Overexpression of Wild-type and Mutant ARF1 and ARF6: Distinct Perturbations of Nonoverlapping Membrane Compartments

Peter J. Peters,*‡ Victor W. Hsu,* Chean Eng Ooi,* Dario Finazzi,* Stephanie B. Teal,* Viola Oorschot,‡ Julie G. Donaldson,* and Richard D. Klausner*

*Cell Biology and Metabolism Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892; ‡Department of Cell Biology, Faculty of Medicine and Institute of Bio Membranes, Utrecht University, 3584 CX Utrecht, The Netherlands

Abstract. The ARF GTP binding proteins are believed to function as regulators of membrane traffic in the secretory pathway. While the ARF1 protein has been shown in vitro to mediate the membrane interaction of the cytosolic coat proteins coatomer (COP1) and γ-adaptin with the Golgi complex, the functions of the other ARF proteins have not been defined. Here, we show by transient transfection with epitope-tagged ARFs, that whereas ARF1 is localized to the Golgi complex and can be shown to affect predictably the assembly of COP1 and γ-adaptin with Golgi membranes in cells, ARF6 is localized to the endosomal/plasma membrane system and has no effect on these Golgi-associated coat proteins. By immunoelectron microscopy, the wild-type ARF6 protein is observed along the plasma membrane and associated with endosomes, and overexpression of ARF6 does not appear to alter the morphology of the peripheral membrane system. In contrast, overexpression of ARF6 mutants predicted either to hydrolyze or bind GTP poorly shifts the distribution of ARF6 and affects the structure of the endocytic pathway. The GTP hydrolysis-defective mutant is localized to the plasma membrane and its overexpression results in a profound induction of extensive plasma membrane vaginations and a depletion of endosomes. Conversely, the GTP binding–defective ARF6 mutant is present exclusively in endosomal structures, and its overexpression results in a massive accumulation of coated endocytic structures.

THE compartmentalization of eukaryotic cells into functionally distinct membrane-bound organelles demands a highly specified and tightly controlled transfer of membrane between compartments, a process referred to as membrane traffic (reviewed in Rothman, 1994; Kreis, 1992; Pryer et al., 1992). The general requirements for traffic include the production of transport intermediates that are directed from their site of origin to specified target organelles, where they undergo fusion to complete a single or unit step of traffic. These requirements are reflected in the distribution of biochemical components of membrane traffic, some of which are used in most, if not all steps, such as NSF and SNAPS (Rothman and Orci, 1992), as well as others whose involvement is limited to specific steps at specific compartments, such as the rab GTPases (Zerial and Stenmark, 1993), and specific v- and t-SNARE interactions (Rothman, 1994).

A successful membrane traffic system consists of a number of highly controlled processes, including the selective sorting of components into and out of transport intermediates, and the correct targeting of these transport intermediates. In addition, membrane movement through organelles demands that such traffic be precisely controlled, on the one hand, to avoid potentially profound dysequilibrium, and on the other hand, to allow for changes in traffic that accompany altered metabolic needs, differentiation, and response to external signals. It is likely that this control is, in part, mediated by the many GTPases that are involved in traffic. The first evidence that GTPases were essential in traffic came from studies in *Saccharomyces cerevisiae* that resulted in the identification of Yptlp and Sec4p, both later characterized as members of the rab family of proteins (Salminen and Novick, 1987; Touchot et al., 1987; Segev et al., 1988). There are more than 30 different mammalian rab proteins known (Chavrier et al., 1992), each of which has a distinct intracellular distribution (Chavrier et al., 1990). Evidence for the importance of rab proteins in specific steps of membrane traffic has been provided by either the overexpression of specific rabs or the overexpression of mutant rab proteins with altered GTP binding or hydrolysis activities (Gorvel et al., 1991; van der Sluijs et al., 1992; Bucci et al., 1992). The current view of the function of rab proteins is that they are involved in the specification of the "correctness" of membrane–membrane interactions at the level of docking, fusion,

Address all correspondence to Richard D. Klausner, Cell Biology and Metabolism Branch, NICHD, National Institutes of Health, Building 18T/101, 9000 Rockville Pike, Bethesda, MD 20892. Tel.: (301)496-6368. Fax: (301) 402-0078. Victor Hsu's current address is Department of Rheumatology and Immunology, Brigham and Women's Hospital and Harvard Medical School, 250 Longwood Ave., #525, Boston, MA 02115.

or both (Zerial and Stenmark, 1993). The identification of lineage specific rab proteins further suggests that they may be involved in the actual specification of traffic pathways (Ayala et al., 1989; Lutcke et al., 1993). While each rab protein has a well-defined location, multiple rabs may be found at the same location. This may reflect any of a number of issues such as the confluence of several traffic pathways at a single organelle, the possibility that different rabs function in different biochemical processes, and the possibility that more than one rab may cooperate in accomplishing a specific traffic step.

The example of the rab proteins may prove to be repeated with other families of small GTPases involved in membrane traffic. A good candidate for a second, distinct family of such GTPases is the ADP-ribosylation factor (ARF)[1] (Tsuchiya et al., 1991; Clark et al., 1993). Currently, five ARF proteins have been identified by cDNA cloning from human cells, and this number is likely to increase (Clark et al., 1993). ARF has received much attention over the recent years. It is abundant on Golgi-derived vesicles coated with coatomer (COP1) (Serafini et al., 1991), and its activation by a membrane-bound guanine nucleotide exchange protein identified in Golgi-enriched membranes appears to be the target of the drug Brefeldin A (BFA) (Donaldson et al., 1992*b*; Helms and Rothman, 1992; Randazzo et al., 1993). Virtually all of the biology of ARF has been studied with ARF1. This protein rapidly cycles between the cytosol (in its GDP form) and specific target membranes (in its GTP form). Current biochemical evidence suggests that ARF1 functions to control the assembly of specific cytosolic coat proteins onto target membranes (reviewed in Donaldson and Klausner, 1994). This appears to involve a stoichiometric requirement for the interaction of ARF-GTP and COP1 with the membrane (Serafini et al., 1991). Inhibition of the activation of ARF with BFA prevents the association of coat proteins with membrane, and it results in redistribution of Golgi membrane into the ER. While the actual effector interactions of ARF1 are not known, its role in the assembly of Golgi-associated coat proteins provides the best described current picture for a function of a GTPase in membrane traffic.

In vitro assays have implicated functions for ARF1 in a number of organelles, including the TGN, where recombinant ARF1 reconstitutes the GTP-dependent assembly of γ-adaptin containing coats onto membranes, precisely analogous to its effects on Golgi COP1 (Stamnes and Rothman, 1993; Traub et al., 1993). In addition, various in vitro assays have implicated ARF1 in ER to Golgi transport (Balch et al., 1992), intra-Golgi transport (Taylor et al., 1992), endosome fusion (Lenhard et al., 1992), and nuclear envelope fusion (Boman et al., 1992). In any reconstitution, however, one must be able to distinguish the ability of a component to affect an assay from the role such a component plays within the cell. For example, ARF1 in vitro may be able to complement a function played by a different, but related protein within the cell. Many of these issues could be resolved by returning to the cell and determining whether ARFs, like rabs, represent a family of proteins, each with its own subcellular localization.

1. *Abbreviations used in this paper*: ARF, ADP ribosylation factor; BFA, Brefeldin A; COP1, nonclathrin coatomer complex; GTPγS, guanosine 5′-[γ-thio]triphosphate; HA, influenza hemagglutinin; TfR, transferrin receptor.

In this paper, we report on the comparison of ARF1 with ARF6 to test the hypothesis that distinct ARFs will have specified locations and functions within the cell. We find that whereas ARF1 is localized to the Golgi region, where its expression can be shown to affect the structure of and association of coat proteins with the Golgi complex, ARF6 is localized to the plasma membrane and early endosomes. ARF6 appears to move through the endocytic pathway rather than between cytosol and membrane as does ARF1, according to its nucleotide cycle. Overexpression of mutants of ARF6 profoundly affects the structures of both the early endosomes and the plasma membrane. Most striking is the accumulation of what may be a novel coat structure on endosomes induced by the expression of an ARF6 mutant that is deficient in nucleotide exchange. These findings suggest that ARF6 plays important roles in the regulation of organelle structure and membrane traffic in the cell periphery.

