CHARACTERIZING ARL1-BINDING PROTEINS*

HILLARY VAN VALKENBURGH, JACK F. SHERN, J. DANIEL SHARER, XINJUN ZHU, AND RICHARD A. KAHN‡

From the Department of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322-3050

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ADP-ribosylation factors (ARFs) and ARF-like (ARL) proteins, distinct functional roles have been inferred from findings that ARLs lack the biochemical or genetic activities characteristic of ARFs. The potential for functional overlap between ARFs and ARLs was examined by comparing effects of expression on intact cells and the ability to bind effectors. Expression of [Q71L]ARL1 in mammalian cells led to altered Golgi structure similar to, but less dramatic than, that reported previously for [Q71L]ARF1 (1). Two previously identified partners of ARFs, MKLP1 and Arfaptin2/POR1, also bind ARL1 but not ARL2 or ARL3. Two-hybrid screens of human cDNA libraries with dominant active mutants of human ARL1, ARL2, and ARL3 identified eight different but overlapping sets of binding partners. Specific interactions between ARL1 and two binding proteins, SCOCO and Golgin-245, are defined and characterized in more detail. Like ARFs and ARL1, the binding of SCOCO to Golgi membranes is rapidly reversed by brefeldin A, suggesting the presence of a brefeldin A-sensitive ARL1 exchange factor. These data reveal a complex network of interactions between GTPases in the ARL family and their effectors and reveal a potential for cross-talk not demonstrated previously.

ADP-ribosylation factors (ARFs)† are highly conserved, ubiquitous, 21-kDa GTP-binding proteins with roles in multiple steps of membrane traffic and other cellular processes (for review, see Refs. 2–6). Within the ARF family are three subgroups that have been defined by sequence and functional relatedness (7). The ARFs share >60% sequence identity and share activities as cofactors in the ADP-ribosylation of Gαs by bacterial toxins ( cholera and Escherichia coli heat-labile toxin) (8, 9), activators of phospholipase D (10, 11), suppression of the lethal double mutation arf1− arf2− in the yeast Saccharomyces cerevisiae (12, 13), and activator of phosphatidylinositol 4-phosphate 5-kinase (14). The ARF-like (ARL) proteins are 40–60% identical to each other or to any ARF and are essentially devoid of the activities described for ARFs (12, 15, 16). A third group of proteins, including yeast SAR1 and CIN4, is included in the ARF family although the proteins share only 25–35% sequence identity and have clearly distinct activities in cells (5, 17).

More than 10 ARLs have been identified in humans, and three in S. cerevisiae (15, 18–21). Although ARFs have been purified repeatedly by laboratories using different biochemical assays, no ARL has ever been purified or cloned based on an activity. The lack of ARL activity in ARF assays has led to the conclusion that ARLs have distinct biochemical activities and thus cellular functions, despite their similarity in sequence and structure (15).‡ The lack of functional overlap between ARFs and ARLs is evident from the findings that ARLs in yeast cannot rescue the synthetic lethality of the arf1− arf2− mutations (12), whereas any of the six mammalian ARFs (13, 23) or ARFs from other organisms (e.g. Drosophila (24), or Giardia (25)) can suppress the lethality of arf1− arf2− in yeast. Similarly, deletion of the Drosophila arl1 gene causes zygotic lethality despite the presence of the full complement of ARF genes in flies (15).

In contrast to the many activities and functions assigned to ARFs, none has yet been assigned to any ARL from any species. The number of binding partners for mammalian ARF proteins has increased dramatically in recent years in large part because of the use of two-hybrid screens that have identified seven new ARF effectors: Arfaptin1 (25), Arfaptin2/POR1 (independently isolated by two groups (25, 26) and named Arfatin 2 and POR1, respectively), MKLP1 (27), Arfophilin (28), and the three GGAs (29–31). The use of carboxyl-terminal fusion proteins and dominant activated ARF mutants has enhanced the ability to select for binding partners that interact preferentially with the activated form of the GTPase (27, 29, 32). Two proteins, “binder of ARL2” (BART (33)) and the subunit of cGMP phosphodiesterase 6 (PDE6 (34)), have been cloned based on their GTP-dependent interaction with either ARL2 or ARL3, respectively. To date, however, no clear biological significance has been attributed to these interactions.

Role(s) for ARF in Golgi functions were evident when expression of the persistently activated mutant allele [Q71L]ARF1 in cells led to expansion and vesiculation of the Golgi compartment (1). Because ARL1 was reported also to bind Golgi membranes in intact cells, we used the same procedures to test for a functional role of ARL1 at the Golgi. We were surprised by the similarities between effects of activated ARF1 and ARL1 on Golgi morphology, and this led us to reexamine the extent of functional overlap between ARF and ARL proteins.

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322-3050. Tel.: 404-727-3561; Fax: 404-727-3746; E-mail: rkahn@emory.edu.

† The abbreviations used are: ARF(s), ADP-ribosylation factor(s); ARL, ARF-like; BART, binder of ARL2; PDE6, δ subunit of cGMP phosphodiesterase 6; NRR, normal rat kidney; GTP-γS, guanosine 5′-3′-O-(thio)triphosphate; BSA, bovine serum albumin; GAP, GTPase-activating protein; SCOCO, short coiled-coil; HBG, human retinal gene; MOPS, 4-morpholinoethanesulfonic acid; bp, base pairs; HA, hemagglutinin; UTR, untranslated region; GEF, guanine nucleotide exchange factor.

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MATERIALS AND METHODS

Data Collection—Every experiment reported herein was repeated at least twice and in most cases additional times with essentially the same results.

Cell Culture—Growth media were prepared, and maintenance of yeast strains was performed as described in Sherman et al. (35). Transformation of yeast was performed using the method of Schiestl and Gietz (36). Plasmids were rescued from yeast (as described from (37)) by further purification on QiaGen mini-preparation columns. DNA was transformed into E. coli strain DH5α prior to plasmid preparation. Normal rat kidney (NRK) cells were obtained from American Type Culture Collection (ATCC; Rockville, MD) and grown and passaged in RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc.) at 37 °C in a humidified atmosphere. RPMI 1640 medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc.) at 37 °C in a humidified atmosphere containing 10% CO2.

Yeast Two-hybrid Screens—Yeast strains Y190 (MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 URA3::GAL-(lacZ) and) Y187 (MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 met- URA3::GAL-(lacZ), plasmids pAS2 and pACT2, and the human B cell library in pACT2 were gifts of Steven J. Elledge (Baylor University). This system utilizes the GAL4 binding and activation domains and allows for two independent read-outs for a positive interaction in the two-hybrid system: histidine synthetase and β-galactosidase expression (38). The human fetal brain cDNA library, in pACT2, was purchased from CLONTECH. Plasmid pBG4D was a gift from Rob Brazas and allowed the expression of proteins fused through their carboxyl termini to the GAL4 binding domain. The open reading frame of each ARL was cloned in-frame into pBG4D via BamHI and NotI sites. Mutations were generated using Stratagene’s QuikChange site-directed mutagenesis kit. All DNA generated by polymerase chain reaction was sequenced both to confirm the presence of designed mutations and prevent the introduction of others.

