ADP-ribosylation factors (ARFs)\(^*\) are a family of small monomeric GTPases. ARFs 1 and 3 function in the recruitment of coat proteins to membranes of the Golgi apparatus, whereas ARF6 is localized to the plasma membrane, where it appears to modulate both the assembly of the actin cytoskeleton and endocytosis. Like other GTPases, ARF activation is facilitated by specific guanine nucleotide exchange factors (GEFs). ARNO (ARF nucleotide-binding site opener) is a member of a growing family of ARF-GEFs that share a common, tryparte structure consisting of an N-terminal coiled-coil domain, a central domain with homology to the yeast protein Sec7p, and a C-terminal pleckstrin homology domain. Recently, ARNO and its close homologue cytohesin-1 were found to catalyze in vitro nucleotide exchange on ARF1 and ARF3, respectively, raising the possibility that these GEFs function in the Golgi. However, the actual function of these proteins may be determined in part by their ability to interact with specific ARFs and in part by their subcellular localization. We report here that in vitro ARNO can stimulate nucleotide exchange on both ARF1 and ARF6. Furthermore, based on subcellular fractionation and immunolocalization experiments, we find that ARNO is localized to the plasma membrane in mammalian cells rather than the Golgi. It is therefore likely that ARNO functions in plasma membrane events by modulating the activity of ARF6 in vivo. These findings are consistent with the previous observation that cytohesin-1 regulates the adhesiveness of αLβ2 integrins at the plasma membrane of lymphocytes.

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\* The abbreviations used are: ARF, ADP-ribosylation factor; GEF, guanine nucleotide exchange factor; PH, pleckstrin homology; GST, glutathione S-transferase; GTPase, guanine 5′-y-thio-triphosphate; PtdIns4,5-P2, phosphatidylinositol 4,5-bisphosphate; PtdIns3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; BFA, brefeldin A; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; BHK, baby hamster kidney.

\* S. Frank, unpublished data.
and the reported effects of cytohesin-1 on cell adhesion, we hypothesized that the ARNO/cytohesin-1 family of proteins would function in vivo in the activation of ARF6 rather than ARF1. However, neither the subcellular localization nor the interaction of these proteins with ARF6 has been analyzed. In this report, we demonstrate that ARNO does not associate with the Golgi apparatus but localizes instead to the plasma membrane. Moreover, we show that the distribution of ARNO overlaps with that of ARF6 in vivo and that ARNO catalyzes GTP loading of ARF6 in vitro. These data are consistent with a role for ARNO in the BFA-resistant activation of ARF6 at the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**cDNA Cloning of ARNO**

Poly(A)+ RNA isolated from CaCo-2 cells was reverse transcribed, and degenerate oligonucleotide mixtures corresponding to regions of identity among previously reported Sec7 domains were then used to amplify sequences encoding this domain. The other encoding a novel sequence. This second product was used as a probe to screen a human fetal brain cDNA library (Uni-ZAP XR, Stratagene, La Jolla, CA). Five positive clones were isolated, the longest of which encoded a protein with 83% sequence identity to cytohesin-1. This sequence was submitted to GenBank under the name cytohe-2 (accession number U70728) and was subsequently found to be identical to that reported for ARNO (13).

**Antibodies and Reagents**

Rabbit polyclonal anti-ARNO antisera were produced in New Zealand White rabbits by the Pocomo Rabbit Farm and Laboratory (Canadensis, PA). Antibodies were affinity purified by passage over a GST-ARNO affinity column.

Polyclonal anti-ARF6 antibody was kindly provided by Victor Hsu (Brigham and Women’s Hospital, Boston, MA), and anti-ARF1 antibody was provided by Paul Melancôn (University of Alberta). The monoclonal antibody 9E10 was used to detect myc-tagged ARNO in all immunofluorescence experiments. Bacteria expressing GST-cytohesin-1 were obtained from Stephen Deitz and Alex Franzusoff (University of Colorado, Denver, CO). Recombinant, myristoylated ARFs were provided by Jennifer Liang and Stuart Kornfeld (Washington University, St. Louis, MO), and recombinant ras, rhoA, and rac were provided by Jeff Settleman (Massachusetts General Hospital Cancer Center).

**Cell Culture and Transient Transfection**

BHK-21, NRK, CaCo-2, and HeP-2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. HeLa cells were grown in the same medium supplemented with 2 mM l-glutamine.

