{167-175}$-Q71L	–	blue	–
$N1^{1-40}$ARF6-$C1^{85-181}$-Q67L	blue[d]	blue	blue
ARF6-$C1^{85-181}$-Q67L	blue[d]	blue	blue

[a] β-Galactosidase activity was determined by a colony-lift filter assay for the 3-day-old SFY 526 yeast transformants containing the indicated plasmids. Two to five independent yeast transformation assays produced similar results. [b] Color development was not observed after incubation for 24 h at 30 °C. [c] Blue color started to develop within 1 h of incubation. [d] pGAD424 vector alone showed blue color when cotransformed with either pGBT9-ARF6-Q67L, pGBT9-$N1^{1-23}$ARF6-Q67L, pGBT9-$N1^{1-40}$ARF6-$C1^{85-181}$-Q67L, or pGBT9-ARF6-$C1^{85-181}$-Q67L, indicating these mutants are autoactivating.

37−80 of ARF6 are critical for binding to arfophilin. This binding was specific because $N1^{1-40}$ARF6-$C1^{85-181}$-Q67L did not bind to GST alone (Figure 4B). However, the chimera lost binding affinity for arfaptin 2 (Figure 4B). Therefore, we also constructed another chimera (ARF6-$C1^{85-181}$-Q67L) that retained the first 40 amino acids of ARF6 (Figure 4A) and determined its level of binding to arfaptin 2. This chimera bound to both arfaptin 2 and arfophilin, but not to GST (Figure 4B). We conclude from all these experiments that the N-terminus of ARF6 is not critical for binding to arfophilin, but that amino acids 37−80 are essential.

Interactions of Different Classes of ARFs with Arfophilin and Arfaptin 2 As Determined by the Yeast Two-Hybrid Assay. We also determined the association between the ARFs and arfophilin by a yeast two-hybrid interaction assay. We employed low-copy number plasmids for the interaction assay because high-level expression of some of the ARF mutants was toxic to the SFY 526 yeast (*28*). Consistent with our GST pulldown experiments, constitutively active ARF1 (ARF1-Q71L), a mutant of this (ARF1-K10S/G11R/Q71L), and three chimeras ($N6^{1-11}$ARF1-Q71L, $N6^{1-11}$ARF1-$C6^{167-175}$-Q71L, and ARF1-$C6^{167-175}$-Q71L) did not interact with arfophilin (Table 1). However, all of these mutants exhibited interaction with arfaptin 2, indicating that they were adequately expressed in a functional form (Table 1). Neither wild-type ARF1, -5, or -6 nor their dominant negative forms exhibited interaction with arfophilin or arfaptin 2 (Table 1). Consistent with the data depicted in Figure 1, constitutively active ARF5 (ARF5-Q71L) bound to both arfaptin 2 and arfophilin (Table 1). ARF6-Q67L, $N1^{1-23}$ARF6-Q67L, $N1^{1-40}$-ARF6-$C1^{85-181}$-Q67L, and ARF6-$C1^{85-181}$-Q67L were all autoactivating when expressed in SFY 526 yeast using a low-copy number plasmid, pGBT9 vector (Table 1), indicating that the central region of ARF6 was also involved in activation of the GAL4 promoter. SFY 526 yeast did not express the GTP-bound mutant form of ARF6 fused to the GAL4 activation domain vector, while it expressed the GTP-bound forms of both ARF1 and ARF5 (data not shown). These data indicated that the yeast two-hybrid binding system was not a proper tool for assaying ARF6 binding.

DISCUSSION

Arfophilin was first identified from a human kidney cDNA library as a binding partner for GTP-bound ARF5. Arfophilin is the only known ARF effector which does not interact with class I ARFs (*28*). In the study presented here, we found that GTP-ARF6 bound to arfophilin. This was surprising because our previous findings had shown that the 17 N-terminal amino acids of ARF5 were essential for binding to arfophilin (*28*), and this sequence is very different in ARF6 (Figure 3). However, studies with ARF1/ARF6 chimeras (Figure 4B) revealed that a different sequence of ARF6 (amino acids 37−80) was involved in its binding to arfophilin. This sequence is different from those of either ARF1 or ARF5 (Figure 3), and also from the sequence (amino acids 2−17) involved in ARF5 binding to arfophilin (Figure 3) (*28*). Because of this, we previously suggested that ARF6 probably does not bind to arfophilin (*28*).

