

ADP-Ribosylation Factor 1 (ARF1) Regulates Recruitment of the AP-3 Adaptor Complex to Membranes

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Abstract. Small GTP-binding proteins such as ADP-ribosylation factor 1 (ARF1) and Sar1p regulate the membrane association of coat proteins involved in intracellular membrane trafficking. ARF1 controls the clathrin coat adaptor AP-1 and the nonclathrin coat COPI, whereas Sar1p controls the nonclathrin coat COPII. In this study, we demonstrate that membrane association of the recently described AP-3 adaptor is regulated by ARF1. Association of AP-3 with membranes *in vitro* was enhanced by GTP γ S and inhibited by brefeldin A (BFA), an inhibitor of ARF1 guanine nucleotide exchange. In addition, recombinant myristoylated ARF1 promoted association of AP-3 with membranes. The role of ARF1 *in vivo* was examined by assessing AP-3 subcellular localization when the intracellular level of ARF1-GTP was altered through overexpression of dominant ARF1 mutants or ARF1-

GTPase-activating protein (GAP). Lowering ARF1-GTP levels resulted in redistribution of AP-3 from punctate membrane-bound structures to the cytosol as seen by immunofluorescence microscopy. In contrast, increasing ARF1-GTP levels prevented redistribution of AP-3 to the cytosol induced by BFA or energy depletion. Similar experiments with mutants of ARF5 and ARF6 showed that these other ARF family members had little or no effect on AP-3. Taken together, our results indicate that membrane recruitment of AP-3 is promoted by ARF1-GTP. This finding suggests that ARF1 is not a regulator of specific coat proteins, but rather is a ubiquitous molecular switch that acts as a transducer of diverse signals influencing coat assembly.

Key words: ARF • adaptin • coat • endosomes • BFA

THE trafficking of integral membrane proteins to their final destinations within the cell is mediated by membrane-bound carrier intermediates. Formation of these intermediates is initiated by deposition of coat proteins on the cytosolic face of the membrane at the appropriate intracellular location (for reviews see Rothman and Wieland, 1996; Schekman and Orci, 1996). Coat proteins participate in two crucial events in the trafficking process, namely the physical formation of transport intermediates and the selection of cargo for transport (for reviews see Bednarek et al., 1996; Kirchhausen et al., 1997; Schmid, 1997). Several protein coats have been identified, and are broadly classified as either clathrin or nonclathrin coats. Clathrin coats comprise clathrin and its associated

adaptor proteins (APs)¹ (Pearse and Robinson, 1990). Two different clathrin coats containing either AP-2 or AP-1 adaptors mediate, respectively, endocytosis from the plasma membrane and transport from the TGN to endosomes and lysosomes (Keen, 1990; Kirchhausen, 1993; Robinson, 1994; Marks et al., 1995). The nonclathrin coats COPI and COPII participate in ER-Golgi pathways and in transport along the endocytic route (for reviews see Cosson and Letourneur, 1997; Kuehn and Schekman, 1997). Other coats present on the TGN have been described but are not as well characterized (Jones et al., 1993; Narula and Stow, 1995; Traub and Kornfeld, 1997).

We (Dell'Angelica et al., 1997a) and others (Simpson et al., 1997) have recently described a third adaptor complex, AP-3, which is involved in the biogenesis of specialized organelles such as pigment granules and synaptic ves-

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1. *Abbreviations used in this paper:* AP, adaptor protein; ARF, ADP-ribosylation factor; BFA, brefeldin A; CB, coat-binding; DOG, 2-deoxyglucose; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; HA, hemagglutinin; HB, homogenization buffer; HMW, high molecular weight.

icles (Ooi et al., 1997; Simpson et al., 1997; Faúndez et al., 1998), and in the delivery of proteins to the yeast vacuole (Cowles et al., 1997; Stepp et al., 1997). The AP-1, AP-2, and AP-3 adaptor complexes have a similar four-subunit composition, each with two large chains (100–160 kD), a medium chain (~50 kD), and a small chain (~20 kD). The corresponding subunits of AP-1, AP-2, and AP-3 have structural homology to each other, and are named, respectively, γ , α , δ and $\beta 1$, $\beta 2$, $\beta 3$ (large chains); $\mu 1$, $\mu 2$, $\mu 3$ (medium chains); and $\sigma 1$, $\sigma 2$, $\sigma 3$ (small chains). The β chains associate physically with clathrin (Ahle and Ungewickell, 1989; Gallusser and Kirchhausen, 1993; Shih et al., 1995; Wilde and Brodsky, 1996; Dell'Angelica et al., 1998), whereas the α , γ , and δ chains are believed to bind to a putative docking site that directs them to the appropriate intracellular membranes (Robinson, 1993; Chang et al., 1993; Page and Robinson, 1995). In addition to providing a link between clathrin and the membrane, adaptor complexes also select transport cargo. This function is imparted by the ability of these complexes to recognize tyrosine- and dileucine-based sorting signals present in the cytosolic tails of integral membrane proteins (Ohno et al., 1995; Boll et al., 1996; Heilker et al., 1996; Dietrich et al., 1997; Marks et al., 1997). In particular, the μ chains have been identified as the subunits of the adaptor complexes that directly recognize tyrosine-based signals (Ohno et al., 1995, 1996). Thus, the adaptor complexes coordinate the multiple functions of coat proteins through the roles of each of their subunits.

Besides their similarity to each other, the adaptor coat complexes also have structural homology, although to a lesser extent, to the components of COPI. Indeed, the β , δ , and ζ chains of COPI are homologous to the β , μ , and σ chains of the adaptor complexes, respectively (Duden et al., 1991; Serafini et al., 1991; Cosson et al., 1996). The observation that many protein coats are structurally related but distinct has led to the concept that the intracellular trafficking machinery uses similar basic mechanisms which are iterated with variations to confer specificity to different transport pathways.

Further support for this concept is provided by small GTP-binding proteins that act as molecular switches in regulating intracellular traffic. The rab family comprises a group of structurally related proteins that participate in vesicle fusion and probably other transport events (for review see Novick and Zerial, 1997). Different rab family members are found at different intracellular locations and act on distinct trafficking pathways. Another group of small GTP-binding proteins regulate the recruitment of different coat proteins from the cytosol to the appropriate membranes (for reviews see Donaldson and Klausner, 1994; Bednarek et al., 1996). In each case, the cycle of GTP binding and hydrolysis controls association of the GTP-binding protein itself to membranes; this association in turn promotes binding of coat. However, in contrast to a model for the rab proteins whereby different family members regulate distinct traffic systems, the picture is more complicated for the GTP-binding proteins that control coat recruitment. A single protein, ADP-ribosylation factor 1 (ARF1), has been shown to regulate at least two different classes of coats, including clathrin (AP-1) and non-clathrin (COPI) coats, and at multiple intracellular locations

(Lenhard et al., 1992; Donaldson and Klausner, 1994; Boman and Kahn, 1995; Dittie et al., 1996; Faúndez et al., 1997), even though there exists other closely-related ARF family members (Kahn et al., 1991; Tsuchiya et al., 1991). In addition, a GTP-binding protein that has structural homology to ARF but which is not from the ARF family, Sar1p, regulates another nonclathrin coat (COPII) (for reviews see Salama and Schekman, 1995; Bednarek et al., 1996), whereas the clathrin coat adaptor AP-2 does not appear to be regulated *in vivo* by an ARF protein (Robinson and Kreis, 1992; Wong and Brodsky, 1992). Thus, studies up to this point have failed to provide a coherent picture regarding control of diverse coat proteins by small GTP-binding proteins.

In this study, we investigate the regulation of membrane recruitment of the newly identified AP-3 adaptor. We find that ARF1, the same protein that regulates two other coats, also regulates AP-3. These results support the concept that ARF1 is a common regulator in the pathways governing recruitment of diverse coat proteins to membranes.