Screening of human cDNA libraries was performed as described previously (38) with the modifications described in Boman et al. (27, 29). Briefly, the dominant activating mutations in each human ARL protein were used as bait to screen human fetal brain or B cell libraries in Y190 by selection for growth on selective plates containing 25 mm 3-amino-1,2,4-triazole (3-AT). Positives were then assayed for β-galactosidase activity, using the nitrocellulose filter binding assay of Breeden and Lasry (39). Filters were incubated at 30 °C for up to 3 h. A strong interaction was defined as the development of a dark blue color within 15 min; a weak interaction required the full 3 h for development of a pale blue color. Further tests for specificity of interactions included: 1) interaction was defined as the development of a dark blue color within 15 min; a weak interaction required the full 3 h for development of a pale blue color. Further tests for specificity of interactions included: 1) interaction was defined as the development of a dark blue color within 15 min; a weak interaction required the full 3 h for development of a pale blue color. Further tests for specificity of interactions included: 1) the interaction was scored as positive if the protein formed a blue precipitate with the 3-AT. Positives were then assayed for β-galactosidase activity, using the nitrocellulose filter binding assay of Breeden and Lasry (39). Filters were incubated at 30 °C for up to 3 h. A strong interaction was defined as the development of a dark blue color within 15 min; a weak interaction required the full 3 h for development of a pale blue color. Further tests for specificity of interactions included: 1) interaction was defined as the development of a dark blue color within 15 min; a weak interaction required the full 3 h for development of a pale blue color. Further tests for specificity of interactions included: 1) interaction was defined as the development of a dark blue color within 15 min; a weak interaction required the full 3 h for development of a pale blue color. Further tests for specificity of interactions included: 1) interaction was defined as the development of a dark blue color within 15 min; a weak interaction required the full 3 h for development of a pale blue color. Further tests for specificity of interactions included: 1) interaction was defined as the development of a dark blue color within 15 min; a weak interaction required the full 3 h for development of a pale blue color. Further tests for specificity of interactions included: 1) interaction was defined as the development of a dark blue color within 15 min; a weak interaction required the full 3 h for development of a pale blue color. Further tests for specificity of interactions included: 1) 3′-galactosidase activity after transfection, the cells were split and diluted into medium containing 100 μM isopropyl-1-thiogalactopyranoside at 30 °C in 20 mM HEPES, pH 7.4, 100 mm NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM MgCl2, 50 μM/ml BSA, and 10 μM [γ-32P]GTP-S (1,000 cpm/ml), as described previously (40).

Gap Assay—ARL1 Gap activity was assayed by a modification of the method of Randazzo and Kahn (41) using 0.18 μM purified, recombinant [17]ARL1-(His)6, as our source of recombinant protein. The addition of 1.8 μM substrate domain of Golgin-245 or SCOCO. Purified human ARL3 and ARL4 GAP (42) served as positive controls for the assay.

GAP Over assay—Direct interaction between human ARL1, 2, or 3 and BART, PDE6, or HRG4 was assayed by gel overlay, as described in Sharer and Kahn (33). Briefly, SDS-solubilized protein lysates were prepared from BL21(DE3) cells containing either the PDE6 open reading frame or the BART coding sequence (pET15b (the clone of Ahmed Zahrour, Compartiments et Dynamique Cellulaires, Institut Curie, Paris, France), HRG4 open reading frame in pT714b at NdeI and BamHI restriction sites, or an empty vector control (Table I). 25 μg of total protein was resolved on a 15% polyacrylamide gel before electrophoretic transfer to 0.2-μm nitrocellulose membrane (Bio-Rad). Proteins adsorbed on the filter were renatured in 10 mM MOPS, pH 7.1, 100 mm potassium acetate, 0.25% Tween 20, 5 mm magnesium acetate, 0.5% BSA, 5 mM dithiothreitol, and incubated with 2 μg of recombinant [17]ARL1-(His)6, ARL2, or ARL3 prebound to 20 μl of [α-32P]GTP. The filter was washed three times with binding buffer (20 mM MOPS, pH 7.1, 100 mm potassium acetate, 0.1% Triton X-100, 5 mm magnesium acetate, 0.5% BSA, 50 μM GTP, 50 μM GDP, and 5 mM dithiothreitol), and specific binding was determined by phosphor imaging.

Affinity Chromatography—10 μl purified recombinant [17]ARL1-(His)6 was loaded with either 100 μl GDP or GTP-S for 15 min at 30 °C on a column of yeast expressing 20 μl of 20 mM HEPS, pH 7.4, 100 mm NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM MgCl2, and 50 μM BSA. SCOCO was covalently attached to Affi-Gel 15 beads according to the manufacturer’s directions (Bio-Rad). SCOCO beads were washed twice in binding buffer containing 10 μM appropriate nucleotide, just prior to the addition of preloaded [17]ARL1-(His)6. SCOCO beads and [17]ARL1-GDP or -GTP-S were incubated with gentle rocking for 15 min at room temperature. The beads were washed twice with binding buffer, containing 20 μM appropriate nucleotide and then mixed with an equivalent amount of 2 μl Laemmli sample buffer. Proteins were resolved on a 15% polyacrylamide gel, and the presence of [17]ARL1-(His)6 was detected using polyclonal rabbit anti-serum raised against ARL1 (R58722-3).

Immunohistochemistry—NRK cells were fixed in 3.7% formaldehyde and permeabilized in 0.2% saponin with 10% goat serum, as described in Zhang et al. (1). Cells were visualized either on an Olympus BX60 fluorescence microscope or a Bio-Rad 1024 laser scanning confocal microscope coupled to a Zeiss Axiovert. For confocal microscopy, a Z-series of images was collected with 5-μm steps and processed using IMAGE J software from NIH IMAGE.

SCOCO Antibody Generation and Affinity Purification—Recombinant SCOCO-(His)6 was purified as described above and used as antigen in rabbits, after conjugation to keyhole limpet hemocyanin through both the NH2 terminus (using glutaraldehyde) and the COOH terminus (using iodoacetamide). Antibodies were affinity purified by sequential protein G-Sepharose and affinity chromatography. 6.5 mg of untagged, recombinant SCOCO was covalently attached to 1 ml Affi-Gel 15 beads, according to the manufacturer’s directions. Serum from rabbit R97679 was enriched for immunoglobulins on a 1 ml protein G column (Amer sham Pharmacia Biotech) and eluted with 0.1 μl glycine HCl, pH 2.7. The eluted antibodies were exchanged into 0.1 M MOPS, pH 7.2, followed and applied to the Affi-Gel 15-SCOCO column. The column was washed with 10 ml of phosphate-buffered saline containing 1 μl NaCl. Antibodies were eluted with 10 ml of 0.1 μl glycine-HCl, pH 2.4, collecting 1-ml fractions into 100 μl of 1 M Tris-HCl, pH 9, to neutralize the glycine buffer. Fractions containing protein were pooled, and the buffer was exchanged for phosphate-buffered saline.

Interferon-inducible ARL1 Cell Lines—COOH-terminal, myc-epitope tagged constructs of ARL1 or [Q7][L]ARL1 were subcloned into pSSE2-2 for expression in mammalian cells under regulation by the interferon-inducible MX1 promoter, as described in Zhang et al. (Table I and Ref. 1). Each of these or the parental vector was cotransfected with pSV2-neo (ATCC), at a 10:1 ratio of DNA, into NRK cells using Fugene 6 (Invitrogen) reagent, following the manufacturer’s directions. 48 h after transfection, the cells were harvested, and the supernatant and the cell pellet were extracted into medium containing 400 μg/ml Genticin disulfate (G418, Sigma). G418-resistant clones were isolated and later cloned by limited dilution. Cell lines carrying stably integrated plasmids directing expression of ARL1-myc or [Q7][L]ARL1-myc with no ARL1 5′-untranslated sequence were NRK-HV1–9 and NRK-HV2–20, respectively. Protein expression was induced with 1,000 units/ml α, β-mercaptoethanol (Lee Biomolecular, San Diego)
and assayed by immunoblot analysis using monoclonal 9E10 (mouse α-myc) antibodies. When lysates were probed with myc antibodies in immunoblots, we consistently observed the interferon-dependent expression of a doublet in which the upper band corresponded to the predicted size of full-length ARL1 (21 kDa) and a second band migrating as a smaller fragment (predicted size of full-length ARL1 (21 kDa) and a second band migrating as a smaller fragment (~17 kDa). Human ARL1 is myristoylated at its NH2 terminus (43), so we tested for the incorporation of [3H]myristic acid into ARL1 by fluorography (44). Only the upper band was labeled using the 12CA5 antibody (mouse 

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Northern Blot Analysis—Northern blotting was performed with the use of the Multiple Human Tissue I Blot (CLONTECH), according to manufacturer’s directions. The SCOCO open reading frame was used as the probe (~250 bp in length) and was labeled by incorporating [α-32P]dCTP (NEN Life Science Products) using the random priming kit from Life Technologies, Inc. Hybridizing bands were detected by exposure to Phosphorscreens.