Materials and Methods

PCR Cloning and Mutagenesis of ARF Proteins

As previously described (Hsu et al., 1992), total RNA from human erythroleukemia cells K562 were extracted, and first-strand cDNA was made using reverse transcriptase and random hexamers as primers (Perkin-Elmer Cetus Instruments, Norwalk, CT). Each 5′ oligonucleotide started with a restriction endonuclease site (EcoRI for ARF1, ARF3, ARF5 and ARF6, and XhoI for ARF4), and then the first 18 bases of the ARF cDNA coding sequence. Each 3′ oligonucleotide also started with a restriction site (XbaI), and then the antisense coding sequence for two tandem stop codons, followed by the hemagglutinin (HA) epitope tag or FLAG epitope tag and the last 24 bases of the various ARFs excluding the stop codon. ARFs without the epitope tags were generated by using the same 5′ primers, and modified 3′ primers that had the antisense sequence for the epitope tags deleted.

For the mutagenesis of ARF6 (Q67L and T27N mutants), single amino acid substitutions in ARF6 were introduced by PCR using a 5′ primer identical to that used for reverse transcription PCR (see above) and a 3′ primer containing the mutation. The following 3′ primers were used: CCCAGCTTAAGCTTGTAGAGGATCGTGTTCTTCCCTGCAGCATCCA for ARF1/T31N, CCAGAGCGGCCGGATCTTGTCTAGACCGCCCACATCCCATACGTTG for ARF6/Q67L, and AATGGTGGTCACCGACTGGCCAAGCTTCAACTTGTACAGGATTGTGTTCTTGCCGGCCGCGTC for ARF6/T27N. The PCR products were digested with EcoRI/AflII for ARF1/T31N, XmaIII for ARF6/Q67L, EcoRI/BstEII for ARF6/T27N, and then used to replace the corresponding fragment in the wild-type, epitope-tagged ARFs. For the G2A mutant, a 5′ primer containing the mutation and a 3′ primer identical to that used for RT-PCR were used. The 5′ primer sequence was GGGGAATTCCATATGGCGAAGGTGCTATCCAAAATCTTCGG. The amplified product was digested with EcoRI and XbaI and subcloned into the expression vector.

All PCR-generated sequences were verified by DNA sequencing (Sequenase; U.S. Biochemical Corp., Cleveland, OH).

Transient Transfections

Human ARFs were subcloned into a modified form of the expression vector pCDLSRα (Takebe et al., 1988), termed pXS, using the restriction sites described above. For transient transfections, cells were transfected using the calcium-phosphate method as previously described (Bonifacino et al., 1989). For 100-mm dishes with 10 ml of medium, 20 μg of plasmid was used. For six-well dishes, each well with 3 ml of medium, 5 μg of plasmid was used.

Cells and Antibodies

Monkey COS and human RD4 and 293 cells were grown in Dulbecco's modified essential medium, 10% FCS, and penicillin/streptomycin.

The following antibodies were used: mouse antibody 12CA5 against HA epitope (BAbCo, Berkeley, CA), rabbit antiserum against HA epitope (BAbCo), mouse monoclonal antibody M2 against the Flag epitope (IBI

Technology, New Haven, CT), rabbit antiserum against Tac antigen (D. Nelson, National Institutes of Health, Bethesda, MD), mouse antibody ID9 against endogenous ARFs (R. Kahn), rabbit antiserum R5 against endogenous ARFs (R. Kahn, NIH), rabbit antiserum against β-COP (J. Lippincott-Schwartz) rabbit antiserum against clathrin (J. Keen, Thomas Jefferson University, Philadelphia, PA), rabbit antiserum against human transferrin (Sigma Immunochemicals, St. Louis, MO), mouse antibody B3/25 against human transferrin receptor (Sigma), rabbit antiserum against horseradish peroxidase (Sigma), mouse antibody 100/3 against γ-adaptin (Sigma), rabbit antiserum against LAMP1 (M. Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA), rhodamine-conjugated donkey antibody against mouse IgG (Jackson ImmunoResearch, Laboratories, Inc., West Grove, PA), fluorescein-conjugated donkey antibody against mouse IgG (Jackson ImmunoResearch), fluorescein-conjugated donkey antibody against rabbit IgG (Jackson ImmunoResearch), and rhodamine-conjugated donkey antibody against rabbit IgG (Jackson ImmunoResearch).

Indirect Immunofluorescence Microscopy

Cells on coverslips were fixed in 2% formaldehyde in PBS for 10 min at room temperature, washed twice with PBS, and then incubated for 5 min in wash medium (PBS/10% FCS/0.02% sodium azide). Coverslips were then incubated in primary antibody (diluted in PBS/10% FCS/0.2% saponin/0.02% sodium azide) for 1 h, washed three times (spaced apart with 5 min incubations) in wash medium, incubated in secondary antibody (conjugated to fluorescent dye and diluted as previously for primary antibody) for 1 h, washed three times (spaced apart with 5-min incubations) in wash medium, and then washed finally with PBS alone. Coverslips were then mounted with fluoromount G (Southern Biotechnologies, Birmingham, AL). Microscopy (Carl Zeiss, Inc., Thornwood, NY) was performed with the 63X oil Planapo lens.

Immunogold Electron Microscopy

The procedures used were as described previously (Peters et al., 1991). The antibodies against the HA epitope, both monoclonal 12CA5 and polyclonal antiserum, have been characterized for EM, and they are described in detail previously (Bosshart et al., 1994). Briefly, samples were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 1 h at room temperature or for clathrin labeling only in paraformaldehyde for 24 h at room temperature. Cells were transferred in 0.2% paraformaldehyde, scraped, and collected. They were then processed for cryoelectron microscopy using an Ultracryo microtome and Diatome diamond knife (Leica, Inc., Deerfield, IL). 45-nm sections were cut at −125°C and collected with a mixture of sucrose and cellulose (Liou, W., manuscript in preparation). Cryosections were incubated at room temperature with antibodies for 30 min, washed, and incubated for 20 min with protein A gold. As a specificity control, untransfected cells were not labeled by either anti-HA antibodies.

Results

ARF1 and ARF6 Are Localized to Distinct Regions of the Cell

Of the five human ARF genes reported in the literature (Kahn et al., 1991; Tsuchiya et al., 1991), ARF1 and ARF6 are most dissimilar in sequence, being only 66% identical at the amino acid level. We cloned each of the reported ARFs from human K562 cells by PCR from RNA, and we confirmed the published sequences. The ARFs were cloned with their normal stop codons or with a carboxy terminal extension corresponding to either the influenza HA (Wilson et al., 1984) or FLAG epitopes (Hopp et al., 1988). The aligned sequences of ARF1 and ARF6 are shown in Fig. 1. The ARFs were transiently expressed in a variety of cells using a modified form of the pCDLSRα vector (Takebe et al., 1988). Using the monoclonal antibody 1D9, which recognizes all known ARFs (Peters, J. P., unpublished observation), we determined by quantitative immunoblotting that pCDLSRα gave between 20- and 80-fold increased levels of

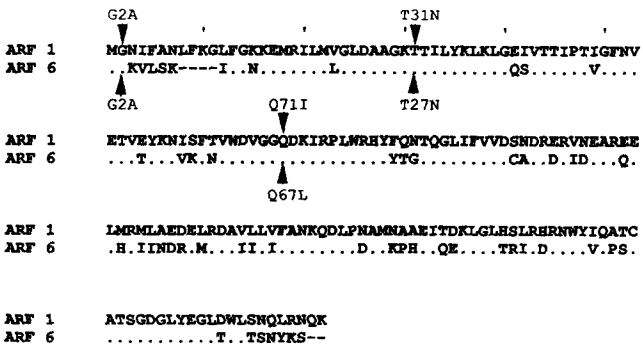


Figure 1. Amino acid sequence comparison of human ARF1 and ARF6. The full sequence of ARF1 is shown along with the aligned differences found in ARF6. The three point mutants used in this study are also shown, at glycine position 2 (both ARF1 and ARF6) substituted by alanine (*G2A*), at threonine positions 31 (*ARF1*) and 27 (*ARF6*) substituted by asparagine (*T31N* and *T27N* respectively), and glutamine positions 71 substituted by isoleucine (Q71I) for ARF1 and 67 substituted by leucine (Q67L) for ARF6.

transfected ARF compared to total ARF on a per-cell transfected basis (data not shown). The actual fold overexpression compared to an individual ARF gene product could not be determined with available reagents.