Materials and Methods

Cells and Reagents

HeLa S3 cells (American Type Culture Collection, Rockville, MD) were cultured in suspension for preparation of membrane fractions. HeLa (American Type Culture Collection) and MDCK II cells (gift of E. Rodriguez-Boulan, Cornell University Medical College, New York) were cultured as monolayers in DME supplemented with 9% FBS, 100U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine. Brefeldin A (BFA) was from Epicentre Technologies (Madison, WI) and trypsin was from Promega Corp. (Madison, WI). GTP γ S, ATP, creatine phosphate, and creatine kinase were from Sigma Chemical Co. (St. Louis, MO).

Recombinant Proteins

GST Fusion with δ -Adaptin Fragment (GG1). DNA encoding residues 752–839 of δ -adaptin was generated by PCR and then cloned into pGEX-5X-1 (Pharmacia Biotech, Inc., Piscataway, NJ). Production and purification of the GST fusion protein using glutathione-Sepharose 4B (Pharmacia Biotech, Inc.) was carried out according to the manufacturer's instructions.

Myristoylated ARF1. Recombinant myristoylated ARF1 was a gift of P. Randazzo and J. Andrade (both from National Cancer Institute, NIH, Bethesda, MD).

Antibodies

The following commercial primary antibodies were used: (a) monoclonal anti-ARF 1D9 and monoclonal anti- α -adaptin AP.6 (Affinity Bioreagents, Inc., Golden, CO); (b) monoclonal anti- γ -adaptin 100/3 (Sigma Chemical Co.); and (c) monoclonal anti-HA 16B12 and rabbit anti-HA 11 (Berkeley Antibody Co., Richmond, CA). Rabbit antiserum to COPI (β -COP) was provided by J. Lippincott-Schwartz (NIH, Bethesda, MD). Preparation of affinity-purified rabbit antibodies to the β , μ , and σ subunits of AP-3 have been described previously (Dell'Angelica et al., 1997a,b). Rabbit antiserum to δ -adaptin was generated using a GST-fusion protein (GG1) containing residues 752–839 of δ -adaptin. The antibody was affinity purified using GG1 immobilized on Affigel-15 beads (Bio-Rad Laboratories, Hercules, CA), and reacted with a major 160-kD species and a minor 90-kD species in total HeLa cell extracts and in bovine brain cytosol.

Membranes

A membrane preparation from HeLa cells enriched in endosomes was prepared as described by Aniento et al. (1996). Cells were grown in suspension to a density of 0.5×10^6 /ml and harvested by centrifugation at 2,000 rpm in a JA-10 rotor (Beckman Instrs., Palo Alto, CA). Cells were

washed twice in homogenization buffer (HB) (250 mM sucrose, 3 mM imidazole, pH 7.4), resuspended in four-pellet volumes of ice-cold HB containing protease inhibitors (0.5 mM 4-[2-aminoethyl]-benzenesulfonyl fluoride, 10 μ g/ml leupeptin, 2 μ g/ml pepstatin), and then homogenized by seven passages through a 23-gauge needle. The homogenate was centrifuged at 800 g to yield the postnuclear supernatant. The postnuclear supernatant was adjusted to 40.6% sucrose, then overlaid sequentially with 35% sucrose, 25% sucrose, and HB (all sucrose solutions contained 3 mM imidazole, pH 7.4). The gradient was centrifuged in a SW41 rotor (Beckman Instrs.) at 40,000 rpm for 90 min. Membrane fractions enriched for endosomal or Golgi membranes were collected from the 35/25% and the 40.6/35% sucrose interface, respectively (Aniento et al., 1996). Membranes were washed with salt by incubation on ice for 20 min after addition of an equal volume of 2M KCl. The stripped membranes were collected by centrifugation at 100,000 g for 90 min, resuspended in coat-binding (CB) buffer (see *In Vitro Membrane-binding Assay*), and then stored in liquid nitrogen. Protein quantitation was performed with the Bradford reagent (Bio-Rad Laboratories) using bovine serum albumin as a standard. Enrichment for endosomal membranes was confirmed by immunoblotting for the early endosomal marker rab5.

Bovine Brain Cytosol Preparation and Fractionation

Bovine brain homogenates were prepared in 0.1 M sodium 2-(N-morpholino)ethanesulfonate, pH 6.5, 1 mM EGTA, 0.5 mM MgCl₂, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.02% (wt/vol) sodium azide, with the aid of a tissue blender. The cytosolic fraction was obtained by centrifugation of the homogenate at 16,000 g for 40 min followed by a second centrifugation step at 105,000 g for 90 min, at 4°C. This fraction (30 mg/ml protein) was designated bovine brain cytosol and used in the *in vitro* membrane-binding assays. To generate a high molecular weight (HMW) fraction of the cytosol that contained AP-3 but not ARF, the cytosol was subjected to gel filtration chromatography on a Superose 6 column (Pharmacia Biotech, Inc.), as described by Donaldson et al., (1992). Fractions were analyzed by immunoblotting with AP-3 (δ -adaptin) and ARF antibodies. AP-3 peak fractions were pooled, concentrated fivefold using a microconcentrator (Centricon 10; Amicon Corp., Beverly, MA), and designated HMW fraction. These AP-3 peak fractions did not contain ARF protein as judged by immunoblotting with ARF antibody.

In Vitro Membrane-binding Assay

The *in vitro* membrane-binding assay for AP-3 and ARF was carried out in CB buffer (25 mM Hepes, pH 7.0, 0.2 M sucrose, 25 mM KCl, 2.5 mM MgCl₂, 1 mM DTT) supplemented with an ATP-regenerating system consisting of 1 mM ATP, 5 mM creatine phosphate, and 10U/ml creatine kinase. In a typical 50- μ l reaction, 10- μ l membranes and 10- μ l cytosol were used. Incubations were carried out for 20 min at 37°C, and the reaction mixture was centrifuged for 10 min at 4°C at 14,000 g. The membrane pellet was rinsed once with buffer, resuspended in Laemmli SDS sample buffer, heated at 95°C for 5 min, and then subjected to SDS-PAGE and transferred to nitrocellulose. Immunoblotting was performed using primary antibodies as described, followed by HRP-conjugated secondary antibodies (Amersham Corp., Arlington Heights, IL). The enhanced chemiluminescence system (Amersham) was used for detection.

Expression of ARF Proteins

All ARF plasmid constructs were in the expression vector pXS (parent vector pCDL-SR α , with modified restriction sites for subcloning) (Peters et al., 1995). Wild-type ARF1 and ARF1/Q71L were expressed using the bovine ARF1 cDNA sequence (there is 100% amino acid identity between human and bovine ARF1). Generation of hemagglutinin (HA)-tagged ARF1, ARF3, ARF5, ARF1/T31N, and ARF6/T27N has previously been described (Peters et al., 1995). For mutagenesis of ARF1 and ARF5 to ARF1/Q71L and ARF5/T31N, respectively, single amino acid substitutions were generated by PCR, using oligonucleotides bearing the mutagenized nucleotide sequence. The PCR products were digested with the appropriate restriction enzymes and then used to replace the corresponding fragment in the wild-type cDNA-encoding proteins bearing the HA epitope at the COOH terminus. Transient transfection of cells was carried out by the calcium phosphate precipitation method 4–6 h after plating on coverslips. Cells were analyzed 36–48 h after transfection.

Confocal Immunofluorescence Microscopy

Cells were fixed in 2% formaldehyde/PBS for 10 min at room temperature, washed twice with PBS, and then incubated for 5 min in 0.1% BSA/PBS. Successive incubations with primary and secondary antibodies (diluted in 0.2% saponin, 0.1% BSA, PBS) were carried out for 1 h at room temperature. Samples were rinsed with 0.1% BSA/PBS after each antibody incubation. After a final rinse with PBS, coverslips were mounted with Fluoromount G (Southern Biotechnologies, Birmingham, AL). Cells were visualized and confocal images acquired using a confocal laser scanning microscope (model LSM 410; Carl Zeiss, Inc., Thornwood, NY). Cy3-conjugated antibodies to rabbit and mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used for visualization of AP-1, AP-2, AP-3, and COPI staining, whereas Cy2-conjugated (Jackson ImmunoResearch Laboratories, Inc.) or Alexa 488-conjugated (Molecular Probes, Inc., Eugene, OR) antibodies were used for detection of the HA and 6-His epitope tags.