Electron Microscopy—After fixation in 1% glutaraldehyde, samples were poststained with 1% osmium tetroxide, dehydrated through a graded series of ethanol, and subsequently embedded in Embed 812 (Electron Microscopy Sciences). Thin sections were stained with uranyl acetate and lead citrate. Electron microscopy was performed on a Philips CM-10 transmission electron microscope at 60 kV.

Guanine Nucleotide Exchange Factor (GEF) Assays—Binding of GTPγS to human ARL1-(His)6 was determined as described in Kahn

**TABLE I**

**Plasmids used in this study**

Columns indicate the name of the plasmid, the insert, parental plasmid, and source. When a fragment of an open reading frame is used, the residues included in the construct are indicated in parentheses after the name of the insert. AD, GAL4 activation domain; BD, GAL4 binding domain.

| Plasmid name | Insert | Vector | Ref. |
|--------------|--------|--------|------|
| pAB155       | ARF3-BD| pBG4D  | 27, 29 |
| pAB157       | [Q71L]ARF3-BD| pBG4D  | 27, 29 |
| pJCH1–11     | BD-[Q71L]ARF1 | pAS1     | 27, 29 |
| pJCH5–2      | BD-[Q71L]ARF3 | pAS1     | 27, 29 |
| pJCH2–14     | BD-[Q71L]ARF4 | pAS1     | 27, 29 |
| pJCH5–12     | BD-[Q71L]ARF5 | pAS1     | 27, 29 |
| pJCH4–13     | BD-[Q71L]ARF6 | pAS1     | 27, 29 |
| pHV330       | ARL1-BD | pBG4D  | This paper |
| pHV628       | [Q71L]ARL1-BD | pBG4D  | This paper |
| pJS100–1     | ARL2-BD | pBG4D  | This paper |
| pJS106       | [Q70L]ARL2-BD | pBG4D  | This paper |
| pJS104       | ARL3-BD | pBG4D  | This paper |
| pJS103       | [Q71L]ARL3-BD | pBG4D  | This paper |
| pAB169       | AD-GGA1(145–639) | pACT     | 29 |
| pAB195       | AD-GGA2(131–613) | pACT     | 29 |
| pAB179       | AD-GGA3(80–690) | pACT     | 29 |
| pAB138       | AD-[E112D]LTA | pACT     | 69 |
| pAB199       | AD-MKLP1 (663–960) | pACT     | 27 |
| pGAD-POR1ΔN  | AD-POR1 (79–341) | pACT2    | 26 |
| pHV859       | AD-Arafaptin/POR1 | pACT    | This paper |
| p2QC-36      | AD-BART | pACT2   | This paper |
| pHV842       | AD-Golg1-245(2025–2083) | pACT    | This paper |
| pJSQB86      | AD-HRG4 | pACT    | This paper |
| pHV844       | AD-MKLP1(456–960) | pACT    | This paper |
| pJSQ6C0B     | AD-PDE5 | pACT2   | This paper |
| pHV841       | AD-SCOCO | pACT    | This paper |
| pHV883       | [AD][Y2032A](Golg1-245(2025–2083)) | pACT    | This paper |
| pHV631       | ARL1-(His)b | pET20   | This paper |
| pHV682       | SCOCO-(O-(His))6 | pET3C   | This paper |
| pHV859       | SCOCO | pET3C   | 33 |
| pBART-(His)b | BART-(His)b | pET3C | This paper |
| pHV870       | (His)b,Golg1-245(2025–2083) | pET14   | This paper |
| pHV877       | [Δ17](ARL1)-(His)b | pET20   | This paper |
| pHV627       | ARL1-myc | pSS2-2  | This paper |
| pHV620       | [Q71L]ARL1-myc | pSS2-2  | This paper |
| pHV820       | 50-bp 5’-UTR-ARL1-HA | pSS2-2/NEO | This paper |
| pHV810       | 50-bp 5’-UTR [Q71L]ARL1-HA | pSS2-2/NEO | This paper |
| pHV873       | Gog1-245(2025–2083)-myc-(His)b | pCDNA3.1 Myc-His | This paper |
| pHV872       | SCOCO-myc-(His)b | pCDNA3.1 Myc-His | This paper |
| pHV885       | [Y2032A](Golg1-245(2025–2083)-myc-(His)b | pCDNA3.1 Myc-His | This paper |
and Gilman (8). Briefly, GTPases (1 µM) were incubated at 30 °C with or without the SEC7 domain (10 µM) in 20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 4.5 mM MgCl₂, 2.5 mM azolectin vesicles, 100 µg/ml BSA, and 10 µM [γ-35S]GTPγS (2,500 cpm/ pmol). Duplicates of 10-μl samples were taken and diluted into 2 ml of ice-cold TNMD buffer (25 mM Tris-Cl, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, and 1 mM dithiothreitol), followed by rapid filtration onto 25-mm BA85 nitrocellulose filters (Schleicher & Schuell).

RESULTS

Expression of [Q71L]ARL1-altered Golgi Structures—A role for ARFs in Golgi function was supported by the finding that the expression of dominant active [Q71L]ARF1 in NRK cells led to expansion and vesiculation of the Golgi compartment (1). Because ARL1, like ARF, localizes to Golgi membranes (21), we used the same procedures to test for a functional role of ARL1 at the Golgi. To enable specific detection of ARL1 in cells, we engineered an epitope (myc) tag at the COOH terminus of both the wild type and [Q71L]ARL1 proteins. Stable NRK cell lines were obtained which were capable of expressing ARL1-myc or [Q71L]ARL1-myc upon induction with interferon, as described under “Materials and Methods.” Localization of each protein to the Golgi was confirmed by indirect immunofluorescence using the monoclonal myc antibody (data not shown). However, the Golgi staining in cells expressing [Q71L]ARL1-myc appeared more diffuse than that seen with the wild type protein. The change in Golgi staining induced by [Q71L]ARL1 was subtle and was not accompanied by the progressive development of large perinuclear vesicles which often surrounded the nucleus, as seen previously for [Q71L]ARF1 (1). When viewed by electron microscopy, it was evident that cells expressing [Q71L]ARL1 contained a Golgi with an engorged lumen (Fig. 1), similar to early time points in the expression of [Q71L]ARF1 and particularly at the ends of stacks. In contrast, the Golgi in cells expressing comparable levels of wild type ARL1 were indistinguishable from those seen in either uninduced or induced control cells. No other differences were seen in these cell lines. Golgi were clearly identified by viewing serial sections and seeing the complete expanded phenotype (shown in Fig. 1) as well as partial expansion in which normal Golgi stacks were contiguous with expanded ones.

The level of expression of [Q71L]ARL1 in these lines was similar to that of [Q71L]ARF1 in the analogous lines. It should be noted that in NRK cells endogenous ARF1 is expressed to ~0.1% of total cell protein, but we cannot detect endogenous ARL1 (estimated level of expression <0.01% total cell protein). Thus, the magnitude of the changes in Golgi structure was considerably less in cells expressing [Q71L]ARL1 than in cells expressing [Q71L]ARF1 even though the fold increase in expression was greater. The similarities in phenotype between cells expressing [Q71L]ARF1 and [Q71L]ARL1 were suggestive of similarities in function of these proteins at the Golgi.