In all cell lines tested, ARF1 was localized to a compact perinuclear structure, as determined by indirect immunofluorescence microscopy (Fig. 2 *A*). In cells that appeared to express particularly high levels of ARF1, staining for the protein appeared in a diffuse reticular pattern, in addition to a Golgi-like structure. The predominant localization of ARF1 coincided with the distribution of β-COP and several intrinsic Golgi proteins (data not shown). ARF6, however, localized clearly to different structures, being primarily at the plasma membrane and internal punctate structures (Fig. 2 *B*). The latter partially colocalized with the distribution of transferrin receptors (see Fig. 5 *D*). Some of these internal structures were stained with antibodies to the cation-independent mannose-6-phosphate receptor (MPR), but the ARF6-positive internal structures were negative for staining with antibodies directed against the late endosomal/lysosomal protein, LAMP1 (not shown). The localization of ARF6 tagged with the FLAG epitope gave similar results (not shown). We also examined the localization of each ARF lacking the carboxy terminal epitope tag. Using either the monoclonal 1D9 or the polyclonal R5 antibody directed against ARF, we were able to detect the overexpressed introduced protein in addition to the normal staining pattern of endogenous ARFs. These reagents demonstrated that essentially the same staining pattern was observed regardless of the presence of the epitope tag, and they assured us that the epitope tags were not altering the intracellular localization of the ARF proteins.

As a further distinction between ARF1 and ARF6, we compared the effect of BFA on the localization of these ARFs. The involvement of ARF1 in the action of BFA was first suggested by the demonstration of the rapid redistribution of ARF from the Golgi to the cytosol upon addition of BFA to cells (Donaldson et al., 1991*a*). As previously reported (Teal et al., 1994), ARF1 in the transfected cells was rapidly redistributed in response to the drug, whereas

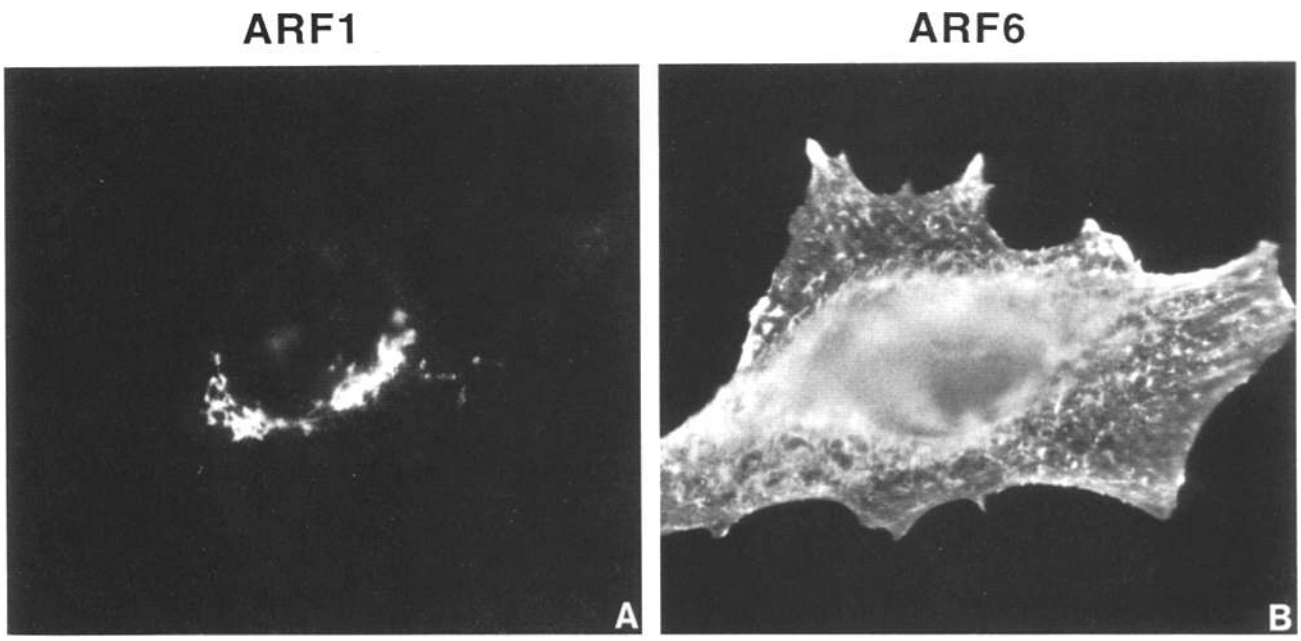


Figure 2. Differential localization of ARF1 and ARF6. Cells were transiently transfected with cDNA encoding HA-epitope-tagged ARF1 or ARF6, and they were stained with mouse anti-HA antibody followed by rhodamine-labeled donkey anti-mouse antibody. Staining for ARF1 (*A*) reveals a tight, juxtanuclear pattern consistent with that of the Golgi apparatus while staining for ARF6 (*B*) is distributed in a widely scattered, peripheral pattern outlining the edges of the cell consistent with plasma membrane. Note the linear structures outlining membrane ruffles, and scattered, internal punctate structures consistent with endosomes.

the membrane distribution of ARF6 was unaffected by the addition of BFA at 10 μg/ml after 1 h (not shown).