Results

The observation that AP-3 is found in both cytosolic and membrane subcellular fractions, and that membrane-associated AP-3 can be extracted with salts (Dell'Angelica et al., 1997a) is consistent with its role as a membrane coat that can be recruited from a cytosolic pool. In addition, the punctate staining of AP-3 by immunofluorescence microscopy, its redistribution to the cytosol by BFA, and the reversibility of the BFA effect (Dell'Angelica et al., 1997a; Simpson et al., 1997) also indicate that AP-3 can cycle between the cytosol and membranes. The sensitivity of AP-3 to BFA in cells suggests that its membrane association may be regulated by ARF1, since BFA inhibits ARF1 guanine nucleotide exchange, and has been shown to prevent ARF1 membrane binding *in vitro* (Donaldson et al., 1992; Helms and Rothman, 1992; Randazzo et al., 1993).

ARF1 Promotes Recruitment of AP-3 to Membranes In Vitro

To study regulation of AP-3 association with membranes, we first designed an *in vitro* membrane-binding assay. The basic assay incubation consisted of salt-washed membranes, bovine brain cytosol as a source of exogenous AP-3 and other cytosolic factors that might be required for membrane binding, as well as an ATP-regenerating system. Since AP-3 has been observed at or near endocytic structures and the TGN (Simpson et al., 1996; Dell'Angelica et al., 1997a, 1998), we first examined AP-3 binding to sucrose gradient membrane fractions enriched in endosomal or Golgi membranes (refer to Materials and Methods) (Aniento et al., 1996). The highest AP-3 binding activity was observed with an endosomal membrane-enriched fraction banding at the 25–35% sucrose interface (data not shown); this fraction was thus used in all subsequent experiments. The membrane binding assays were carried out in the absence or presence of GTP γ S, a nonhydrolyzable analogue of GTP that would render ARF1 and other GTP-binding proteins in a constitutively active state. Membranes incubated alone in the absence or presence of GTP γ S had no detectable AP-3 as judged by immunoblotting with antibodies to all four subunits of AP-3 (δ , β 3, μ 3, and σ 3) (Fig. 1 A), indicating that these salt-washed membranes do not contain any endogenous AP-3. Upon incubation of membranes with cytosol, AP-3 was found associated with membranes in a GTP γ S-enhanced manner (Fig.

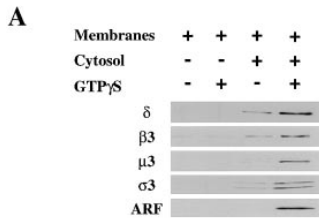
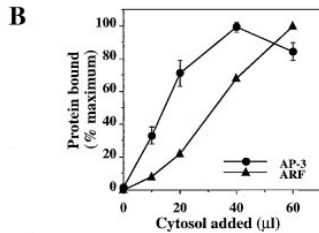


Figure 1. Association of AP-3 and ARF with membranes in vitro. (A) The in vitro membrane-binding assay was carried out as described in Materials and Methods. Incubations were performed either in the absence or presence of cytosol (10 μ l cytosol/50 μ l reaction) and/or 25 μ M GTP γ S. Bound proteins were analyzed by immunoblotting using antibodies to all four subunits of AP-3 (δ , β 3, μ 3, and σ 3) and to ARF (antibody 1D9). The two bands detected with the σ 3 antibody probably represent the σ 3A and σ 3B isoforms. (B) The in vitro membrane-binding



assay was carried out in 120- μ l incubations containing the indicated amounts of cytosol, and in the presence of 25 μ M GTP γ S. Bound proteins were quantified by densitometry analysis of bands on immunoblots and the amount bound is presented as percent of maximum bound. Values shown for AP-3 are the average of values obtained for all four subunits (δ , β 3, μ 3, and σ 3) \pm SEM. Under conditions near saturation, 5–10% of the added AP-3 in the cytosol bound to membranes.

1 A), implicating involvement of a GTP-binding protein for AP-3 recruitment to membranes. In addition, using an ARF antibody,² we determined that the binding of AP-3 to membranes paralleled the binding of ARF (Fig. 1 A), consistent with ARF binding being required for AP-3 binding. Binding of AP-3 to membranes was saturable, with maximum binding occurring when 20–40 μ l cytosol was present in a 120- μ l incubation mixture (Fig. 1 B). Under these conditions, binding of ARF to membranes still increased with increasing amounts of cytosol added, and did not appear to be saturated (Fig. 1 B). This difference in AP-3 and ARF membrane binding is consistent with previous reports of the existence of two different populations of membrane-bound ARF, only one of which is required for coat binding (Helms et al., 1993; Finazzi et al., 1994).

The saturability of AP-3 binding to membranes suggested that it interacts with a limiting component on membranes. To determine if a protein factor(s) on the membranes was required for AP-3 association, we inactivated proteins by either treating membranes with an exogenous protease or by exposure to heat. Membranes were preincubated with 0–50 μ g/ml trypsin before addition of cytosol. Binding of AP-3 was abolished upon incubation of membranes with \geq 10 μ g/ml trypsin, but not if the trypsin inhibitor TPCK was present during exposure to trypsin (Fig. 2). Inactivation of membrane proteins by incubation at 65°C for 10 min also abolished AP-3 binding (Fig. 2).

2. The ARF antibody used (clone 1D9) recognizes all five known human ARF proteins, but ARF1 and/or ARF3 is by far the most abundant ARF species in cells, comprising >90% of total cellular ARF.

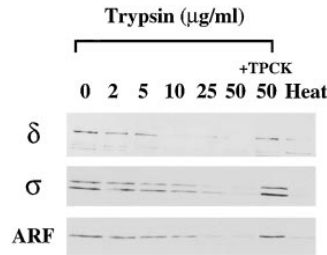


Figure 2. Membrane proteins are required for AP-3 and ARF membrane association. Membrane proteins were proteolyzed by exposure to 0, 2, 5, 10, 25, and 50 μ g/ml trypsin or inactivated by heat (65°C, 10 min). Trypsin digestion of membrane proteins was carried out for 10 min at room temperature, in 40- μ l reactions

comprising 10- μ l membranes and 30 μ l of CB buffer. Trypsin inhibitor TPCK was added after trypsin treatment, except for an additional sample in which TPCK was added before addition of 50 μ g/ml trypsin (+ TPCK). The treated membranes were then used in the in vitro membrane-binding assay with cytosol and GTP γ S. Bound AP-3 (δ and σ 3) and ARF were detected by immunoblotting. The apparent higher sensitivity of the delta subunit to trypsin treatment of membranes as compared with the sigma subunit is reproducible and probably reflects the greater sensitivity of the delta subunit to digestion by residual proteolytic activity after trypsin inactivation.

Immunoblotting of ARF in the same samples showed that ARF binding to membranes was also sensitive to trypsin and heat treatment. Thus, binding of both ARF and AP-3 to membranes depends on the presence of a protein factor(s) on the membrane.

We next tested for the involvement of ARF1 in AP-3 membrane association. First, the effect of BFA on binding of AP-3 to membranes was assessed. Membrane and cytosol were either preincubated with BFA for 5 min before addition of GTP γ S for the usual assay incubation, or were incubated with BFA for an additional 5 min after the assay incubation in the presence of GTP γ S. Pre-incubation with BFA resulted in \sim 70% inhibition of ARF binding as compared with samples in which BFA was added after GTP γ S or where no BFA was added (Fig. 3 A). Binding of AP-3 to membranes was similarly inhibited by BFA, with \sim 50% inhibition observed (Fig. 3 A). The inhibition of AP-3 binding to membranes by BFA suggested that ARF1 is required for AP-3 membrane association.