A Subset of ARF Effectors Also Binds to ARL1—We next asked whether a set of known ARF-binding proteins could also bind to any of three ARLs. Toward this end, we applied the yeast two-hybrid system (8) to test binding of LTA, Arfaptin2/ POR1, GGA1, 2, 3, and MKLP1 to each of three human ARL proteins, ARL1, ARL2, and ARL3. The results are summarized in Table II. Two proteins, Arfaptin2/POR1 and MKLP1, each interacted as well with [Q71L]ARL1 as with any ARF protein (similar activities with activated human ARF1, ARF3, ARF4, ARF5, or ARF6). Binding to ARL1 was predicted to be GTP-dependent because neither Arfaptin2/POR1 nor MKLP1 bound to wild type ARL1. None of the GGAs or GGA1 bound ARL1 or [Q71L]ARL1, although each interacted with all five activated ARFs. The wild type and activated mutants of ARL2 and ARL3 lacked binding activity to any of the ARF effectors tested. Thus, ARL1 was the only one of these three ARLs which bound to a subset of ARF binding partners in two-hybrid assays.

ARL1 and Arfaptin2/POR1 Bind Directly—Several ARF-binding proteins have recently been shown to increase the stoichiometry of GTP binding to ARFs (32). This property extends to Arfaptin2/POR1, which can increase the equilibrium binding of GTPγS to ARF3 by 3–8 fold (Fig. 2 and Ref. 32). Similar effects were found for Arfaptin2/POR1 on the binding of GTPγS to purified ARL1 (Fig. 2). The addition of Arfaptin2/POR1 led to a 4-fold increase in the amount of GTPγS bound to ARL1 at steady state. Binding reached a maximum of 0.42 mol of GTPγS bound/mol of ARL1, very similar to the amount of GTPγS bound to ARF1 under the same conditions (Fig. 2). In the absence of Arfaptin2/POR1, these preparations of ARL1 and ARF1 bound 0.08 and 0.09 mol of GTPγS/mol of GTPase, respectively. This increase in GTP binding induced by Arfaptin2/POR1 was specific because neither the BSA present in the assay nor other proteins tested (e.g. GGA1; data not shown) had any effect on nucleotide binding to ARL1. Thus, ARFs and ARL1 share the ability to bind directly and specifically to Arfaptin2/POR1.

Identification and Characterization of ARL1–3 Binding Partners—We initiated a series of yeast two-hybrid based screens to identify novel ARL-binding proteins. Human ARL1, ARL2, and ARL3 were each engineered as wild type or dominant activated mutants, fused at their carboxyl termini with the binding domain of GAL4. Human fetal brain and B cell cDNA libraries were screened with the activated mutant form of each human ARL, as described previously (27, 29) and under “Materials and Methods.” As predicted from the direct tests described above, the [Q71L]ARL1 screens led to the cloning of fragments of Arfaptin2/POR1 twice and MKLP1 once, and these cDNAs
were not pulled from either of the other ARL screens ([Q70L]ARL2 or [Q71L]ARL3).

Screens of human cDNA libraries with the activating mutants of ARL1–3 led to the identification of six binding partners that were not identified by previous screens with activated ARF proteins, and each failed to bind ARFs (wild type or activated mutants) when tested individually in two-hybrid assays. These proteins include two previously identified ARL-binding partners: the delta subunit of rod-specific cGMP phosphodiesterase 6 (PDEδ; accession number AF045999 (47)) and "binder of ARL2" (BART; accession number AF126062 (33)), as well as four newly identified ARL-binding proteins: human retinal gene 4 (HRG4; accession number U40998 (48)), Golgin-245 (accession number U31906 (49)), RanBP2a (50), and a short coiled-coil protein, termed SCOCO (accession number AF330205). These six human proteins bound the three activated ARLs with different specificities (Table II).

**PDEδ and HRG4 Bind ARLs 1, 2, and 3—**A direct and GTP-dependent binding of ARL2 and ARL3 to PDEδ has been reported previously (34). Our results extend the likely set of binders to include ARL1 (Table II). The complete open reading frame of PDEδ was cloned once from the human fetal brain library using [Q71L]ARL1 as bait and 21 times using [Q70L]ARL2. Direct tests for specificity also revealed an interaction between [Q71L]ARL3 and PDEδ (data not shown). The open reading frame of PDEδ is 450 bp and encodes a predicted 17-kDa protein. We expressed the full-length protein but found it was insoluble in bacterial extracts under a variety of growth and extraction conditions. When this protein was resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes it was able to refold at least partially into a structure that bound ARL2α[^32P]GTP or ARL3α[^32P]GTP in the gel overlay assay (data not shown). Weak signals, compared with BART in the same assays, suggest that refolding was inefficient. No binding of ARL1α[^32P]GTP was observed, but the stoichiometry of GTP binding to ARL1 is at least 4-fold below that of the other ARLs. Thus, although we were able to confirm the binding of ARL2 and ARL3 to PDEδ, we cannot yet confirm that ARL1 also binds. Although originally described as a component of a tetrameric phosphodiesterase complex with high expression in retinal cells (51), PDEδ is actually expressed in many other tissues (including brain and

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**Table II**  
**Interactions between GTPases in the ARF family and binding partners**

| Partner          | ARF1–6 WT | ARF1–6 Q71L | ARF1 Q71L | ARF1 Q70L | ARF1 Q71L | ARF2 Q70L | ARF2 Q71L | ARF3 Q70L | ARF3 Q71L |
|------------------|-----------|------------|----------|----------|----------|----------|----------|----------|----------|
| Arfaptin2/POR1   | –         | +         | –        | –        | –        | –        | –        | –        | –        |
| MKLP1            | –         | +         | –        | –        | –        | –        | –        | –        | –        |
| GGA1–3           | –         | +         | –        | –        | –        | –        | –        | –        | –        |
| LTA              | –         | +         | –        | –        | –        | –        | –        | –        | –        |
| PDEδ             | –         | –         | –        | –        | –        | –        | +        | +        | +        |
| HRG4             | –         | –         | +        | +        | +        | +        | +        | +        | +        |
| Golgin-245-GRIP  | –         | –         | –        | –        | –        | –        | –        | –        | –        |
| [Y→A]Golgin-245-GRIP | –     | –         | –        | –        | –        | –        | –        | –        | –        |
| RanBP2a          | –         | –         | –        | –        | +        | –        | –        | –        | +        |
| BART             | –         | –         | –        | –        | –        | ND       | ND       | ND       | ND       |
| SCOCO            | –         | +         | –        | –        | –        | –        | –        | –        | –        |

**FIG. 2.** Arfaptin2/POR1 binds to ARL1 and ARF1 and increases the binding of GTPγS. The binding of 10 μM [γ-[^35S]GTP to 1 μM purified recombinant ARL1-(His)₆ (squares) or 1 μM ARF1 (circles) was determined at 30 °C in the presence (closed symbols) or absence (open symbols) of 3.6 μM MBP-POR1, as described under “Materials and Methods.” Each time point contains 10 pmol of ARL1-(His)₆ or ARF1.

β-Galactosidase activities were determined using the filter lift assay in yeast carrying the designated pairs of proteins, as described under “Materials and Methods.” Activities when coexpressed with the wild type and activating mutants of each GTPase are shown. ARF1–6 are shown collectively because no differences were seen when human ARF1, ARF3, ARF4, ARF5, and ARF6 were assayed independently. Only the GRIP domain of Golgin-245 (residues 2025–2083) was included for those proteins, one of which included the point mutation Y2032A. Interactions were scored by eye as the time and intensity of blue color development at 30 °C. Activities were defined as a strong (+ +; dark blue within 15 min), intermediate (+; blue within 30 min), or weak (+; pale blue within 3 hs). Lack of any detectable blue color development within 3 h is shown as no interaction (–). Negative controls (see “Materials and Methods”) were included in every assay but are not shown. Each result was obtained multiple (≥3) times. ND, not determined.
adrenal gland (52)) and is not only found associated with the phosphodiesterase complex, implicating a more general role for the protein in cells and tissues.