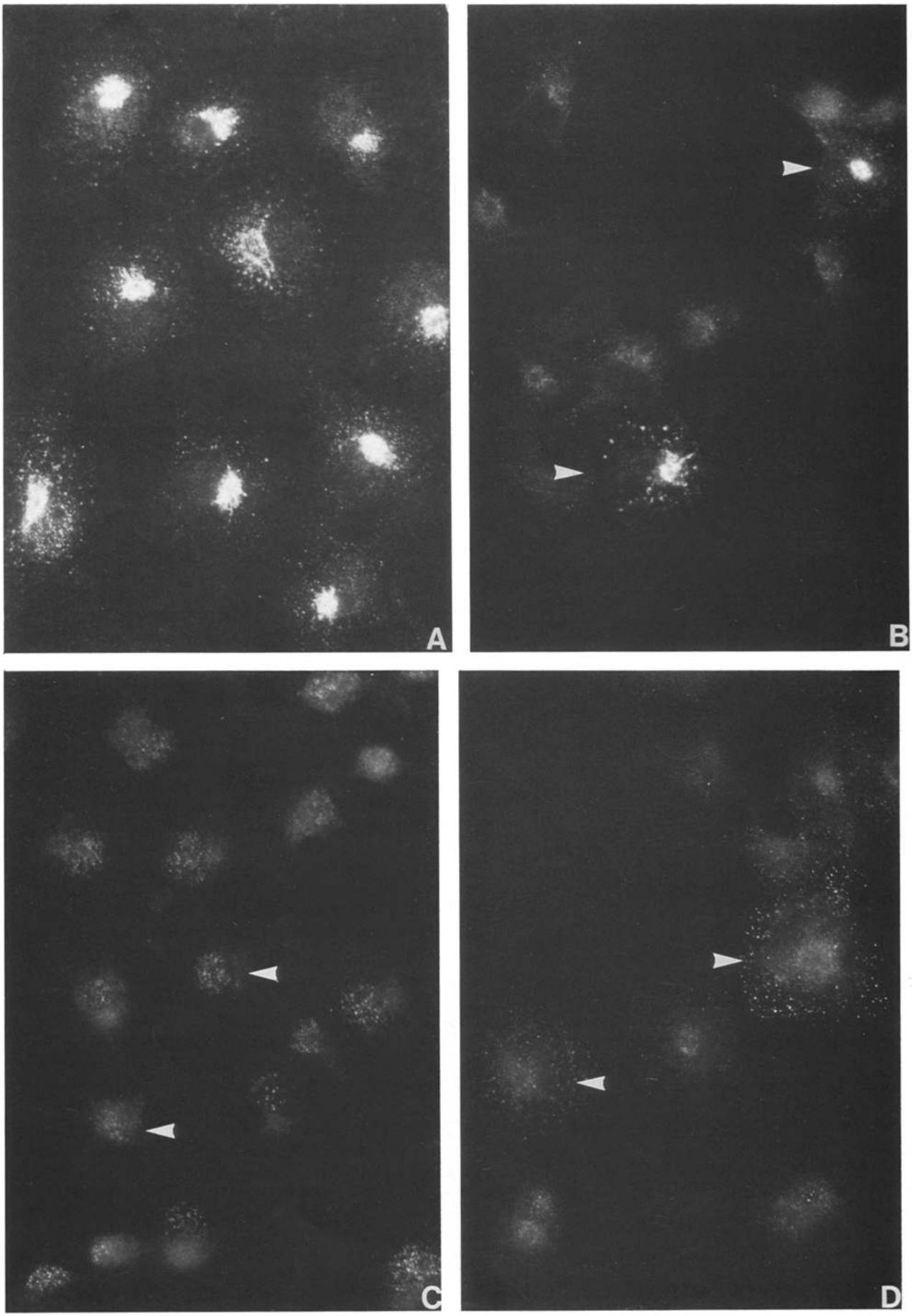
ARF1, but Not ARF6, Supports Coatomer Binding to Golgi Membranes

The distinct localization of ARF1 and ARF6 led us to examine the effects of overexpression of the two proteins on the Golgi and its associated coat proteins. ARF1 has been shown to support the in vitro GTP-dependent association of COP1 with Golgi membranes (Donaldson et al., 1992*a*; Palmer et al., 1993). ARF1 localization to this organelle is consistent with such a function in vivo. Two pharmacologic treatments of intact cells that result in the release of COP1 from the Golgi complex are BFA and agents that cause energy depletion (Donaldson et al., 1991*b*). The mechanism of action of the latter is likely to reflect a drop in GTP levels. We reasoned that if an ARF protein is critical for coatomer binding in vivo, overexpression of that ARF, although not capable of rendering the cell resistant to BFA, might allow coat assembly under conditions of limiting amounts of GTP. We thus used this bioassay to further distinguish ARF1 and ARF6 function.

When COS cells in culture were treated with 0.04% sodium azide and 50 mM 2-deoxy glucose in media containing 10 mM glucose, a redistribution of the β-COP component of COP1 was observed in 100% of untransfected cells (Fig. 3, *A* vs *B*). However, in cells overexpressing ARF1, β-COP remained Golgi associated under these conditions (Fig. 3 *B*, *arrowheads*), indistinguishable from untreated cells. This effect was seen whether or not the carboxy terminal epitope tag was present (not shown). NH_2-terminal myristoylation of the protein was required for this effect since cells overexpressing the nonmyristoylated ARF1 (created by mutation of the second residue from glycine to alanine) showed no protection of the β-COP distribution upon energy depletion (Fig. 3 *C*). Although overexpression of ARF1 could protect β-COP from these moderate energy depletion protocols, it failed to protect after more extreme treatments, such as an increase in the concentration of 2-deoxyglucose and sodium azide, or the use of glucose-free media in the incubation. The shift in the sensitivity of a β-COP redistribution induced by the overexpression of wild-type ARF1 provides in vivo evidence for the ability of this protein to mediate β-COP association with the membrane.

We next looked at the effect of overexpression of ARF6 in similar experiments. In contrast to ARF1, no alteration in the sensitivity of β-COP distribution to energy depletion was ob-

Figure 3. Differential effects of ARF1 and ARF6 in regulating the Golgi coat protein β-COP. COS cells were transiently transfected with cDNAs encoding HA-tagged ARF1, ARF1/G2A, or ARF6. Cells were treated with 50 mM 2-deoxy glucose and 0.04% sodium azide in media containing 10 mM glucose for 30 min at 37°C to deplete energy levels in the cell, fixed, and then double labeled with mouse anti-HA and rabbit anti-β-COP antibodies, followed by fluorescein-labeled donkey anti-mouse and rhodamine-labeled donkey anti-rabbit antibodies. Staining for β-COP is shown, with arrowheads indicating cells that are transfected with the various ARF constructs in *B–D*. In the control experiment (*A*) without energy depletion, untransfected cells stained for β-COP in a Golgi-like pattern. After energy depletion, staining for β-COP is in a Golgi-like pattern in cells overexpressing ARF1 (*B*, *arrowheads*), as compared to untransfected cells or cells transfected with ARF1/G2A (*C*) or ARF6 (*D*), where staining for β-COP is changed to a diffuse cytosolic pattern.


A
B
C
D

served at any level of ARF6 expression, again regardless of the presence of the epitope tag (Fig. 3 *D*). The failure of ARF6 to stabilize the membrane association of β-COP was consistent with its distinct non-Golgi localization. ARF1 also supports the assembly of AP1 adaptor molecules (containing γ- and β′-adaptins) to the TGN (Stamnes and Rothman, 1993; Traub et al., 1993) in vitro. The membrane localization of γ adaptin is lost when cells are treated with energy-depleting regimens (Robinson and Kreis, 1992). Similar to that observed for β-COP, we found that ARF1, but not ARF 6, shifted the dose response of γ-adaptin redistribution in cells to the energy depletion regimen (data not shown). In contrast to energy depletion, overexpression of wild-type ARF1 had no protective effect against the addition of BFA for either β-COP or γ-adaptin.

Expression and Localization of ARF6 Mutants

Because the function of all low molecular weight GTP binding proteins is determined by their GTP binding and hydrolysis cycle, the use of specific pharmacologic reagents that block defined parts of that cycle has proven to be extremely valuable. For ARF1, the ability of BFA and GTPγS, the poorly hydrolyzable analogue of GTP, to inhibit GTP exchange and GTP hydrolysis, respectively, greatly facilitated the elucidation of ARF1 function in cells and in cell-free assays (Donaldson and Klausner, 1994). Additionally, mutations in ARF1 that are predicted, based on sequence alignments with ras (Bourne et al., 1990, 1991), to result in proteins defective in GTP binding and GTP hydrolysis have been generated. These mutant ARF1 proteins behave as predicted both in vivo and in vitro according to the model of ARF action. The expression of an ARF1 mutant (T31N) defective in GTP binding results in an inability of COP1 to bind to Golgi membranes, as well as in the disassembly of this organelle, recreating the BFA phenotype (Dascher and Balch, 1994). Conversely, the mutation in ARF1 (Q71I or Q71L) predicted to slow the rate of GTP hydrolysis results in a constitutively active ARF1. Expression of this mutant in cells results in irreversible binding of coatomer to Golgi membrane, and it renders the coatomer association with Golgi membranes resistant to the effects of BFA (Tanigawa et al., 1993; Teal et al., 1994; Zhang et al., 1994).