To directly investigate the requirement for ARF1, we assessed the ability of purified recombinant myristoylated ARF1 to promote binding of AP-3 to membranes. An AP-3-enriched fraction from bovine brain cytosol that was devoid of ARF was prepared by subjecting cytosol to gel filtration chromatography on a Superose 6 column. Fractions comprising the AP-3 peak were pooled, designated high molecular weight (HMW) fraction, and used as a source of AP-3 in the membrane-binding assay. When HMW fraction was incubated with membranes, no binding of AP-3 was detected either in the absence or presence of GTP γ S, as assessed by immunoblotting for the σ 3 subunit (Fig. 3 B). Addition of purified recombinant myristoylated ARF1 (50 μ g/ml) to the incubation mixture resulted in binding of AP-3 to membranes in the presence, but not in the absence, of GTP γ S (Fig. 3 B). Immunoblotting with the ARF antibody showed that AP-3 binding in this experiment paralleled ARF binding to membranes (Fig. 3 B). The effect of the recombinant protein indicates that ARF1 promotes association of AP-3 with membranes in vitro.

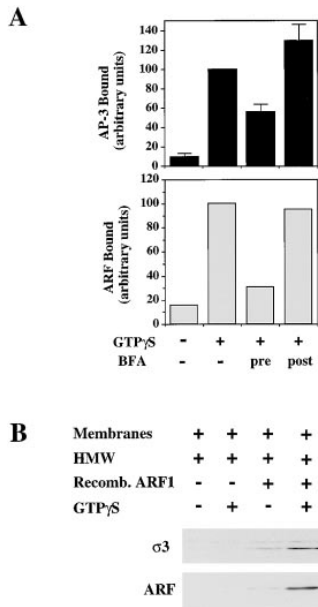


Figure 3. ARF1 promotes membrane association of AP-3. (A) BFA inhibits membrane association of AP-3. The in vitro membrane-binding assay with cytosol and membranes (refer to Material and Methods) was carried out in the absence or presence of 25 μ M GTP γ S, and also in the presence of GTP γ S and 200 μ M BFA. BFA was added either before (*pre*) GTP γ S, or after (*post*) GTP γ S (refer to Results). AP-3 (δ and σ subunits) and ARF bound to membranes were quantified by densitometry of protein bands from immunoblots, and the mean values (with standard error for AP-3) in arbitrary units are shown ($n = 5$ for AP-3; $n = 2$ for ARF). The amounts of protein bound in

the presence of cytosol and GTP γ S are considered 100% binding. (B) Recombinant ARF1 promotes membrane association of AP-3. The in vitro membrane binding assay was carried out with membranes and a high molecular weight Superose 6 fraction of bovine brain cytosol (HMW) containing AP-3 but not ARF (refer to Materials and Methods). Incubation reactions of 75 μ l contained 15- μ l membranes, 30 μ l HMW, and were performed in the absence or presence of 50 μ g/ml recombinant myristoylated ARF1, with or without 25 μ M of GTP γ S. Bound protein (σ 3 for AP-3) was assessed by immunoblotting.

ARF1 Regulates the Localization of AP-3 in Cells

The above experiments indicate that ARF1 is required for the membrane association of AP-3 in vitro. To determine if ARF1 similarly regulates AP-3 in cells, we altered the ARF1 activity in vivo by expressing dominant ARF1 mutants, or by overexpressing either ARF1 or an ARF1 regulatory ARF1-GAP. We then assessed the effects of these manipulations on AP-3 localization. The subcellular localization of AP-3 was visualized by indirect immunofluorescence microscopy, using an affinity-purified antibody to the δ -adaptin subunit of AP-3. In HeLa cells, this antibody gave punctate staining that was slightly more pronounced in a juxtannuclear region, but which extended to the periphery of the cell. Many of these structures colocalized with early endocytic structures stained with the transferrin receptor, as well as with clathrin, but showed little colocalization with a transfected TGN marker, furin (data not shown). This staining pattern of AP-3, and its sensitivity to BFA (see Fig. 5 D), is similar to that previously observed using antibodies to the σ 3 and β 3 subunits of AP-3 (Dell'Angelica et al., 1997a, 1998).

We first expressed an HA epitope-tagged ARF1 dominant-negative mutant, ARF1/T31N, by transient transfection of HeLa cells. This mutant renders endogenous ARF1 inactive, presumably by binding to and sequestering an ARF1 guanine nucleotide exchange factor (ARF GEF), and produces a BFA-like phenotype (Dascher and Balch, 1994; Peters et al., 1995). When expressed at high levels,

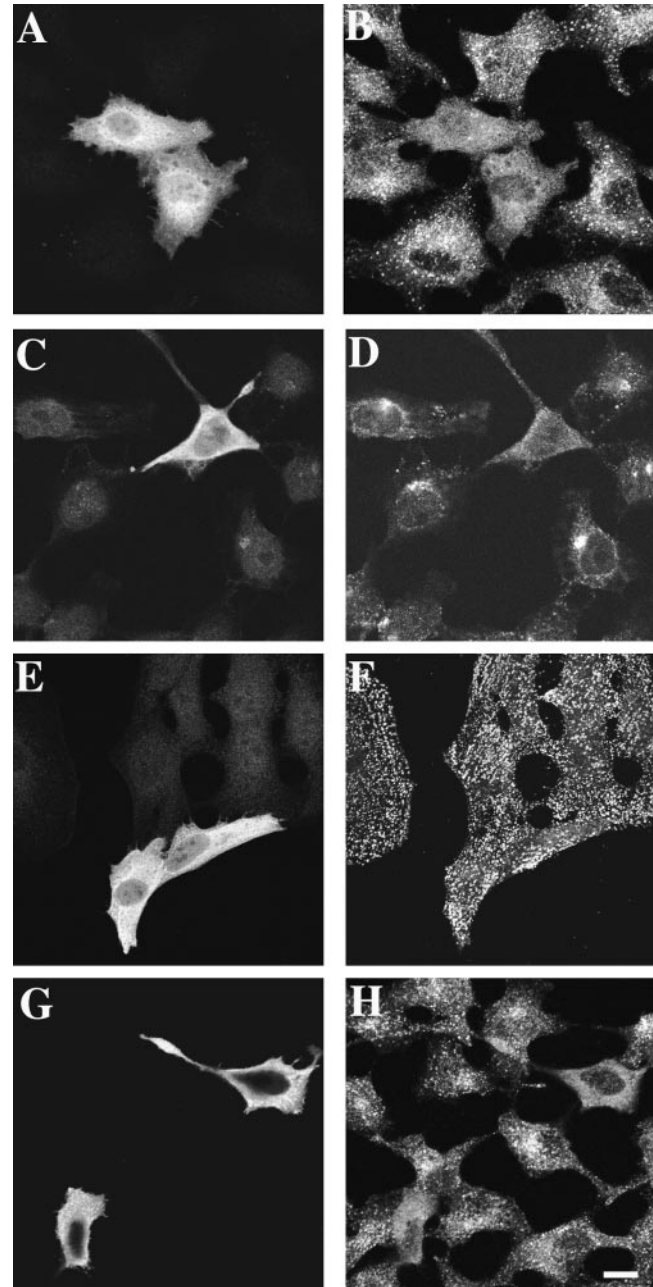


Figure 4. Effect of ARF1 inactivation on subcellular localization of AP-1, AP-2, and AP-3. HeLa cells were transiently transfected with HA-tagged ARF1/T31N (A-F) or 6-His-tagged ARF1-GAP (G and H), fixed, and then double labeled for the HA (A, C, and E) or 6-His (G) epitopes and AP-3 (rabbit anti- δ) (B and H), AP-1 (mouse anti- γ -adaptin) (D), or AP-2 (mouse anti- α -adaptin) (F). Fluorophore-conjugated secondary antibodies were used as described in Materials and Methods. Cells were visualized by confocal immunofluorescence microscopy. Bar, 10 μ m.