The open reading frame of HRG4 is 720 bp in length, encoding a protein of 240 residues with a predicted mass of 27 kDa. Sequence homology between PDEδ and HRG4 is evident; they are 30% identical (40% homologous) in their COOH-terminal regions (residues 159–240) and 23% identical overall (53). Like PDEδ, HRG4 is expressed in photoreceptor cells (54), but in contrast to PDEδ, little or no HRG4 message has been detected in other tissues tested (54). Plasmids encoding the portion of HRG4 from residue 96–240 (HRG4[96–240]) were isolated seven times from the human B cell library screen with [Q70L]ARL2. The presence of the HRG4 message in the B cell cDNA library is evidence that expression is not limited solely to photoreceptor cells. HRG4[96–240] was later tested directly and shown to interact with both [Q71L]ARL1 and [Q71L]ARL3 but not ARFs in two-hybrid assays (Table II). HRG4 (residues 96–240) bound indistinguishably to both wild type and activated mutant forms of each ARL in two-hybrid assays (Table II) but failed to bind to any of the ARF constructs.

**BART Binds ARL2 and ARL3—** BART is a 19-kDa protein, shown previously to bind ARL2-GTP with high affinity (Kd ~ 15 nM (33)). Library screens led to the isolation of cDNAs encoding BART a total of 33 times using [Q70L]ARL2 as bait and once using [Q71L]ARL3. Neither [Q71L]ARL1, nor any ARFs, bound BART when tested directly in two-hybrid assays. The gel overlay assay was used to confirm the direct and GTP-dependent binding between BART and ARL3 (data not shown).

**Golgin-245 and RanBP2α—** Fragments of Golgin-245 were isolated 35 times from library screens using [Q71L]ARL1 as bait and once using [Q71L]ARL3. Golgins are Golgi-associated antigens in a number of autoimmune diseases. Golgin-245 is a 245-kDa protein that is predicted to contain an extensive coiled-coil domain (residues 1–2010) and is peripherally associated with the cis-Golgi (49, 55). Alignment of Golgin-245 with another golgin, Golgin-97, revealed a conserved domain, termed GRIP. GRIP domains are found at the COOH terminus of at least five proteins (Golgin-245, Golgin-97, RanBP2α, and two uncharacterized open reading frames) and are capable of directing the association of attached proteins to the Golgi apparatus (56, 57). All library inserts included the entire COOH-terminal GRIP domains, and some included very little else. Thus, the GRIP domain of Golgin-245, which includes the last 60 residues, binds [Q71L]ARL1 and [Q71L]ARL3 in two-hybrid assays (Table II). Stronger signals were observed when fragments of Golgin-245 were paired with the activated mutants of ARL1 or ARL3, compared with either wild type protein, but preference for the activated proteins was less marked than those seen with other partners (Table II).

Because ARL1, like ARFs, binds activating guanine nucleotides to only low stoichiometry in vitro, we designed an additional screen, using the NH2-terminal truncation mutation, [Δ17]ARL1 (46), coupled with the activating mutation, in efforts to maximize the amount of activated ARL1 in cells. A screen of the human fetal brain library with [Δ17,Q71L]ARL1 resulted in the cloning of each of the binding partners found with [Q71L]ARL1, but in addition we cloned a COOH-terminal fragment of RanBP2α, containing the entire GRIP domain. The GRIP domain of RanBP2α gave a strong signal when paired with [Q71L]ARL1 or [Δ17,Q71L]ARL1 but did not interact with ARL1. Thus, at least two of the five GRIP-containing proteins bind to ARL1, though with apparent differences in GTP-dependence when tested as the isolate GRIP domains.

Because rat ARL1 and murine Golgin-245 have been found independently to localize to the Golgi (21, 49, 56), we wanted to confirm the localization of the human orthologs in the same cell and test their interdependence of Golgi membrane binding. Murine and human Golgin-245 are only 66% identical overall, although the GRIP domains are 97% identical. We expressed an epitope (myc)-tagged GRIP domain of human Golgin-245 in NRK cells that had previously been stably transfected with plasmid-directing expression of ARL1-HA and determined the location of each protein by indirect immunofluorescence. Extensive overlap between the staining of ARL1 and the GRIP domain of Golgin-245 at the Golgi was apparent (Fig. 3).

One hallmark of ARF binding to Golgi membranes is its sensitivity to brefeldin A, an inhibitor of ARF GEFs. We tested the sensitivity of ARL1 to brefeldin A and found that, like ARFs, and as described previously (21), the binding of ARL1 to the Golgi is lost rapidly upon exposure to brefeldin A (see Fig. 8). This effect clearly precedes the changes in Golgi morphology and integrity which result from long term exposure to brefeldin A and the eventual fusion of Golgi and endoplasmic reticulum elements (data not shown). Although ARL1 localization to the Golgi was disrupted by treatment with brefeldin A within 3 min (see Fig. 8 and Ref. 21), the GRIP domain of Golgin-245 remained localized at the Golgi even after 5 min of brefeldin A treatment (data not shown). Thus the GRIP domain can bind to the Golgi independently of ARL1, and its association is insensitive to brefeldin A.

The ARL1-independent binding of the GRIP domain of human Golgin-245 to Golgi membranes suggested that instead of being recruited to membranes by the GTPase it may be acting as a docking site for activated ARL1. Mutation of a tyrosine residue (Y2032A) in Golgin-245, conserved in all GRIP domains, results in the loss of Golgi binding by this domain (56, 57). The relationship between binding of the GRIP domain to Golgi and ARL1 was investigated further by testing for effects of the homologous mutation in human Golgin-245 on the localization of the expressed protein in mammalian cells and on the binding of [Q71L]ARL1 in two-hybrid assays. The GRIP domain of

![Fig. 3. Human ARL1 and the GRIP domain of human Golgin-245 colocalize in mammalian cells.](http://www.jbc.org/)

NRK-HV820–9 cells were transiently transfected with plasmids containing the COOH-terminal GRIP domain of Golgin-245 controlled by the constitutive cytomelagovirus promoter. 16 h after cells were transfected and protein expression was induced with interferon, the cells were fixed and prepared for indirect immunofluorescence, as described under “Materials and Methods.” Mouse monoclonal antibody 9E10 (α-myc) and rabbit polyclonal antibody SC-605 (α-HA) were used to visualize the expressed ARL1-HA (left panel) or Golgin-245 GRIP domain (center panel). The merged images are shown on the right. The extensive overlap in staining is evident in all cells expressing both proteins.
human [Y2032A]Golgin-245 (residues 2025–2083) was expressed in NRK cells using the same method described above for the nonmutated domain. Mutation of the single tyrosine residue was sufficient to prevent the binding to Golgi membranes because the mutant protein was seen located diffusely throughout the cytosol (data not shown), consistent with previously published observations made with the murine GRIP domain (56, 57). The expression of [Y2032A]Golgin-245(2025–2083) in NRK cells had no effect on the localization of ARL1-HA (data not shown). When tested in two-hybrid assays, the mutated domain was also unable to bind [Q71L]ARL1 (Table II). Thus, it appears that this tyrosine in the GRIP domain is involved in the binding to both Golgi membranes and ARL1.

Like ARF proteins, ARL1 has no detectable intrinsic GTPase domain (56, 57). The expression of [Y2032A]Golgin-245(2025–2083) in NRK cells had no effect on the localization of ARL1-HA (data not shown). When tested in two-hybrid assays, the mutated domain was also unable to bind [Q71L]ARL1 (Table II). Thus, it appears that this tyrosine in the GRIP domain is involved in the binding to both Golgi membranes and ARL1.