Since overexpression of the mutants in the ARF1 protein behaved as predicted from in vitro biochemical studies, recreating BFA and GTPγS treatment, we reasoned that the analogous mutations in ARF6, when expressed in cells, could begin to elucidate the ARF6 cycle by demonstrating the consequences of blocking activation (GTP binding) and termination of activation (GTP hydrolysis). For this reason, we produced two point mutants of ARF6. One mutant, termed Q67L, altered the conserved glutamine at position 67 to a leucine. This mutant is predicted to be defective in GTP hydrolysis and thus "locked" into the active GTP-bound state. Another mutant, T27N, contained a change of a conserved threonine at position 27 in ARF6 to an asparagine, and it is predicted to be defective in GTP binding. The behavior of the T27N mutant vis-a-vis GTP binding has been confirmed (D'Souza-Schorey, C., and P. Stahl, personal communication). Finally we mutated the glycine at position 2 to an alanine (G2A) to test whether the predicted NH_2-terminal myristoylation of ARF6 was necessary for its intracellular localization.

Before examining the consequences of the overexpression of epitope tagged ARF6, we further tested the validity of using overexpressed, epitope-tagged ARFs in these cells by comparing the effects of the GTP binding defective mutants of ARF1 (T31N) and ARF6 (T27N) on the distribution of β-COP. As expected (Dascher and Balch, 1994), overexpression of ARF1/T31N acted as an inhibitor of endogenous ARF1 activation and fully mimicked the effects of BFA on the Golgi and the TGN. The mutant protein itself was diffusely distributed throughout the cell as was β-COP (Fig. 4, *top row*). In cells expressing ARF1/T31N the Golgi marker enzyme mannosidase II was also localized in an ER-like pattern and a complete block in the exit of newly synthesized proteins from the ER was observed (data not shown; see also Dascher and Balch, 1994). In contrast, overexpression of the analogous ARF6/T27N mutant, at similar levels, had none of these effects. In transfected cells, β-COP was localized in the perinuclear region, indistinguishable from that of β-COP in untransfected cells (Fig. 4, *bottom row*). Additionally, in cells expressing the ARF6/T27N, there was no block in the transport of newly synthesized membrane glycoproteins to the cells surface (not shown). Thus, despite the very high levels of protein attained by overexpression, the action of the overexpressed ARFs appears to be specific.

We expressed both wild-type and mutant ARF6 by transient transfection, and we examined the intracellular location of the epitope-tagged proteins by indirect immunofluorescence microscopy. Compared to the wild-type ARF6, which was localized to both the plasma membrane and punctate endosomal-like structures (Figs. 2 *B* and 5 *A*), the distribution of the Q67L mutant was more limited to the cell surface (Fig 5 *B*). In contrast, the distribution of the T27N mutant was shifted to internal structures. The T27N-positive internal structures were heterogeneous in size; there were large granular structures as well as small punctate structures (Fig. 5 *C*). Double labeling experiments revealed that many of these structures colocalized with the transferrin receptor (Fig. 5 *D*). These experiments demonstrate that the localization of ARF6 within the endosomal system can be perturbed by mutations that affect the GTP cycle, and they suggested that the GTP cycle of this protein may be coupled to its localization at different sites in this recycling membrane pathway.

Effects of ARF6 Mutants on the Plasma Membrane and Endosomes at the Ultrastructural Level

We analyzed the intracellular distribution of ARF6 and its mutants at the ultrastructural level by immunogold labeling of cells that were transiently transfected with the different ARF6 constructs. The distributions of the various forms of ARF6 were consistent with the immunofluorescence observations, and they were easily and repeatedly distinguished from each other in double-blind investigations.

Quantitation of the specific immunogold signal revealed that ~40% of the wild-type ARF6 was found on the plasma membrane and ~40% was found on internal membrane structures, with the remaining label found scattered throughout the cytoplasm and nucleus (Fig. 6 *A*). Under higher magnification, we could appreciate that wild-type ARF6 was evenly distributed on the plasma membrane and was neither concentrated in nor excluded from coated pits (Fig. 6 *A*, *inset*). The internal structures labeled were tubulovesicular organelles (Fig. 6 *B*), morphologically characteristic of early

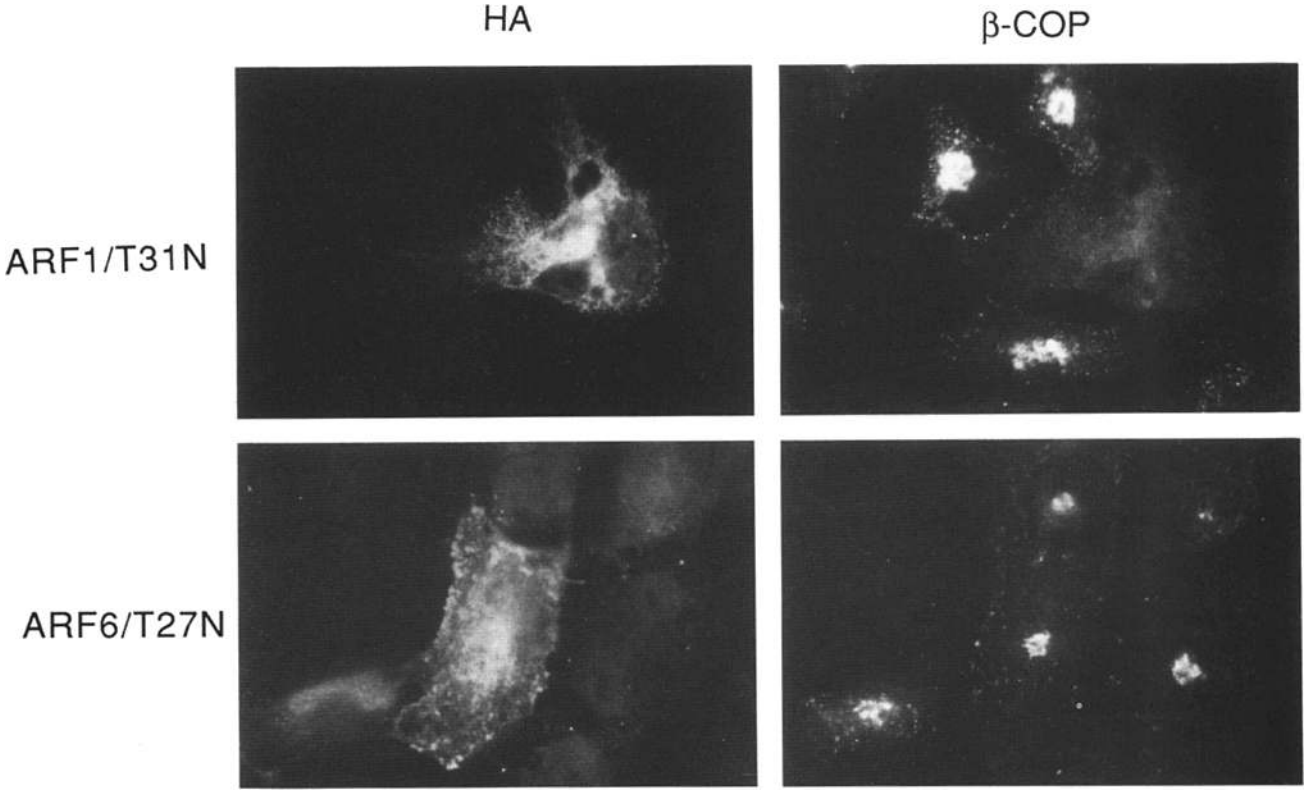


Figure 4. Effects of ARF1 and ARF6 GTP binding defective mutants on β-COP distribution. RD4 cells were transiently transfected with cDNA encoding for HA-tagged ARF1/T31N or ARF6/T27N. Cells were stained with mouse anti-HA and rabbit anti-β-COP antibodies followed by fluorescein-labeled donkey anti-mouse and rhodamine-labeled donkey anti-rabbit antibodies. Compared to untransfected cells, the staining for β-COP in cells expressing HA-ARF1/T31N is changed from its normal Golgi associated pattern to a diffuse, cytosolic pattern, while the staining for β-COP in cells expressing ARF6/T27N remains unaffected.

endosomes, or the previously described CURL (Geuze et al., 1983). While no anti-human transferrin receptor (TfR) antibodies were available for cryoimmunoelectron microscopy, double labeling experiments indicated that these structures were primarily negative for LAMP-1, a marker of late endosomes and lysosomes (data not shown). The endosomal structures contain a vacuolar region surrounded by tubulovesicular extensions that are believed to represent recycling structures (Geuze et al., 1983, 1984). Labeling for ARF6 in these structures was preferentially seen in these extensions. Compared to untransfected cells, cells expressing the wild-type ARF6 had similar numbers of coated pits and clathrin-coated vesicles. The Golgi cisternae appeared unaffected.