ARF1/T31N shows cytosolic staining as detected by an antibody to the HA epitope. In these cells, AP-3 distribution was no longer punctate but appeared cytosolic (Fig. 4, A and B), reminiscent of the effect of BFA (Dell'Angelica et al., 1997a; Simpson et al., 1997). The distribution of AP-1 was similarly affected by ARF1/T31N (Fig. 4, C and D), whereas AP-2 distribution, which is not BFA sensitive

(Robinson and Kreis, 1992; Wong and Brodsky, 1992), was not affected (Fig. 4, *E* and *F*).

As another way of inactivating ARF1 *in vivo*, we overexpressed ARF1-GAP, an ARF1 regulatory protein that activates hydrolysis of ARF1-bound GTP (Cukierman et al., 1995). High levels of this protein would drastically reduce the intracellular concentration of ARF1-GTP, and has been shown to result in redistribution of coatamer from membrane to the cytosol (Aoe et al., 1997). Cells overexpressing a 6-His epitope-tagged ARF1-GAP were identified by staining with antibody to the 6-His epitope. In these cells, AP-3 distribution was cytosolic (Fig. 4, *G* and *H*), strongly suggesting, again, that ARF1-GTP was required for membrane localization of AP-3.

To further confirm the role of ARF1 in the membrane association of AP-3, we tested the ability of a constitutively active ARF1 mutant, ARF1/Q71L, to prevent dissociation of AP-3 from membranes. ARF1/Q71L has a markedly lowered rate of hydrolysis of bound GTP (Dascher and Balch, 1994). Expression of such a mutant thus effectively increases the intracellular concentration of ARF1-GTP, and has been observed to protect coatamer from BFA-induced membrane dissociation (Teal et al., 1994; Zhang et al., 1994). In HeLa cells, expression of ARF1/Q71L did not visibly alter the distribution of AP-3 (Fig. 5, *A* and *B*). Some of the AP-3-containing structures appeared coincident with those stained with ARF1/Q71L (Fig. 5, *A* and *B*, *arrows*), suggesting that AP-3 bound to membranes at sites where ARF1/Q71L was present. HeLa cells transiently transfected with HA-tagged ARF1/Q71L were then subjected to two different treatments that induced dissociation of AP-3 from membranes. These manipulations were BFA treatment and energy depletion (by a combination of 50 mM 2-deoxyglucose [DOG] and 0.04% sodium azide). In untransfected cells, treatment with either BFA (Fig. 5, *C* and *D*) or DOG/azide (Fig. 5, *E* and *F*) caused redistribution of AP-3 to the cytosol. In cells expressing ARF1/Q71L, however, AP-3 was retained on punctate structures present throughout the cell (Fig. 5, *C–F*). Thus, ARF1/Q71L protects AP-3 from membrane dissociation. These results strongly suggest that ARF1-GTP promotes membrane association of AP-3, consistent with the findings using ARF1/T31N and ARF1-GAP.

We have so far deduced a role for ARF1 in AP-3 membrane association using ARF1 mutants and an ARF1 regulator. In addition, we found that we could observe a direct effect of overexpressed wild-type ARF1 on AP-3 distribution in cells subjected to energy depletion. In cells overexpressing wild-type HA-tagged ARF1, AP-3 was found on punctate structures upon energy-depletion, whereas in untransfected cells AP-3 was cytosolic (Fig. 5, *G* and *H*). However, this effect was only observed under extremely high expression levels of ARF1; thus, wild-type ARF1 was not as potent as ARF1/Q71L in conferring protection of AP-3 from membrane dissociation.

Effects of Other ARF Family Members on AP-3

ARF1 is one member of a family of highly conserved proteins, with four other ARFs having been identified in humans (Kahn et al., 1991; Tsuchiya et al., 1991). The existence of three classes of ARF proteins has been proposed

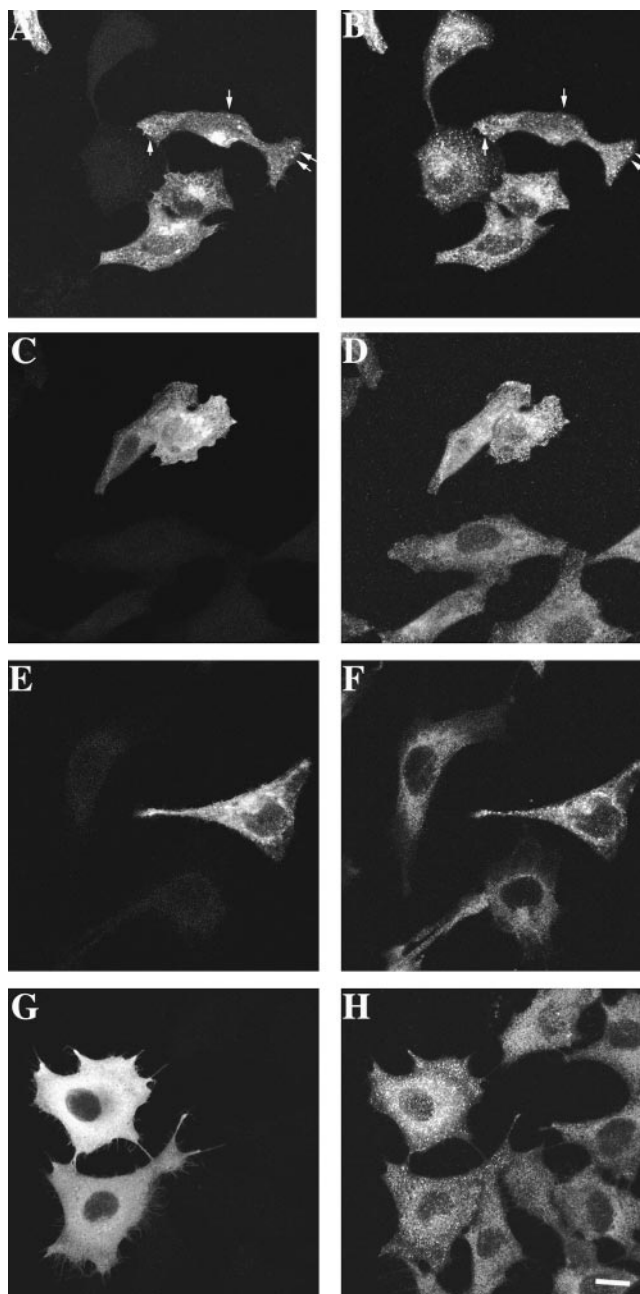


Figure 5. ARF1/Q71L and wild-type ARF1 prevents redistribution of membrane-bound AP-3 to the cytosol. HeLa cells were transiently transfected with HA-tagged ARF1/Q71L (*A–F*) or HA-tagged wild-type ARF1 (*G* and *H*), and then fixed without further treatment (*A* and *B*), or after exposure for 30 min to 2 μ g/ml BFA (*C* and *D*) or 50 mM DOG/0.4% sodium azide in RPMI/5% FBS (*E–H*). Cells were double labeled for the HA epitope (mouse anti-HA) (*A*, *C*, *E*, and *G*), and AP-3 (rabbit anti- δ) (*B*, *D*, *F*, and *H*). Cy2- or Alexa 488-conjugated anti-mouse, and Cy3-conjugated anti-rabbit secondary antibodies were used. Cells were visualized by confocal immunofluorescence microscopy. Bar, 10 μ m.

based on structural relationships: class I (ARF1 and ARF3), class II (ARF4 and ARF5), and class III (ARF6) (Tsuchiya et al., 1991). Except for ARF6, which regulates plasma membrane dynamics (D'Souza-Schorey et al., 1995;

Peters et al., 1995; Radhakrishna et al., 1996; Radhakrishna and Donaldson, 1997), the other ARF family members have not been functionally characterized. To determine if AP-3 is regulated specifically by ARF1, we tested other ARFs for regulation of AP-3 membrane localization in cells.