**Fig. 4.** Human SCOCO is predicted to form an extended coiled-coil structure. Panel A, the protein and cDNA sequences of human SCOCO are shown, using one-letter abbreviations. Panel B, the probability for coiled-coils structures, predicted by the COILS program (59), is shown.
Enriched RNA (2 μg/ lane) was isolated from eight different human tissues, resolved by agarose-gel electrophoresis, and transferred to nylon membranes. A human SCOCO cDNA probe was labeled by random priming and hybridized, as described under "Materials and Methods." A single hybridizing band was seen in all tissues surveyed except lung. The mobility of size standards, in kilobases, is shown on the left.

![Fig. 5. SCOCO is widely expressed in human tissues. Poly(A)^+ -enriched RNA (2 μg/ lane) was isolated from eight different human tissues, resolved by agarose-gel electrophoresis, and transferred to nylon membranes. A human SCOCO cDNA probe was labeled by random priming and hybridized, as described under "Materials and Methods." A single hybridizing band was seen in all tissues surveyed except lung. The mobility of size standards, in kilobases, is shown on the left.](http://www.jbc.org/)

activity (58) and is predicted to depend upon interaction with an ARL1 GAP for hydrolysis of bound GTP. The GRIP domain of Golgin-245 was assayed and found to lack any ARL1 GAP activity. The addition of a 10-fold molar excess of the purified Golgin-245 GRIP domain did not alter the rate of GTP hydrolysis by ARL1 when assayed under the same conditions in which ARF GAP activity can be readily detected (“Materials and Methods” and data not shown). This same assay has been used recently to detect and purify an ARL2 GAP activity.3

SCOCO—The full-length human homolog of SCOCO (mouse accession number NP062682) was identified from the yeast two-hybrid B cell library as a specific binder of [Q71L]ARL1. The entire human SCOCO protein is 82 amino acids in length, 100% identical at the amino acid level to the mouse probe and predicted by the COILS program to fold predominantly into a coiled-coil structure (Fig. 4). Northern blot analyses of human tissues with a cDNA probe from human SCOCO revealed a single ~2.2-kilobase message, present in all tissues tested, except perhaps lung (Fig. 5). SCOCO mRNA was most abundant in brain, heart, and skeletal muscle.

Homology searches of sequence data bases revealed only three proteins with high homology to SCOCO, one in humans and two in yeast (S. cerevisiae). Human Golgin-95, yeast IMH1, and yeast VPS30 have 26%/51%, 22%/58%, and 30%/64% identity/homology over a stretch of ≥52 residues in the middle of each protein. Interestingly, all three have predicted roles in vesicle traffic at the Golgi; one is another Golgin (Golgin-95), and one also contains a GRIP domain at the COOH terminus (IMH1) (56, 57, 60–63).

Human SCOCO was expressed as a soluble protein in bacteria and purified to >95% homogeneity, taking advantage of a hexahistidine sequence added to the COOH terminus. Recombinant human SCOCO migrated in SDS-polyacrylamide gels with the mobility expected of a 9-kDa protein, but its mobility in nondenaturing gel filtration medium was faster than predicted, more consistent with that of a protein of ~54 kDa (data not shown). This observation is consistent with the formation of an extended coiled-coil with one longer axis.

Binding of guanine nucleotides to purified ARL1 was similar to ARFs, with low stoichiometry that is partially relieved by added lipids or detergents. To assist in the biochemical characterization of ARL1 and its binding partners, we expressed an NH2-terminal truncation mutant of ARL1, [Δ17]ARL1-(His)6, comparable to the truncation of ARF1 described previously (46). [Δ17]ARL1-(His)6 bound guanine nucleotides to higher stoichiometry than the full-length protein and did so independently of added lipids or detergents (data not shown). The nucleotide binding site of [Δ17]ARL1-(His)6 was filled with either GDP or GTP•γS by prior incubation in saturating concentrations of the nucleotide and then incubated with SCOCO or BART (serving as a negative control) that had been covalently attached to Affi-Gel beads, as described under “Materials and Methods.” The beads were collected by centrifugation, and the amount of ARL1 retained on the beads after washing was determined by immunoblot analysis. [Δ17]ARL1-GDP and [Δ17]ARL1-GTP•γS were specifically retained on the SCOCO affinity column, with approximately twice as much [Δ17]ARL1 bound to the column in the presence of the activating nucleotide, GTP•γS, as in its absence (Fig. 6). No binding to BART was detected under these same conditions (data not shown). Preferential binding to the activated conformation of a regulatory GTPase has been observed previously with effectors and GAPs (25, 26, 29, 64). Purified SCOCO had no detectable ARL1 GAP activity, under the same conditions used for ARF GAPs (see “Materials and Methods”).

The cellular location of SCOCO and its relationship to ARL1 were next analyzed in cultured mammalian cells. Rabbit polyclonal antibodies were raised against human SCOCO-(His)6 and affinity purified, as described under “Materials and Methods.” These antibodies were sufficiently sensitive and specific (see “Materials and Methods”) to allow detection of endogenous SCOCO in NRK cells. A punctate, perinuclear staining pattern, very similar to that seen for markers of the Golgi, was observed (Fig. 7), along with punctate staining in cytosol, and some staining at the plasma membrane. Double labeling with SCOCO and either β-COP or ARF antibodies revealed extensive overlap in staining at the Golgi membranes (data not shown). Because our ARL1-specific antisera are not sufficiently sensitive to detect endogenous ARL1 by indirect immunofluorescence, we used stably transfected NRK cells expressing inducible human ARL1-HA to assess the extent of colocalization of ARL1 and SCOCO. Confocal microscopy of the induced cells revealed extensive overlap in staining of ARL1-HA and SCOCO (Fig. 7) at the Golgi.

Because the activating mutations of ARF and ARLs block the actions of GAPs and lead to increased abundance of the GTP-bound proteins in cells, these mutations confer a limited degree of resistance to brefeldin A, as described in Zhang et al. (1) for [Q71L]ARF1. The ability of [Q71L]ARL1 to provide such resistance to the actions of brefeldin A on itself or SCOCO were examined in NRK cells expressing comparable levels of ARL1 or the activated mutant. As a control, comparisons were also made between these cells and those expressing wild type or [Q71L]ARF1 (1). The binding of endogenous or overexpressed ARF1 and its effector, COP-1, were clearly diminished within 1 min of exposure to 10 μM brefeldin A and were essentially absent by 3 min (Ref. 1 and data not shown). Expression of [Q71L]ARF1 delayed this response such that it took 5 min or longer for complete dissociation of the signal from Golgi membranes (1). We observed a very similar level of resistance to brefeldin A provided to SCOCO by expression of [Q71L]ARL1 (Fig. 8). Both [Q71L]ARL1 and SCOCO were still present on Golgi membranes after 3 min of brefeldin A treatment (Fig. 8). The activating mutation only delayed the release of the GTPase and binding partner from the Golgi, it did not prevent it. By 5 min of exposure to the drug, both [Q71L]ARL1 and SCOCO appeared fully dissociated from Golgi membranes (Fig. 8). The expansion and vesiculation of the Golgi which resulted from expression of [Q71L]ARF1 also

3 J. D. Sharer, J. Shern, and R. A. Kahn, unpublished observation.
resulted in a dilution of the staining of SCOCO which makes comparisons technically more demanding. However, we saw no indication of protection of SCOCO localization to Golgi membranes when such cells were treated with brefeldin A (data not shown).

ARF GEFs each contain a common structural motif, termed the SEC7 domain, which binds to both ARFs and brefeldin A (for review, see Ref. 65). We assayed for ARL1 GEF activity of the brefeldin A-sensitive SEC7 domain of the SEC7 protein, using ARL1 and ARF3 as substrates in the absence or presence of the SEC7 domain. As seen in Fig. 9, the SEC7 domain increased the binding of GTPγS to ARF3 in a linear fashion over time but had no effect on the binding of GTPγS to ARL1 (also see Ref. 66).