We next evaluated the effect of the mutation predicted to prevent myristoylation (G2A) on the distribution of ARF6 (Fig. 6 *C*). A similar mutation in ARF1 had been previously shown to lead to a loss of myristoylation, membrane localization, and effector function. By immunoelectron microscopy, the distribution of gold particles for this mutant seemed dispersed, with a loss of membrane localization. This protein could be found mainly in the cytoplasm, and to a lesser extent, in the nucleus and mitochondria. The labeling intensity is higher than with wild-type ARF6 because the epitope is most likely better exposed in a cytosolic location. Membrane compartments appeared unperturbed in cells expressing this mutant.

We next examined the results of cells expressing the ARF6 hydrolysis-defective mutant, Q67L. In this case, gold particles were localized almost exclusively (~90%) to the plasma membrane (Fig. 7). Most of the remaining staining was still membrane bound, but they were in internal structures that have yet to be characterized. The structure of the cell surface was profoundly altered in cells overexpressing the Q67L mutant. When compared to untransfected cells whose surface was smooth, the Q67L cells exhibited a marked increased in plasma membrane surface area with tortuous invaginations and sheetlike extensions. These extensive areas of the cell surface were largely devoid of coated pits. These invagination, to a lesser extent, were also seen in cells overexpressing the wild-type ARF6. On the rest of the cell surface, the Q67L mutant appeared to be evenly distributed along the plasma membrane with no particular concentration in coated pits. Strikingly, compared to untransfected cells, cells expressing this mutant appeared to be depleted of structures that are characteristic of early endosomes. The number and the morphologic appearance of coated pits, clathrin-coated vesicles, lysosomes, and the Golgi apparatus in transfected cells did not appear to be altered compared to untransfected cells.

Finally, we examined the distribution of the T27N mutant (Figs. 8 and 9). Consistent with the impression given by immunofluorescence microscopy, at the ultrastructural level, >90% of the gold particles labeling for this mutant was in intracellular structures. We did not find any significant proportion of the gold labeling in the cytoplasm or the nucleus. Only a few gold particles were seen in the mitochondria and on the plasma membrane, and occasionally

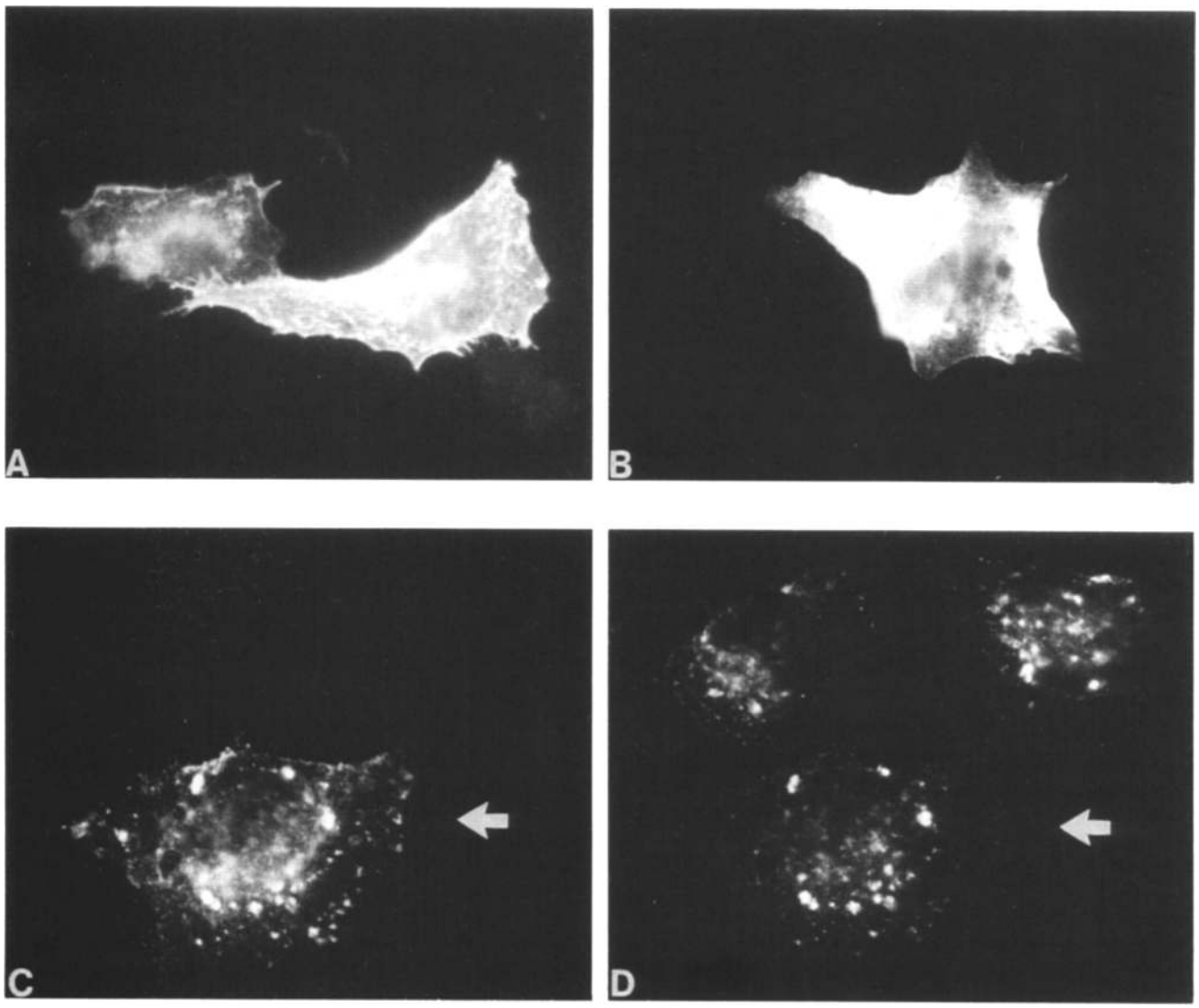
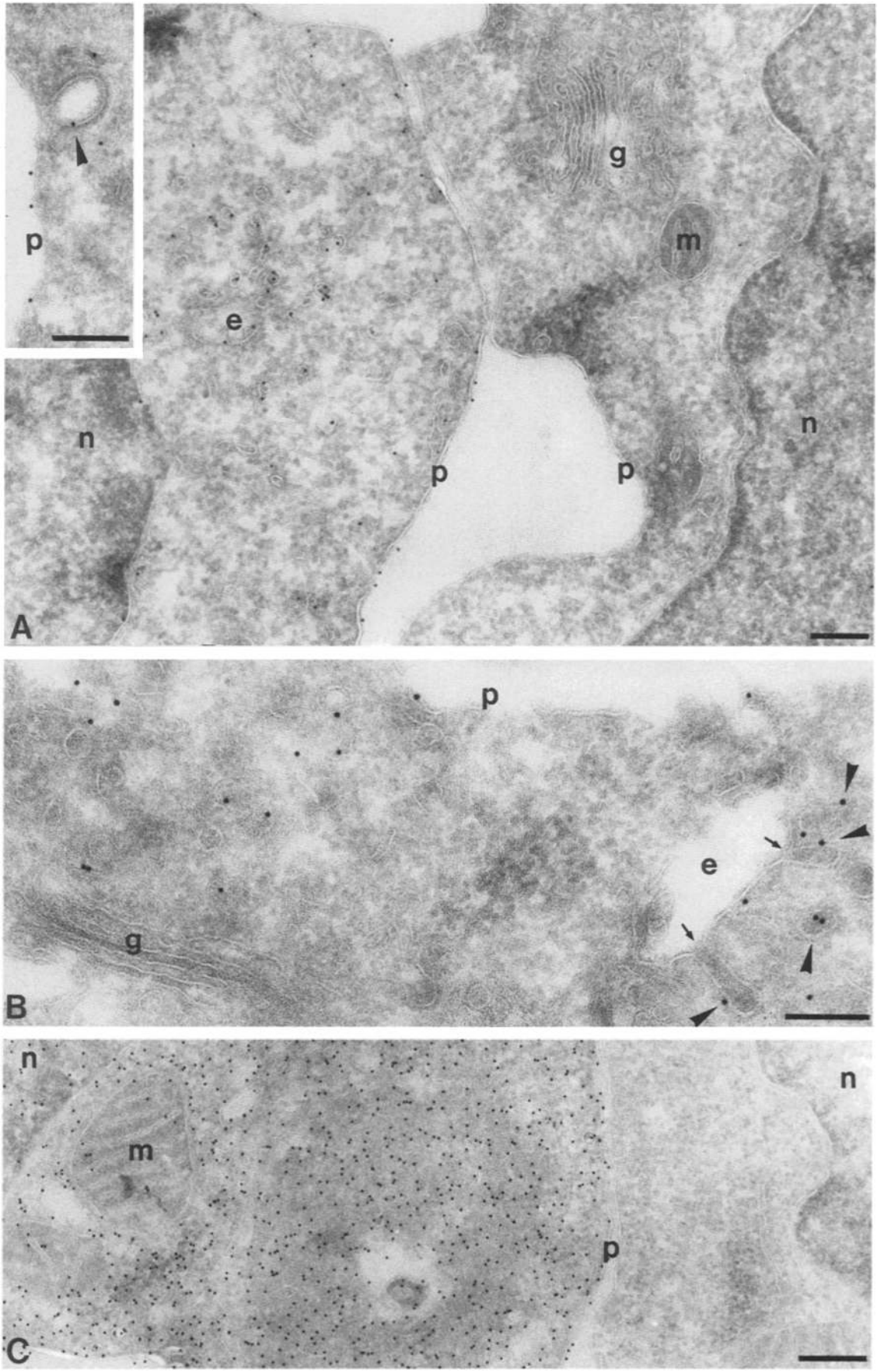