ARF3 is the ARF family member most closely related to ARF1 (96% amino acid identity between the two proteins) and a function has not been ascribed to it. One scenario is that ARF3 also regulates AP-3 in cells, but even more potently than ARF1, and could thus be a physiological regulator of AP-3. To test this possibility, we assessed the effect of overexpressed wild-type ARF3 on AP-3 localization in energy-depleted cells. Since wild-type ARF1 was not very potent in this setting (see above, Fig. 5, *G* and *H*), this assay would allow revelation of higher effectiveness of ARF3 relative to ARF1, if such were the case. We found, however, that ARF3 had a barely discernible protective effect on AP-3 in this assay (data not shown); thus, its effect was weaker than that of ARF1.

We next examined the role of ARF5, a class II ARF, in AP-3 localization. A dominant-negative mutant of ARF5, HA-tagged ARF5/T31N, was transiently transfected in HeLa cells to inactivate endogenous ARF5. In cells expressing ARF5/T31N at levels comparable to those seen for ARF1/T31N in Fig. 4 *A*, AP-3 distribution was unaffected (Fig. 6, *A* and *B*). However, in cells expressing very high levels of this mutant, AP-3 showed some cytosolic staining, although punctate structures were still visible (Fig. 6, *A* and *B*). Thus, ARF5/T31N apparently has the ability to affect AP-3 distribution, but is less potent than ARF1/T31N. To further examine the role of ARF5, we tested the effect of a constitutively active mutant of ARF5 analogous to ARF1/Q71L. HeLa cells were transfected with HA-tagged ARF5/Q71L, and then subjected to either BFA treatment or energy-depletion. Treatment of ARF5/Q71L-expressing cells with BFA resulted in retention of COPI on Golgi/vesicular membrane structures (data not shown), but did not prevent AP-3 membrane dissociation (Fig. 6, *C* and *D*). Similarly, expression of ARF5/Q71L did not protect AP-3 from membrane dissociation induced by energy depletion (Fig. 6, *E* and *F*).

Finally, we assessed the effect of inactivating endogenous ARF6. As expected, expression of the dominant-negative mutant ARF6/T27N, even at very high levels, did not have any effect on AP-3 distribution (Fig. 6, *G* and *H*).

These results indicate that although other ARF family members may affect AP-3 distribution in some settings, their effects are generally much weaker as compared with ARF1. Thus, ARF1 is most likely the ARF species that regulates AP-3 in cells.

Visualization of ARF1 on AP-3-containing Structures

The visualization of ARF1 on AP-3-associated intracellular structures would constitute another piece of evidence in support of a role for ARF1 in regulation of AP-3 membrane association. To specifically detect ARF1 in cells, we transfected cells with HA epitope-tagged ARF1 and stained for the HA epitope. HA-tagged ARF1 was seen both at the Golgi and in the cytosol, and also on weakly stained punctate structures distributed throughout the cell, especially in areas where cytosolic staining was less in-

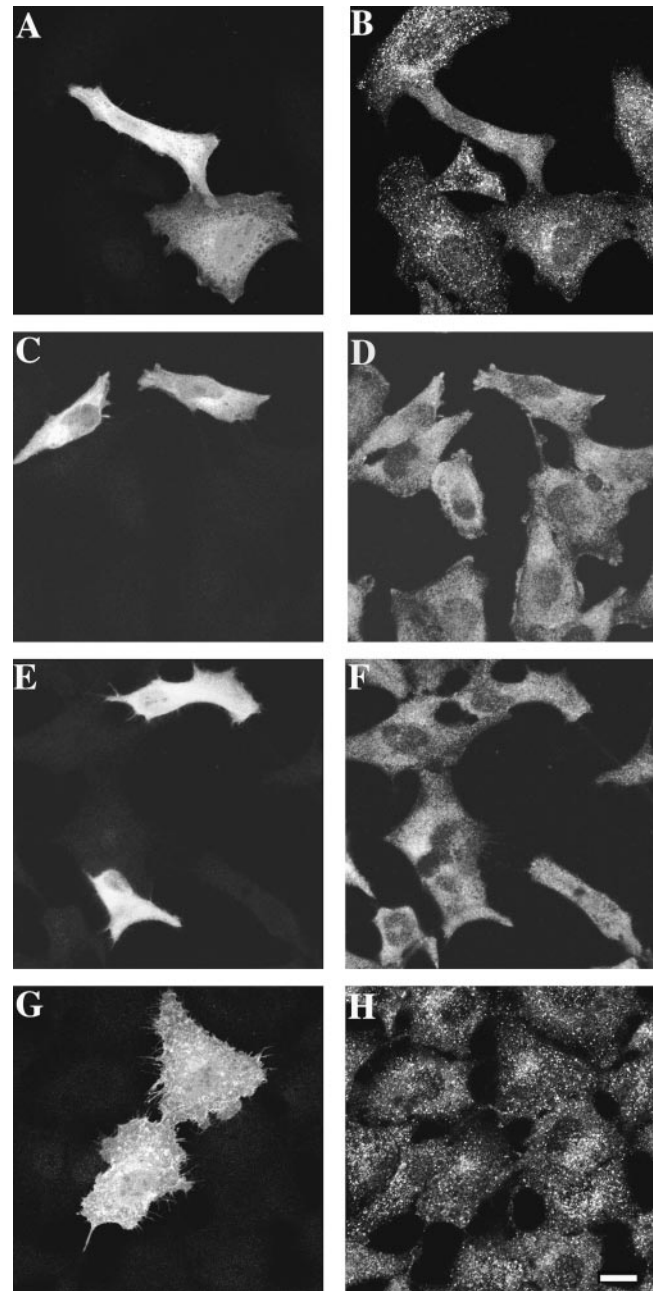


Figure 6. Effect of ARF5 and ARF6 mutants on subcellular distribution of AP-3. HeLa cells were transiently transfected with HA-tagged ARF5/T31N (*A* and *B*), ARF5/Q71L (*C–F*), or ARF6/T27N (*G* and *H*), and then fixed without further treatment (*A*, *B*, *G*, and *H*), or after exposure for 30 min to 2 μ g/ml BFA (*C* and *D*) or 50 mM DOG/0.4% sodium azide in RPMI/5% FBS (*E* and *F*). Cells were double labeled for the HA epitope (mouse anti-HA) (*A*, *C*, *E*, and *G*) and AP-3 (rabbit anti- δ) (*B*, *D*, *F*, and *H*). Cy2- or Alexa 488-conjugated anti-mouse, and Cy3-conjugated anti-rabbit secondary antibodies were used. Cells were visualized by confocal immunofluorescence microscopy. Bar, 10 μ m.

tense. Some of these structures appeared to stain with AP-3 antibodies, but assessment of colocalization was hampered by difficulty of detecting the weak ARF1 punctate staining superimposed upon the more intense cytosolic and Golgi staining. We found, however, that we could enhance visu-