FIG. 6. [Δ17]ARL1-(His)₆ binds directly to SCOCO in a GTP-dependent manner. Purified recombinant human SCOCO was covalently attached to Affi-Gel 15 beads, as described under “Materials and Methods.” These beads were then incubated with [Δ17]ARL1-(His)₆, that had been equilibrated previously with either GDP or GTPγS for 15 min at room temperature. Beads were washed twice with binding buffer containing 20 μM appropriate nucleotide, and proteins were then eluted by boiling in an equivalent amount of 2 × Laemml sample buffer. Proteins were resolved on a 15% polyacrylamide gel, and the presence of [Δ17]ARL1-(His)₆ was detected using polyclonal ARL1 antiserum (R85722-3). Equivalent volumes of [Δ17]ARL1-(His)₆ that was loaded onto the Affi-Gel 15-SCOCO beads (L), the supernatant from the second wash (W2), and the eluted protein from the beads (E) are shown.

FIG. 7. ARL1-HA and endogenous SCOCO colocalize in mammalian cells. NRK-HV820–9 cells were treated with interferon to induce expression of ARL1-HA for 16 h prior to fixing and processing for indirect immunofluorescence using confocal microscopy. Affinity-purified rabbit SCOCO (left panel) and monoclonal 12CA5 (α-HA; center panel) antibodies were used to label the SCOCO and ARL1, respectively. Each panel represents a flattened stack of 18 images taken in 5-μm steps. The SCOCO and ARL1-HA images were merged using Image-Pro Plus software, and the overlap appears as yellow in the panel on the right.

FIG. 8. Binding of SCOCO to Golgi is made partially resistant to brefeldin A by expression of [Q71L]ARL1. NRK-HV820–9 (ARL1-HA) and NRK-HV810–13 ([Q71L]ARL1-HA) cells were treated with 10 μM brefeldin A for 0, 1, 3, or 5 min. Cells were then fixed in 3.7% formaldehyde and prepared for indirect immunofluorescence, as described under “Materials and Methods.” Cells were labeled with monoclonal HA and affinity-purified SCOCO antibodies and visualized using confocal microscopy. This figure represents a flattened stack of 24 images taken in 5-μm steps. The retention of perinuclear staining of ARL1 and SCOCO is evident at 3 min after brefeldin A addition, only in NRK-HV810–13 cells.
revealed a number of functional similarities between human ARL1 and ARF1–6. This apparent overlap in functions with ARFs did not extend to either human ARL2 or ARL3. Similarities included changes in Golgi morphology with expression of the dominant activated mutant, brefeldin A-sensitive localization to the Golgi, and a subset of shared binding partners. The extent to which different ARF or ARL proteins shared common effectors was investigated further, using two-hybrid technology, to reveal both specificity and extensive overlap between binders of ARL1, ARL2, and ARL3, but only ARL1 was found to share binding partners with ARFs. Thus, the functional overlap between ARF and ARLs may be limited to ARL1. Two-hybrid screens of human cDNA libraries identified five novel and specific binding partners of ARL1, none of which bound ARFs. The presence of GRIP domains at the COOH termini of two of these reveals this domain to be an ARL binding structure. The distinct specificities in binding proteins for ARFs and each ARL suggest that signaling by members of the ARL family involves complex networks of multiple overlapping effectors.

Expression of [Q71L]ARL1 led to an engorgement of the Golgi apparatus, most evident at the ends of stacks. This expansion was similar in appearance to that seen with [Q71L]ARF1 (1) but was less dramatic. We saw no evidence of expansion of the endoplasmic reticulum lumen. The effect on Golgi morphology was specific to the activating mutant, [Q71L]ARL1 (1) but was less dramatic. We saw no evidence of expansion was lost at lower levels of expression, resulting from the actions of brefeldin A (Fig. 8 and Ref. 1). One of three likely explanations may account for these observations: 1) there exists at least one ARL1-specific GEF that is brefeldin A-sensitive; 2) one or more of the brefeldin A-sensitive ARF GEFs can also bind and activate ARL1; or 3) the binding of ARL1 to Golgi membranes is dependent on the activation of one or more ARFs. The finding that at least one SEC7 domain, active as a brefeldin A-sensitive GEF for human ARFs, is inactive when human ARL1 is the substrate, tempers support for explanation 2 above (Fig. 9 and Ref. 66) but does not exclude it. The similarities in the time course of responses of ARF1 and its effector (COP-I) and ARL1 and its binding partner (SCOCO) to brefeldin A similarly run counter to explanation 3 (Fig. 8 and Ref. 1). We therefore favor the possibility that there exist one or more brefeldin A-sensitive ARL1 GEF, although more definitive tests of these three models are required. The identification of such an ARL1 GEF would provide further evidence that at least some of the brefeldin A-sensitive activities in cells, currently ascribed to ARFs, may actually result from the actions of ARL1.

Ultimately, the functional description of the actions of a regulatory GTPase will be determined by the molecules with which it specifically interacts. Current descriptions of specific GTPases, e.g. Ras, can include as many as a dozen distinct downstream binding partners (for review, see Ref. 68). The identity and specificity of these regulated protein interactions will define the signaling pathways that may be activated in response to the activation of each GTPase. Toward this end we and others have identified more than 10 direct, GTP-dependent, ARF-binding partners (10, 11, 14, 25–31, 64, 69–71). Biochemical and genetic evidence indicate a lack of functional overlap between ARF and ARL actions. It was surprising, therefore, to discover that two-hybrid assays revealed ARL1 interacting with two ARF-binding proteins, Arfaptin2/POR1 and MKLP1, in a specific and GTP-dependent fashion. The presence of an extensive coiled-coil domain revealed a number of functional similarities between human ARL1 and ARF1–6. This apparent overlap in functions with ARFs did not extend to either human ARL2 or ARL3. Similarities included changes in Golgi morphology with expression of the dominant activated mutant, brefeldin A-sensitive localization to the Golgi, and a subset of shared binding partners. The extent to which different ARF or ARL proteins shared common effectors was investigated further, using two-hybrid technology, to reveal both specificity and extensive overlap between binders of ARL1, ARL2, and ARL3, but only ARL1 was found to share binding partners with ARFs. Thus, the functional overlap between ARF and ARLs may be limited to ARL1. Two-hybrid screens of human cDNA libraries identified five novel and specific binding partners of ARL1, none of which bound ARFs. The presence of GRIP domains at the COOH termini of two of these reveals this domain to be an ARL binding structure. The distinct specificities in binding proteins for ARFs and each ARL suggest that signaling by members of the ARL family involves complex networks of multiple overlapping effectors.

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Similarities between ARL1 and ARF extended to the ability of the activated form of each protein to become partially resistant to the actions of brefeldin A (Fig. 8 and Ref. 1). One of three likely explanations may account for these observations: 1) there exists at least one ARL1-specific GEF that is brefeldin A-sensitive; 2) one or more of the brefeldin A-sensitive ARF GEFs can also bind and activate ARL1; or 3) the binding of ARL1 to Golgi membranes is dependent on the activation of one or more ARFs. The finding that at least one SEC7 domain, active as a brefeldin A-sensitive GEF for human ARFs, is inactive when human ARL1 is the substrate, tempers support for explanation 2 above (Fig. 9 and Ref. 66) but does not exclude it. The similarities in the time course of responses of ARF1 and its effector (COP-I) and ARL1 and its binding partner (SCOCO) to brefeldin A similarly run counter to explanation 3 (Fig. 8 and Ref. 1). We therefore favor the possibility that there exist one or more brefeldin A-sensitive ARL1 GEF, although more definitive tests of these three models are required. The identification of such an ARL1 GEF would provide further evidence that at least some of the brefeldin A-sensitive activities in cells, currently ascribed to ARFs, may actually result from the actions of ARL1.

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* J. D. Sharer and R. A. Kahn, unpublished observations.