Figure 5. Localization of ARF6 and mutants by indirect immunofluorescence microscopy. RD4 cells were transiently transfected with HA-epitope tagged forms of ARF6 wild-type, Q67L, or T27N mutants. Cells were stained with mouse anti-HA antibody followed by rhodamine-labeled donkey anti-mouse antibody. For colocalization with TfR, cells were stained with rabbit anti-HA and mouse anti-TfR antibodies, followed by fluorescein-labeled donkey anti-rabbit and rhodamine-labeled donkey anti-mouse antibodies. Cells transfected with either ARF6 wild-type (*A*) or Q67L (*B*) revealed diffuse staining of the cell surface consistent with the plasma membrane. In cells expressing ARF6 wild-type (*A*), there is also staining for some internal punctate structures consistent with endosomes. In many cells expressing the T27N mutant (*C*), on the other hand, staining of this mutant ARF6 is mainly in scattered internal structures. These are heterogeneous in size, some being large granular structures, and others being small punctate structures. Both structures show significant costaining for TfR (*D*).

Figure 6. Localization and effects of ARF6 wild-type and G2A mutant at the ultrastructural level. 293 cells were transiently transfected with either HA-tagged ARF6 wild-type or G2A mutant, and were then processed for ultrathin cryosections. Samples were stained with an anti-HA antibody followed by protein A–conjugated gold particles (10 nm). (*A*) A transfected cell (*left*) shows specific gold labeling on the plasma membrane and internal vesicles. Some labeling in the cytoplasm is also seen. The inset for *A* shows a profile of the plasma membrane with a coated pit (*arrowhead*). Note that there is no particular enrichment in the coated pit. An untransfected cell (on the right) is devoid of labeling. (*B*) A higher magnification of a cell expressing ARF6 wild-type, shows specific labeling (*arrowheads*) present on tubular extensions of an endosomal structure. Labeling is also present on some vesicles and on the plasma membrane. (*C*) In a cell expressing the ARF6 G2A mutant, labeling is randomly distributed over the cytoplasm, nucleus, and mitochondria. The Golgi complex and endosomes appear unperturbed. An untransfected cell on the right shows no labeling. *n*, nucleus; *e*, endosome; *p*, plasma membrane; *g*, Golgi complex. Bar, 200 nm.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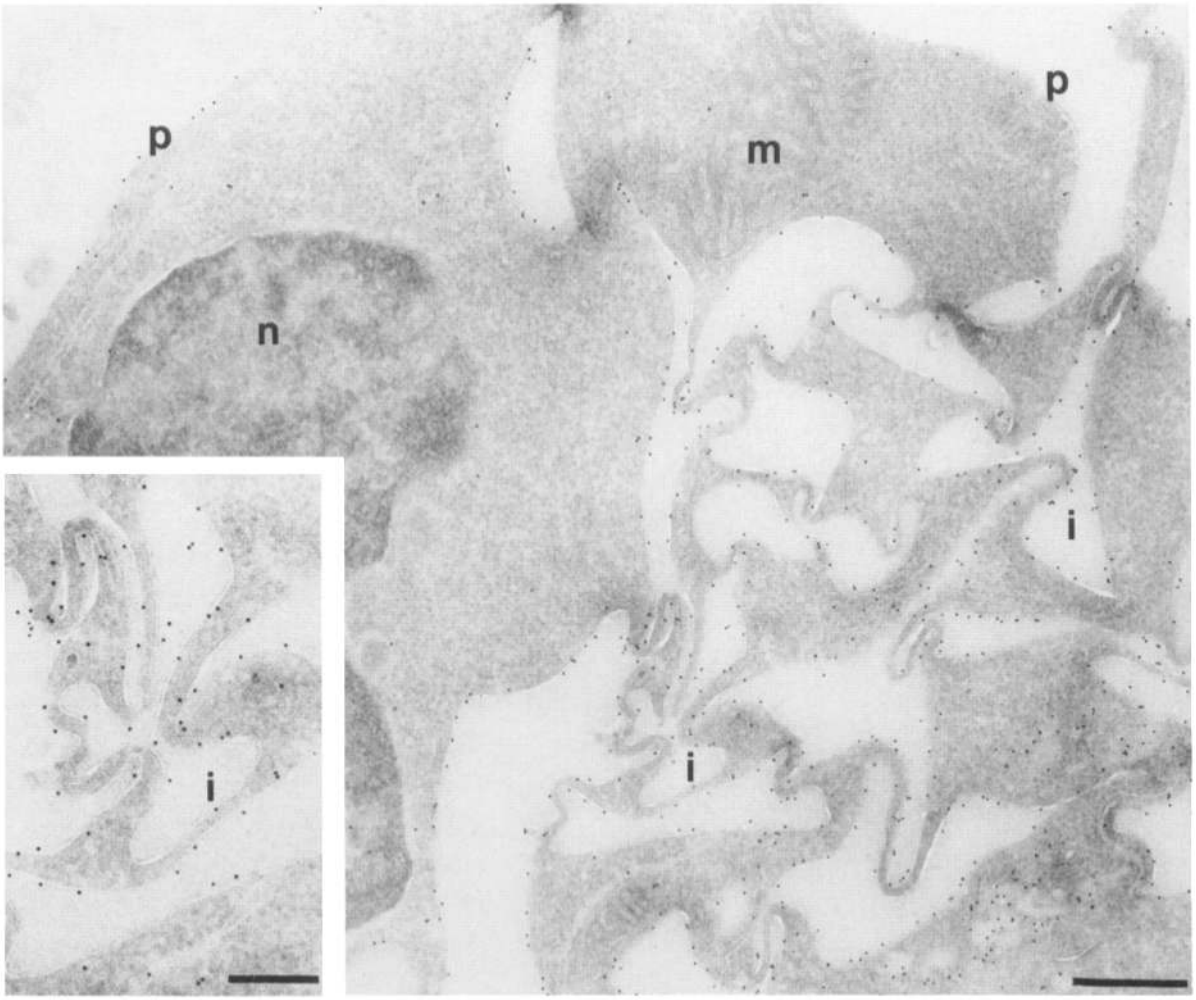