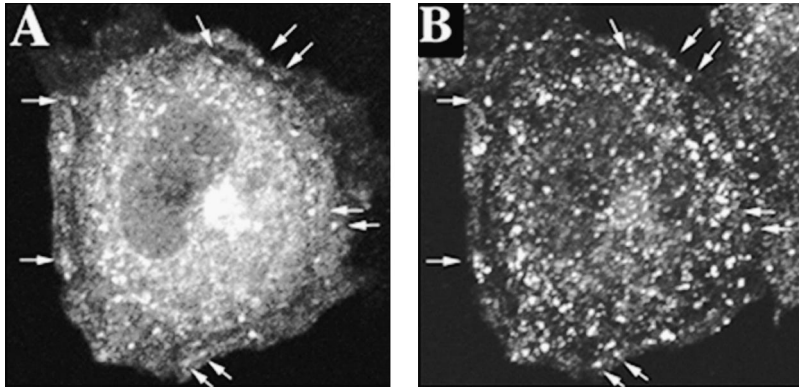


Figure 7. Visualization of ARF1 on AP-3 containing structures. HeLa cells were transiently transfected with HA-tagged wild-type ARF1 and treated with 50 μ M AlCl_3 /30 mM NaF for 30 min at 37°C, fixed, and then stained with antibodies to HA (mouse anti-HA) (A) or AP-3 (rabbit anti- δ) (B), followed by Alexa 488-conjugated anti-mouse and Cy3-conjugated anti-rabbit antibodies. Cells were visualized by confocal immunofluorescence microscopy. Arrows, structures that stain for both ARF1 and AP-3. Bar, 5 μ m.

alization of the ARF1 punctate structures by treatment of cells with aluminum fluoride before fixation. Aluminum fluoride is a G protein activator that affects behavior of ARF1 and ARF6 vis-à-vis their distribution or effect on coat proteins (Donaldson et al., 1991; Finazzi et al., 1994; Radhakrishna et al., 1996), either via an indirect effect on upstream G proteins or via a direct effect on ARF itself. Treatment of HeLa cells with aluminum fluoride increased the number and size of the ARF1-associated punctate structures, allowing us to observe the presence of AP-3 on many of them (Fig. 7, A and B). Similar experiments performed with HA-tagged ARF3 and ARF5 staining revealed predominant staining in the cytosol and Golgi region with little or no colocalization with AP-3 (data not shown). Thus, of the three ARF species examined, colocalization with AP-3 was most readily observed for ARF1.

Differential Effects of BFA on ARF1-regulated Coats in MDCK Cells

The three distinct coats (COPI, AP-1, and now AP-3) that are regulated by ARF1 are involved in different transport pathways, and thus need to be independently regulated. In MDCK cells, the Golgi coat COPI is resistant to the effects of BFA (Hunziker et al., 1991), presumably due to insensitivity of the Golgi ARF1 GEF to this drug. This thus provides a setting in which to explore independent behavior of different ARF1-regulated coats. When MDCK cells were treated with BFA, COPI Golgi association was unaffected (Fig. 8, A and B), as expected. In contrast, AP-3 was BFA sensitive, and was redistributed from its punctate structures to the cytosol (Fig. 8, C and D). AP-1 was also sensitive to BFA in these cells, as has been reported (Wagner et al., 1994), and was redistributed from the Golgi to the cytosol (Fig. 8, E and F). The differential effects of BFA on these different coats suggest that their ARF1-dependent regulation may be mediated by distinct ARF1 GEFs with differential sensitivity toward this drug.

Discussion

In this study, we demonstrate that ARF1 regulates association of the recently identified adaptor AP-3 with membranes. Using an *in vitro* biochemical approach, we found that AP-3 could be recruited from cytosol to membranes.

Binding of AP-3 to membranes was saturable, implying recognition by a limiting protein component on membranes. Consistent with this, a protein factor(s) was found to be required for binding of AP-3 to membranes. Such a factor could be an ARF1 receptor (e.g., ARF1 GEF) since we also find that ARF1 binding to membranes is abolished upon inactivation of membrane proteins. Another candidate for the required membrane protein is a putative receptor or docking protein for AP-3. Binding of AP-3 to membranes was stimulated by GTP γ S and inhibited by BFA, consistent with it being regulated by ARF1. The inhibition by BFA was partial, as has been observed for other coat proteins (Donaldson et al., 1992; Palmer et al., 1993; Stammes and Rothman, 1993; Dittie et al., 1996); this could be due to nonoptimal assay conditions or some ARF-independent binding of AP-3. Direct demonstration of a requirement for ARF1 was provided by the ability of purified recombinant myristoylated ARF1 to promote binding of AP-3 to membranes.

The role of ARF1 *in vivo* was assessed by altering intracellular levels of ARF1-GTP through overexpression of ARF1, or of dominant mutants or regulators of ARF1. Inactivation of intracellular ARF1 by high-level expression of either the dominant-negative mutant ARF1/T31N or of ARF1-GAP resulted in dissociation of AP-3 from membrane-bound structures. Although these experiments implicate involvement of ARF1 in membrane association of AP-3, it is possible that the observed effects reflect inhibition of a similar but distinct ARF1-related regulator of AP-3. To address this issue, we demonstrated a direct role for ARF1 by showing that high level expression of a constitutively active ARF mutant (ARF1/Q71L) could overcome the effects of pharmacologic agents or treatments that induced dissociation of AP-3 from membranes. The protective effect of ARF1/Q71L was seen in experiments using two distinct treatments for promoting AP-3 dissociation from membranes, namely BFA, and energy depletion. Furthermore, expression of wild-type ARF1 itself, albeit at extremely high levels, was able to confer protection from the effects of energy depletion. Although BFA acts by affecting ARF1-GTP status, it is not known how energy depletion causes AP-3 dissociation. The ability of ARF1/Q71L to overcome the effects of energy depletion, however, does not necessarily point to a direct effect of this manipulation on ARF1 status (see below). Rather, since

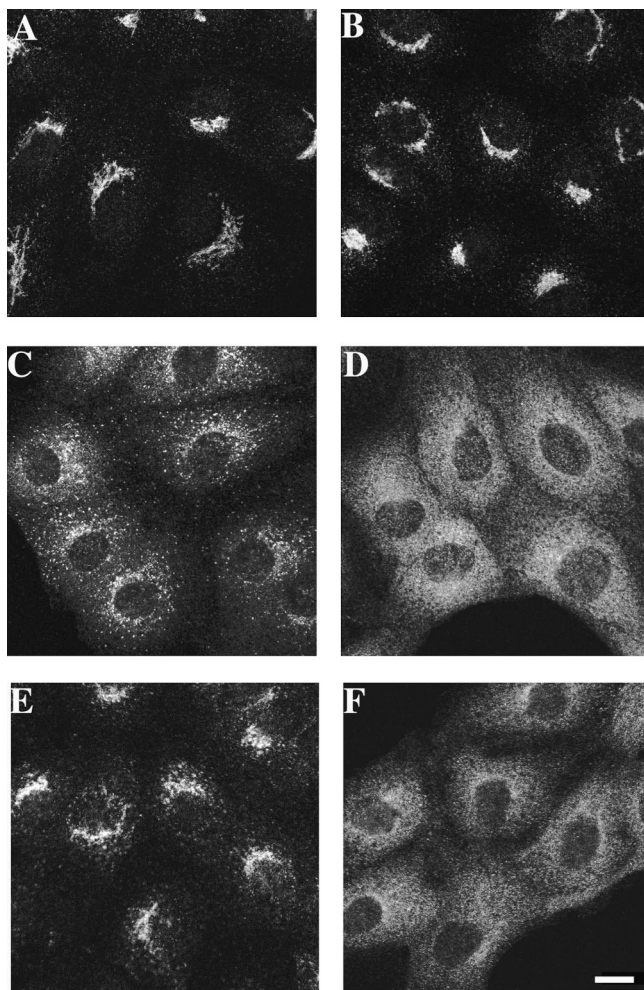


Figure 8. Comparison of the effect of BFA on different ARF1-dependent coat proteins in MDCK cells. MDCK II cells were fixed without previous treatment (A, C, and E) or after exposure to 2 $\mu\text{g/ml}$ BFA for 30 min at 37°C (B, D, and F). Cells were stained with antibodies to COPI (rabbit anti- β -COP) (A and B), AP-3 (rabbit anti- δ) (C and D), and AP-1 (mouse anti- γ) (E and F), followed by Cy3-conjugated anti-mouse or anti-rabbit antibodies. Cells were visualized by confocal immunofluorescence microscopy. Bar, 10 μm .

the interplay of multiple factors (Le Borgne et al., 1996; Rapoport et al., 1997) probably determines coat binding, ARF1/Q71L expression could compensate indirectly by shifting the equilibrium of one of the factors, i.e., ARF1-GTP.