For transient transfection, BHK cells in 6-well plates (1—2 × 10⁶ cells/well) were transfected with 2 µg of plasmid using Lipofectase reagent (Life Technologies, Inc.). HeLa cells were transfected using the calcium phosphate method.

**Subcellular Fractionation and Marker Enzyme Analysis**

**Analysis of Cytosol and Total Membranes**—Separation of cells into membrane and cytosolic fractions was performed essentially as described by Hansen and Casanova (17), and ARNO was immunoprecipitated from detergent-solubilized fractions using anti-ARNO antisera covalently coupled to protein A-Sepharose. Samples were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and probed with affinity purified anti-ARNO antibody.

**Analysis of Linear Density Gradients**—Density gradient centrifugation of BHK cell membranes was performed essentially as described (18). Briefly, cells from 10 subconfluent 100-mm plastic dishes were homogenized, and the membrane fraction was layered on top of continuous iodixanol density gradients (2.5—25% w/v OptiPrep) (Life Technologies, Inc.). Gradients (35 ml) were centrifuged for 3 h at a max 130,000 × g (27K in SW28 rotor) and fractionated into 2-ml samples, starting from the top. For Western blotting, gradient fractions were first concentrated by trichloroacetic acid precipitation, with 25 µg/ml bovine serum albumin added as carrier. Samples were resolved by 10% SDS-PAGE and immunoblotted using ARNO antisera (1:500), rabbit anti-Na+,K+-ATPase (1:1000), gift of Dr. Guido Guidotti, Harvard Uni-

**FIG. 1. Identification and subcellular distribution of endogenous ARNO. a, characterization of ARNO antibody. 50 ng of either GST-ARNO or GST-cytohesin-1 was immunoblotted with affinity purified ARNO antibody, demonstrating specificity of the antibody for ARNO. b, subcellular distribution of endogenous ARNO. Three confluent 10-cm plates of each cell line were separated into membrane (M) and cytosol (C) fractions as described under “Experimental Procedures.” ARNO was concentrated from each fraction by immunoprecipitation with ARNO antiserum, and the resulting immune complexes were analyzed by immunoblotting using affinity purified antibody. ARNO migrates as a 47-kDa band just below IgG heavy chain. 25 ng of recombinant His6-ARNO was run in an adjacent lane (R) for comparison.

**Indirect Immunofluorescence**

30—36 h after transfection, cells on glass cover slips were fixed with 2% paraformaldehyde, permeabilized with cold methanol, and preincubated in blocking buffer (phosphate-buffered saline/10% normal goat serum/0.2% saponin). Cover slips were then incubated with primary antibody diluted in blocking buffer for 30 min, followed by the appropriate secondary antibody for 30 min.

**GEF Assays**

Nucleotide exchange assays were performed essentially as described by Chardin et al. (13). Briefly, recombinant, myristoylated ARF6 was diluted to a final concentration of 1 µM into reaction buffer containing 50 mM Hepes, pH 7.5, 1 mM MgCl₂, 100 mM KC1, 1 mM dithiothreitol, 4 µM [35S]GTPyS (∼3 × 10⁶ cpm), and 1.5 mg/ml azolectin vesicles as a source of lipid. Reactions were initiated by addition of 50 mM His₆-ARNO and continued for 3 min at 37°C. Reactions containing ARF6 (20% myristoylated) and ARF6 (50% myristoylated) were normalized to contain 1 µM myristoylated ARF6. Control experiments with nonmyristoylated ARFs demonstrated that the nonmyristoylated fraction bound negligible amounts of [35S]GTPyS over the course of the assay.

**RESULTS**

**Preparation and Characterization of Anti-ARNO Antisera**—To facilitate the analysis of endogenous ARNO in mammalian cells, polyclonal antiserum were raised in rabbits. His₆-tagged recombinant human ARNO was used as antigen, and antiserum were subsequently affinity purified by adsorption to a GST-ARNO fusion. The specificity of affinity purified antibody was tested by Western blot analysis using GST-ARNO and GST-cytohesin-1. Surprisingly, although ARNO and cytohe-1 share 83% sequence identity, affinity purified anti-ARNO antibody recognized GST-ARNO but not react with GST-cytohesin-1 (Fig. 1a). The same antibody detected a single polypeptide species of 47 kDa in a total lysate of the human cell line HeP2 (not shown), precisely the size predicted by the ARNO coding sequence. The lack of cross-reactivity with cytohe-1 indicates that this band represents the product of the ARNO gene. It should be noted that the level of endogenous ARNO expression is very low in all cell lines tested.