The central regions of class I ARFs (ARF1 and -3) and ARF5 are identical except for a single amino acid difference (S to C) (Figure 3). If this region were involved in binding, class I ARFs would bind to arfophilin as is seen for ARF5. In addition, we previously showed that an ARF3 construct with N-terminal ARF5 amino acids (N5-ARF3) bound to arfophilin (*28*), further suggesting that the single amino acid difference in the central region of class I ARFs and ARF5 is not critical for binding to arfophilin. If this difference were critical, N5-ARF3 would not bind to arfophilin. On the basis of these observations, we conclude that only the N-terminus of ARF5 is involved in binding to arfophilin. It is interesting to note that the structural differences between the GDP- and GTP-bound forms of ARF1 are localized to amino acids 38−83 as well as the N-terminus (amino acids 2−17) (*18*), and that residues 35−94 in ARF1 are required for efficient activation of phospholipase D1 and recruitment of adaptor protein 1 (*36*). Thus, there is consistency between our findings and the proposed model for ARF1 interaction with its effectors. The GTP-bound forms of ARF5 and ARF6 bind to the same C-terminal domain of arfophilin (amino acids 612−756) even though different sequences in the ARFs are involved. This suggests that there may be two different binding sites in the amino acid sequence of residues 612−756.

The finding that arfophilin may be a common target for the GTP-bound form of ARF5 and ARF6 suggests that the GTPases may influence cellular pathways through arfophilin as a common downstream effector. GTP-ARF5 specifically translocates to Golgi in rat brain homogenates (*6*), while it translocates to Golgi, endoplasmic reticulum, and endosomes in CHO-K1 cells (*12*). ARF6 overexpressed in a CHO-K1 cell line is mainly localized to the perinuclear region (*37*), although it is not clear whether it is in a GDP- or GTP-bound form. Some studies suggest that both GTP-ARF6 and GDP-ARF6 are always associated with membranes (*12*, *13*), while other studies indicate that only GTP-ARF6 is associated with membranes and GDP-ARF6 is cytosolic (*14*, *15*).

Most evidence indicates that GTP-ARF6 acts in the peripheral region of cells and is involved in vesicle trafficking in this region (*7*, *13*, *38*).

Arfophilin overexpressed in CHO-K1 cells was associated with the perinuclear region, including the Golgi and possibly endosomes (data not shown). Some portion also localized to the peripheral region. Unfortunately, we could not determine whether this arfophilin is associated with either GTP-ARF5 or GTP-ARF6 because the constitutively active forms of these GTPases were extremely toxic to the CHO-K1 cells, inducing severe cell shrinkage. On the basis of previous studies (*6*, *13*, *37*, *38*), we propose that arfophilin bound to the Golgi region is associated with GTP-ARF5, and that arfophilin in the peripheral region is associated with GTP-ARF6.

The cellular function of arfophilin is not clear. Structural analysis (not shown) indicates that it may act as an adaptor in class II and III ARF signaling pathways. Both the N-terminal proline-rich region (amino acids 1−200) and the C-terminal coiled-coil domain (amino acids 531−694) may be involved in protein−protein interactions with downstream targets. The positively charged R/K-rich region (amino acids 460−680) close to the ARF binding domain (amino acids 612−756) may associate with phosphatidylinositol 4,5-bisphosphate (*43*) after translocation to membranes with the GTP-bound form of either ARF5 or ARF6. In human pancreas, the strength of the arfophilin message is increased during the development of cancer (*34*), indicating that the arfophilin pathway may be involved in secretion because this is usually upregulated for metastatic spreading. This is also consistent with one of the proposed cellular roles of ARF6 (*7*, *13*, *38*, *39*).

In this study, we also examined the interaction of different classes of ARFs with arfaptin 2. Arfaptin 2 was identified as a GTP-ARF3 binding partner in our laboratory (*24*), and another group reported the identification of a form of arfaptin 2 lacking the 38 N-terminal amino acids named POR1 (partner of Rac1) (*25*). POR1 was identified as a physiological downstream effector of ARF6 involved in cytoskeletal rearrangements in the peripheral region of cells (*26*). POR1 may be a differently spliced isoform of arfaptin 2 possessing cellular functions different from that of arfaptin 2. This study (Figure 1) indicates that arfaptin 2 binds strongly to ARF1 compared with either ARF5 or ARF6. This is similar to what has been reported for arfaptin 1 (*24*, *42*). The cellular concentration of class I ARFs is 3−10-fold higher than those of class II or class III ARFs (*12*). On the basis of both the specific affinity (*24*) and cellular concentration of ARFs (*12*), ARF1 may be a cellular target for arfaptin 2. Overexpression of arfaptin 2 in CHO-K1 cells is associated with the disappearance of the Golgi and subsequent translocation of arfaptin 2 to the periphery (data not shown).

In summary, we have demonstrated that arfophilin binds to either GTP-ARF5 or GTP-ARF6 and could be a common physiological target of these ARFs. To understand the cellular role of arfophilin, we need to determine its role in the cellular actions of ARF5 and ARF6 and to identify other proteins that may be involved in its actions.

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