Taken together, the biochemical evidence and in vivo studies indicate that ARF1-GTP promotes the association of AP-3 with membranes. Our observation that activated ARF1 can be found on intracellular AP-3-associated structures provides circumstantial evidence in further support of this conclusion. Since AP-3 is involved in transport within the endosomal-lysosomal system (Cowles et al., 1997; Ooi et al., 1997; Stepp et al., 1997; Faúndez et al., 1998), the punctate ARF1 structures that also stain for AP-3 probably correspond to endosomal structures.

The finding that AP-3 is regulated by ARF1 contributes to the notion that, of the clathrin adaptor complexes, AP-2

is in a separate class distinct from AP-1 and AP-3. Although AP-2 can be recruited to membranes by ARF1 or GTP γ S, this can only be achieved in vitro, and appears to reflect mistargeting under nonphysiological conditions (Seaman et al., 1993; Traub et al., 1993; West et al., 1997). Previous studies (Robinson and Kreis, 1992; Wong and Brodsky, 1992) have pointed to differences between AP-1 and AP-2 recruitment in vivo with respect to sensitivity to BFA and GTP γ S. This is probably a reflection of differences in ARF1 requirement in vivo. We have found that membrane recruitment of AP-2 also differs from that of AP-3 in that conditions which induce AP-3 dissociation from membranes, namely ARF1 inactivation, energy depletion, and lowered temperature (15°C), have no effect on AP-2 membrane association (data not shown). Using similar criteria, we also find that requirements for AP-1 membrane recruitment differ from that for AP-3, even though both of these complexes are regulated by ARF1. AP-1, like AP-3, was rendered cytosolic by energy depletion, but unlike AP-3, remained mostly membrane bound at 15°C (data not shown). Thus, the three adaptor complexes each display a distinct profile when assessed using a panel of factors affecting membrane localization. The sensitivity of AP-3 to energy depletion in vivo (which lowers both ATP and GTP levels) may reflect involvement of events such as protein phosphorylation as well as the requirement for ARF. The effect of lowered temperature could be a change in the properties of proteins or lipids that interact with AP-3 or are specific to the AP-3 pathway. In fact, the observation that 15°C treatment blocks traffic out of endosomal intermediates but not endocytosis from the plasma membrane (Singer and Riezman, 1990; Desnos et al., 1995) may be explained in part by our finding that AP-2 and AP-3 respond differently to 15°C treatment, and by a possible role for AP-3 in the endosomal transport pathway.

The existence of several ARF family members initially led to the idea that different ARF proteins might regulate distinct protein coats and thus different transport pathways. However, ARF1, which was found initially to promote binding of the nonclathrin coat COPI to membranes (for reviews see Donaldson and Klausner, 1994; Boman and Kahn, 1995) was then found to have the same function for a clathrin coat (AP-1) (Stammes and Rothman, 1993; Traub et al., 1993; Dittie et al., 1996). In addition, ARF1 has been reported to be required for endosome-endosome fusion (Lenhard et al., 1992) and for synaptic vesicle biogenesis from endosomes (Faundez et al., 1997). Here, we find that ARF1 is also required for membrane association of another adaptor, AP-3, believed to function in the endosomal-lysosomal system. Indeed, ARF1 has been detected in endosomal fractions, and may be responsible for recruiting COPI to endosomes (Whitney et al., 1995; Aniento et al., 1996). Thus, the emerging picture is that ARF1 regulates many different coat proteins, refuting a 'one ARF-one coat' paradigm. As a consequence of its effect on multiple coats, ARF1 appears to be a regulator of diverse membrane transport systems within the cell. Such effects of BFA on the Golgi and the endosomal-lysosomal system (Lippincott-Schwartz et al., 1991), as well as from recent studies with a yeast *arf* mutant, which indicate that

the orthologue of ARF1 in yeast is required for maintenance of both Golgi and endosome structure and function (Gaynor et al., 1998).

The finding that ARF1 regulates three different coats, COPI, AP-1, and now AP-3, reinforces the idea that ARF1 is not a regulator of specific coats, but rather a common molecular switch that transduces signals from upstream modulators of coat assembly. A candidate for such a modulator is the ARF1 GEF that activates ARF1 by guanine nucleotide exchange. Four different cytosolic and/or Golgi ARF1 GEFs have recently been identified (Chardin et al., 1996; Rosa et al., 1996; Meacci et al., 1997; Morinaga et al., 1997), and a Golgi membrane-associated ARF1 GEF has been described but not yet isolated (Donaldson et al., 1992; Helms and Rothman, 1992; Randazzo et al., 1993). It is possible that activation of ARF1 by these distinct GEF proteins leads to recruitment of different coat proteins. In support of such a scenario, we find in MDCK cells that two ARF1-regulated coats, COPI and AP-3, display differential sensitivity to BFA. Since BFA appears to be an inactivator of ARF1 GEF, this implies the existence of distinct ARF1 GEFs that control different coats via ARF1. In addition, two Golgi-localized ARF1-regulated coats, COPI and AP-1, also display differential sensitivity to BFA (Wagner et al., 1994; this study). Thus, different subdomains of an organelle can exhibit independent ARF1-regulated events.

The GTPase-activating protein ARF1-GAP is another ARF1 regulatory protein that may represent a modulator of ARF1 activity that is specific for a particular coat. We cannot distinguish at present whether the effect of the Golgi-localized ARF1-GAP on AP-3 in our experiments is due to a direct or indirect effect of ARF1-GAP. Conceivably, the latter case could arise from sequestration of the pool of available intracellular ARF1 by cytosolic overexpressed ARF1-GAP. Finally, the finding that the KDEL receptor ERD2 regulates ARF1-mediated events by recruiting ARF1-GAP (Aoe et al., 1997) raises the possibility that other as yet unidentified transmembrane receptors modulate different coat proteins via ARF1-GAP.

The molecular mechanism by which ARF1 recruits coat proteins is not known. Two prevailing views are (a) ARF is a stoichiometric receptor for the coats themselves; and (b) ARF acts catalytically through activation of the enzyme phospholipase D, locally remodeling lipids to generate sites favorable for coat protein binding (Ktistakis et al., 1996). The finding that ARF1 is found on isolated coat vesicles in a 3:1 molar ratio relative to COPI (Serafini et al., 1991), and report of a direct interaction of ARF1 with the β -COP subunit of COPI (Zhao et al., 1997) supports the first model. Evidence for the second model is provided by the finding that exogenous phospholipase D can recruit coat proteins to membranes and also stimulate release of nascent secretory vesicles from the TGN (Ktistakis et al., 1996; Chen et al., 1997). The two models are not mutually exclusive, and both mechanisms may be in operation.

Do other ARF family members regulate AP-3? ARF6 is clearly distinct from all the other ARFs, based on its structural, morphological, and functional characteristics. From our studies, it appears that ARF3, while apparently able to affect AP-3 localization, is a 'weaker' version of ARF1. We also found that ARF5 mutants had limited or no effect

on AP-3. The much less evident colocalization of ARF3 and ARF5 with AP-3 is consistent with the results of the functional assays. Thus, although different ARFs appear to be able to substitute functionally for ARF in yeast *arf* mutants (Stearns et al., 1990; Kahn et al., 1991), and have similar in vitro activities (Liang and Kornfeld, 1997), they do not appear to be strictly redundant in mammalian cells in vivo.

The recruitment of coat proteins to their appropriate intracellular membranes is a critical step in the initiation of a transport event. It is dependent upon the interplay of multiple molecular interactions that are coordinated by the adaptor complex which itself is a component of the coat. The conclusions of this study contribute to the concept of ARF as a common molecular switch in the different regulatory pathways that govern this process for distinct coats.

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