**ARNO Exists in Both Cytoplasmic and Membrane-bound Pools**—To determine the subcellular distribution of endogenous ARNO, homogenates of several cell lines (HeP2, BHK-21, HeLa, NRK, and CaCo-2) were separated into membrane and...
cytosolic fractions by centrifugation at 100,000 × g. Each sample was then immunoprecipitated with anti-ARNO antiserum to concentrate the antigen, resolved by SDS-PAGE, and immunoblotted using the antibodies indicated. Na⁺,K⁺-ATPase was used as a marker of the plasma membrane, and EEA1 was used as a marker of early endosomes.

Endogenous ARNO Does Not Cofractionate with Golgi Membranes by Density Gradient Centrifugation—To further define the subcellular distribution of endogenous ARNO, a postnuclear membrane pellet from BHK-21 cells was resolved by centrifugation through continuous Iodixanol gradients (2.5–25% OptiPrep®). Gradients were fractionated from the top to bottom and assayed by either marker enzyme analysis or Western blot. a, distribution of enzymatic markers. Mannosidase II activity (△) was measured as a marker for Golgi membranes, and glucosidase II activity (•) was measured as a marker for the endoplasmic reticulum. b, immunoblotting of membrane fractions. Aliquots of each fraction were trichloroacetic acid precipitated, resolved by SDS-PAGE, and immunoblotted using the antibodies indicated. Na⁺,K⁺-ATPase was used as a marker of the plasma membrane, and EEA1 was used as a marker of early endosomes.

membrane-derived vesicles, whereas ARNO is less restricted in its distribution. Clearly, however, ARNO does not associate with Golgi membranes, as would be expected for an ARF1-GEF. Neither does it appear to associate with early endosomal membranes (defined by EEA-1 staining), which have also been reported to contain coatomer components (19). These data combine to suggest that ARNO may actually function at the plasma membrane as an exchange factor for ARF6 rather than ARF1.

Localization of ARNO in Transfected Cells—To confirm the association of ARNO with the plasma membrane, indirect immunofluorescence microscopy was used to localize the protein in intact cells. However, initial attempts to detect the endogenous protein in a variety of cell lines were unsuccessful due to the low level of expression. Therefore ARNO was transiently expressed in either HeLa or HeLa cells using the pCIB vector, which utilizes a cytomegalovirus promoter (20). To facilitate double-labeling studies, this construct was modified with a c-myc epitope tag at the N terminus. In agreement with the fractionation studies described above, immunostaining of cells expressing c-myc-tagged ARNO revealed a primarily cytosolic distribution (Fig. 3e, HeLa cells, and Fig. 3f, BHK cells). However, membrane staining was clearly observed at the extreme periphery of most cells, particularly in lamellar extensions and protrusive structures (arrowheads). No concentration of ARNO was observed in the perinuclear region, suggesting that even when overexpressed little of the protein associates with Golgi membranes in intact cells.

To confirm that the observed distribution of ARNO was similar to that of ARF6, BHK cells were transfected with cDNAs encoding either ARF1 (Fig. 3c) or ARF6 (Fig. 3d). As described by others (2, 3, 5), ARF6 is distributed diffusely on the plasma membrane and is readily detectable at the cell periphery. In contrast, ARF1 is detected almost exclusively in a perinuclear pattern typical of the Golgi with no detectable expression on the plasma membrane. Cells cotransfected with plasmids encoding both ARNO and ARF6 (Fig. 3, e and f)
These authors also localized the catalytic activity of the protein to the Sec7 domain and demonstrated that the C-terminal PH domain positively regulates nucleotide exchange in response to binding of inositol polyphosphates. However, no other ARFs were tested as potential substrates, and the subcellular localization of ARNO was not examined.

In this study we provide several lines of evidence supporting a role for ARNO as a GEF for ARF6 rather than ARF1 in vivo. First, endogenous ARNO does not cofractionate with Golgi markers by density gradient centrifugation but instead associates with light membrane fractions enriched in plasma membrane. Second, ARNO is readily observed at plasma membrane sites by immunofluorescence microscopy but does not appear to concentrate in the Golgi region of transfected cells. Moreover, ARNO and ARF6 appear to colocalize in doubly transfected BHK cells. Third, we found that ARNO was capable of catalyzing nucleotide exchange on ARF6 in the presence of acidic phospholipids. In contrast, ARNO did not detectably enhance nucleotide exchange on ras, rac, or rhoA (data not shown). Combined with previous findings that the ARF-related GTPase ARL3 is also not a substrate (13), these data indicate that ARNO is highly specific for members of the ARF family. We have subsequently found that the ARNO homologue, cytohesin-1, shows in vitro substrate specificity that is identical to that of ARNO (not shown).