* J. D. Sharer, J. F. Shern, H. Van Valkenburgh, D. C. Wallace, and R. A. Kahn, submitted for publication.
Fig. 10. Summary of ARF and ARL binding partners. The interactions detected by two-hybrid technologies between ARF and ARL binding partners are depicted. The upper panel marked SHARED indicates those binding partners that bind to more than one GTPase (ARF1–6 and ARL1–3 are shown collectively because no partner has yet been described which does not bind them all). The lower panel marked SPECIFIC indicates the binding partners that appear to interact with only one GTPase. The question mark following ARFaptin1 and ARFophilin indicates that they have not been tested for interaction with ARLs 1–3. Not shown in this figure are the five or more proteins (e.g. COP-I, AP-1, AP-3, PLD1, phosphatidylinositol 4-phosphate, 5-kinase) that bind directly to ARFs but do not work in two-hybrid assays because of their oligomeric nature or tight membrane binding.

Interactions of Arf and Arl Binders

| SHARED | SPECIFIC |
|--------|----------|
| MKLP1  | LTA      |
| POR1   | SCOCO    |
| Golgin-245 | ARFaptin1? |
| HRG4   | ARFophilin? |
| PDEδ   |          |
| BART   |          |

Arnl-6 Arl1 Arl2 Arl3

In MKLP1 likely contributes to its inability to be expressed as a recombinant protein, precluding biochemical analyses. We focused instead on Arfaptin2/POR1 and have confirmed its direct binding to ARL1, using the fact that GTPlS binding stoichiometry is sensitive to the addition of binding proteins, first established for ARFs (32). Indeed, Arfaptin2/POR1 increased the stoichiometry of GTPlS binding to ARL1 and ARF1 with the same sensitivity, to the same extent, and with the same kinetics (Fig. 2). With its previous identification as a partner of RAC, Arfaptin2/POR1 may now be viewed as an ARF/RAC/ARL1-binding protein.

Screens of three different human cDNA libraries (B cell, kidney, and fetal brain) led to the isolation of seven different cDNAs, encoding proteins predicted to bind ARL1. Two of these were Arfaptin2/POR1 and MKLP1. Cross-reactivity between ARF and ARL1 partners prompted tests for specificity between these novel partners with both ARFs and ARL1–3 (Fig. 10). PDEδ was described previously as binding ARL2 and ARL3 (34). Results from two-hybrid assays confirmed those results and extended the binding specificity to include ARL1 but not ARFs.

Golgins are a group of autoimmune antigens that share a localization at the Golgi, and though they lack defined primary sequence homology they typically contain predicted coiled-coil domains (for review, see Ref. 55). Two of the Golgins, Golgin-97 and Golgin-245, share a sequence motif, termed the GRIP domain, which is also found in three other proteins, RanBP2α and two proteins predicted from ESTs sequences (57). GRIP domains are always found at the COOH terminus, are 50–60 residues in length, and are autonomously folding and acting domains that are sufficient to direct localization of fusion proteins to Golgi membranes (22, 57). The GRIP domain of Golgin-245 was pulled from screens of human B cell and fetal brain cDNA libraries using the activated mutant of ARL1 as bait. Tests of specificity in two-hybrid assays indicated that this GRIP domain also binds to activated ARL3 but not ARL2 or any of the ARFs. Although a preference for the [Q71L]ARL1 mutant was observed, it was less dramatic than was found for ARFs with their partners (27, 29; Table II). A related screen of the brain library also led to the cloning of a fragment of RanBP2α, including the GRIP domain, as a binder of ARL1 which is highly dependent on the activation state of the GTPlase. Thus, we independently cloned two of the three known GRIP-containing proteins using activated ARL1 as bait and conclude that GRIP domains are involved in both binding to ARL1 and Golgi membranes. It is possible that the use of full-length Golgin-245 or RanBP2α would yield different results with regard to the activation dependence on ARL1 binding or Golgi binding, but the previous demonstration of GRIP domains as autonomously folding and acting domains (22, 57) makes it likely that these activities are retained and biologically relevant in the full-length proteins. Indeed, one theme that has been repeated several times (MKLP1, GGA1–3, Golgin-245, SCOCO) is the binding of ARF family members to domains close to or overlapping predicted coiled-coils. The presumed binding of RanBP2α to the nuclear pore may suggest that the GRIP domain and ARL1 binding may have functions independent of Golgi membranes. Because no full-length sequence for human Golgin-245 is available and the GRIP domain had already been shown to bind Golgi membranes and ARL1, we focused on the relationship between these two activities for the GRIP domain of Golgin-245.

Tests of the interdependence between binding of Golgin-245 and ARL1 to Golgi membranes were initiated. Expression of the GRIP domain of Golgin-245 in NRK cells revealed colocalization with ARL1 when viewed by confocal microscopy. A tyrosine residue that is conserved in all GRIP domains and is required for Golgi binding (22, 57) was mutated in the GRIP domain of human Golgin-245 and found to be required for both Golgi and ARL1 binding. This overlap in ARL1 and Golgi binding domains suggests that the binding of one protein to the Golgi is likely to require or be promoted by the other. The insensitivity of the GRIP domain to short term treatments with brefeldin A suggested that it may represent a docking site for ARL1 to bind Golgi, rather than the alternative. The large size of both Golgin-245 and RanBP2α (358 kDa) makes them candidate scaffolding proteins, and the identification of an ARL1 binding site at their COOH termini likely represents a site of protein recruitment and assembly, whether on nuclear pores or Golgi membranes.

Screens of several human cDNA libraries resulted in the isolation of the ARL1-specific binding partner, SCOCO. The name was based on the prediction that >75% of the 82 residues in the full-length protein exist in a coiled-coil (Fig. 4). A small subset of coiled-coil-containing proteins have short (50–80 residues) regions of high homology to SCOCO which may serve as Golgi or ARL1 binding domains. Affinity-purified SCOCO antibodies were used to localize endogenous SCOCO to the Golgi and plasma membrane in NRK cells (Figs. 7 and 8). There was extensive overlap in the staining of ARL1 and SCOCO at the Golgi, and each was sensitive to brefeldin A. Sensitivity of a...
GoLgi membrane protein to brefeldin A has previously been taken as evidence for a role for ARF proteins because the drug binds directly to ARF GEFs (65, 67). However, the brefeldin A-induced dissociation of SCOCO from GoLgi was slowed by the presence of the activated ARL1 protein (see Fig. 8) but not by activated ARF1. The simplest interpretation of these data is that there exists a brefeldin A-sensitive ARF1 GEF whose inhibition is sufficient to cause the rapid release of both ARF1 and ARL1-dependent binding proteins from the GoLgi. Whether such an ARF1 GEF represents a novel protein or is an ARF GEF with substrate specificity that includes ARL1 is currently under investigation. Thus, earlier conclusions that ARFs and ARLs lack functional overlap must now be altered to include ARL1 as a GTPase with the potential to signal through interactions with ARF GEFs and effectors shared by one or more ARFs. Similarly evidence of brefeldin A sensitivity must now be interpreted as evidence for ARF or ARL1 involvement.

Overlap in binding partners is essentially complete among the ARF proteins, which share >60% sequence identity, and is nearly absent between ARFs and ARLs, with the notable exception of ARL1. Given that the sequence relatedness between ARFs and ARLs is the same as between any two ARFs, we also predicted very limited, if any, overlap in binding partners between ARLs. However, we found instead a more extensive set of shared partners than specific ones between ARL1, ARL2, and ARL3. These interactions, defined originally by two-hybrid assays, are summarized in Fig. 10. This evidence for greater potential overlap in signaling between members of the ARF family indicates that more extensive tests for specificity should be performed in future characterizations of effectors, GEFs, and GAPs for members of the ARF/ARL family.

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ADP-ribosylation factors (ARFs) and ARF-like 1 (ARL1) Have Both Specific and Shared Effectors: CHARACTERIZING ARL1-BINDING PROTEINS
Hillary Van Valkenburgh, Jack F. Shern, J. Daniel Sharer, Xinjun Zhu and Richard A. Kahn

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