Several other ARF-GEFs have recently been identified, each of which contains a Sec7 domain. In yeast, genetic screens have identified two large (160 kDa), closely related proteins named Gea1p and Gea2p (10), which were shown to catalyze nucleotide exchange on ARF1 in vitro. Importantly, mutant yeast strains exhibit temperature-sensitive defects in ER to Golgi transport, indicating that Gea1p functions in the Golgi in vivo. Moreover, the catalytic activity of Gea1p is sensitive to BFA, suggesting that it may be a yeast homologue of a mammalian BFA-sensitive, Golgi-associated ARF-GEF. Such a protein has recently been identified and shares significant sequence homology with both Gea1p and Gea2p. Our yeast Sec7p is also necessary for ER to Golgi and intra-Golgi transport and has been found to exhibit genetic interactions with ARF (22–25). Although the catalytic activity of yeast Sec7p has yet to be tested directly, a putative 200-kDa bovine Sec7p homologue has been isolated and partially characterized (9). This protein is BFA-sensitive and can apparently utilize ARF1 and ARF3 but not ARF5 as substrates for nucleotide exchange. Except for the ~200-amino acid Sec7 domain, Sec7p (which is 230 kDa) shares little additional homology with Gea1p or Gea2p. In addition, Gea1p expression is unable to suppress the sec7 growth defect, nor can sec7 rescue gea1 mutant growth (10). Therefore, despite the fact that these proteins share a common ARF-GEF catalytic domain, they have distinct and nonoverlapping functions in vivo that are presumably dictated by sequences outside the Sec7 domain.

In contrast to these large Golgi GEFs, our data indicate that the ARNO/cytohesin-1 family is likely to function in the activation of ARF6 rather than ARF1. The precise function of ARF6 at the plasma membrane is as yet unclear; however, pronounced effects of ARF6 activation on the cortical actin cytoskeleton have been observed (5, 26). Overexpression of constitutively active ARF6 (ARF6(Q67L)) results in rounding of the cells and the formation of deep invaginations of the plasma membrane (2). In addition, Donaldson and colleagues have shown that acute treatment of ARF6-expressing cells with aluminum fluoride (which is thought to inhibit the activity of one or more ARF-GTPase-activating proteins) leads to the assem-

**Discussion**

ARNO has been shown by Chardin et al. to function as a guanine nucleotide exchange factor for ARF1 in vitro (13).
ibly of actin into protrusive membrane structures (5). These authors have also found that treatment of cells with the actin-depolymerizing drug cytochalasin D results in redistribution of ARF6 from the plasma membrane to novel endosomal structures (27). Taken together, these data suggest that ARF6 may regulate the assembly of cortical actin in a manner similar to the rhoA family GTPases rhoA, rac, and cdc42 (28). In fact, recent evidence suggests that ARF6 and rac1 share at least one common downstream effector, POR1. Expression of POR1 mutants inhibits the cytoskeletal rearrangements induced by either activated rac1 or ARF6 (26).

As described above, members of the ARNO/cytohesin-1 family contain a C-terminal PH domain, which appears to be a common feature of GEFs. Exchange factors specific for ras (sos), rac (tiam-1), rhoA (dbl), and cdc42 (cdc24), to name a few, all contain PH domains that are apparently required for their activity (29). The PH domain of ARNO has been shown to mediate the enhancement of GEF activity in the presence of polyphosphoinositides (13). Inclusion of PtdIns-4,5-P2 in phospholipid vesicles both increased the binding of ARNO to the vesicles and significantly stimulated nucleotide exchange activity. Whether this is simply a mechanism for bringing ARNO into close proximity to membrane-bound ARF6 or induces a conformational change resulting in enhanced catalysis remains to be determined. Recently, both cytohesin-1 and GRP-1 have been found to bind PtdIns-3,4,5-P3 in preference to PtdIns-4,5-P2 in vitro (14), and because the PH domain of ARNO is nearly identical in sequence to those of cytohesin-1 and GRP-1, it is likely that all three proteins share the same lipid specificity. PtdIns-3,4,5-P3, which is normally present at low levels in resting cells, is produced acutely in response to receptor-activated PI 3-kinases, raising the intriguing possibility that ARNO/cytohesin/GRP1 (and subsequently ARF6) may be subject to regulation by extracellular signals.

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