Figure 7. Localization and effects of the Q67L mutant at the ultrastructural level. 293 cells were transiently transfected with the HA-tagged Q67L mutant, and they were then processed for ultrathin cryosections. Samples were stained with an anti-HA antibody followed by protein A–conjugated gold particles (10 nm). In cells overexpressing the Q67L mutant, the plasma membrane is transformed into areas of deep invagination (*i*). Few, if any, endosomal structures are observed. The Q67L mutant appears to reside mainly on the plasma membrane and its deep invaginations. *n*, nucleus; *m*, mitochondria; *p*, plasma membrane; *i*, invaginations. Bar, 500 nm; bar in inset, 200 nm.

clustered in coated vesicles (Fig. 8, *arrow*). The plasma membrane and the Golgi complex appeared normal in these cells, despite of the marked transformation of the endosomal compartment. What appeared as large granular staining patterns seen by immunofluorescence microscopy represented, at the EM level, concentrations of the ARF in regions of $\leqslant 2$ μm in diameter within the cell (Fig. 8). These areas contained vesicular membrane structures of heterogeneous sizes (100–300 nm in diameter) and shapes (Figs 8, *inset*, and 9), suggesting that they are tubulovesicular in nature. In fact, occasionally, profiles of short tubules could be appreciated. These structures were negative for LAMP-1, MPR, and cathepsin D (data not shown). The most prominent feature of these membrane structures was that they were heavily coated on their cytoplasmic face, where gold particles labeling for the mutant ARF were seen (Fig. 8, *inset*). In Fig. 9 we compared the appearance of these coated structures with clathrin-coated vesicles. Using double-immunogold labeling, we found that most (>99%) of these coated structures that were positive for ARF were not labeled for clathrin (Fig. 9). These coats often appeared thinner and less electron dense than the classical clathrin coats, and they were more reminiscent of the COPI coats seen on Golgi-derived vesicles after incubation with GTPγS and cytosol.

Discussion

In this study, we have made use of transient expression of epitope-tagged wild-type and mutant ARF proteins to ascertain the localization and function of these proteins in the cell. We show that ARF1 and ARF6 have distinct effects on membrane traffic and organelle structure and function; the effects of ARF1 are limited to the Golgi apparatus and the TGN, while those of ARF6 are apparent in the endosomal and plasma membrane system.

The considerable data accrued on the biochemical function of ARF1 provides an excellent test for the validity of using overexpressed, epitope-tagged wild-type and mutant ARF proteins to ascertain function. Indeed, the defined roles of ARF1 vis-à-vis membrane traffic (Donaldson and Klausner, 1994) are reiterated by the results of the transient overexpression of mutant forms of epitope-tagged ARF1. First

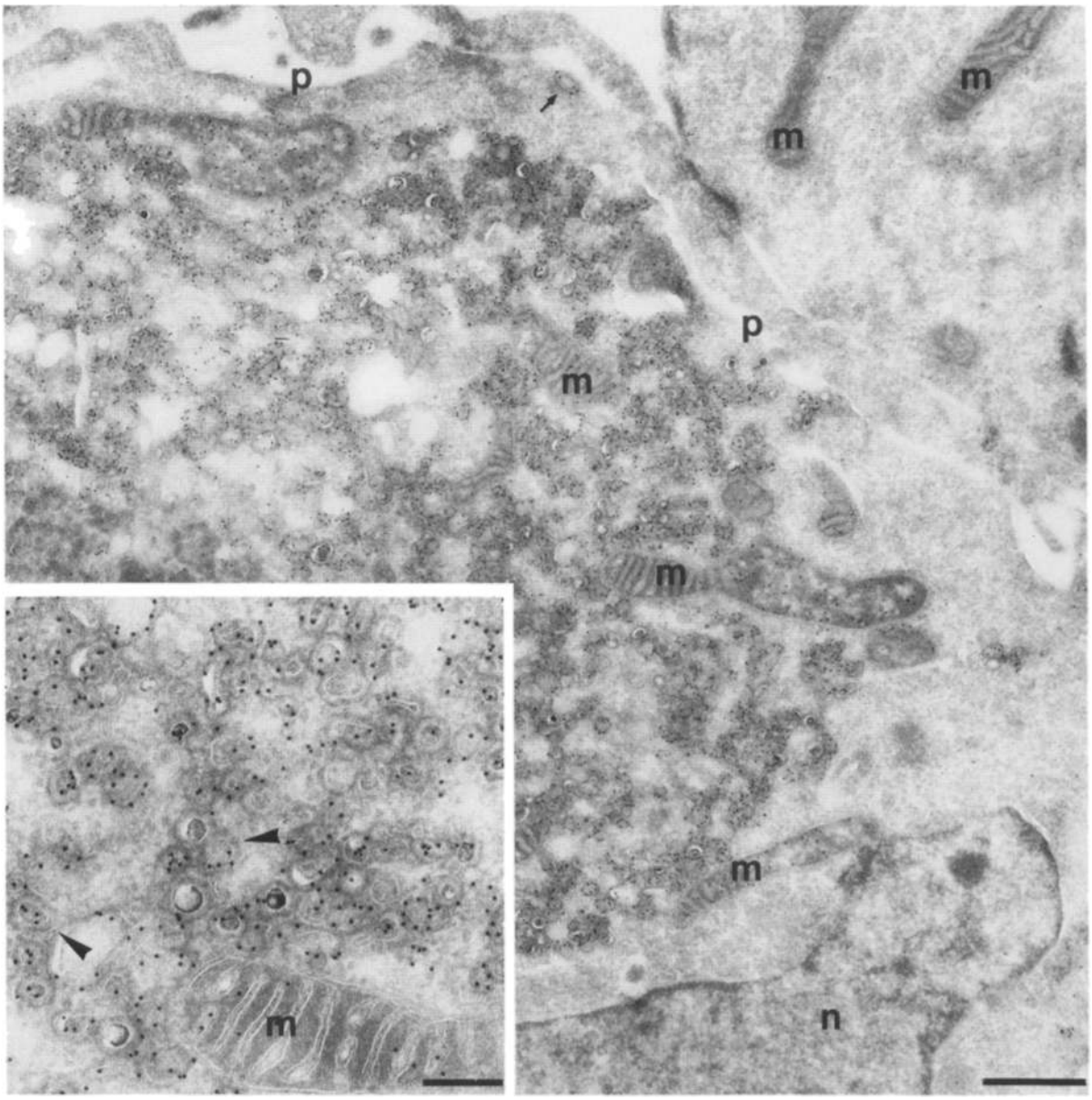

Figure 8. Localization and effects of the T27N mutant at the ultrastructural level. 293 cells were transiently transfected with the T27N mutant, and they were then processed for ultrathin cryosections. Samples were stained with an anti-HA antibody followed by protein A–conjugated gold particles (10 nm). Large aggregations of membranous structures that are labeled by the T27N mutant appear in the transfected cell. These regions are ≤2 μm in diameter. The inset shows a higher magnification of this area and reveals multiple-coated (*arrowhead*) tubulovesicular structures. Labeling for the T27N mutant is mainly on these coated structures. There is also some labeling in the mitochondria. The plasma membrane is sparsely labeled, with occasional labeling of a coated structure (*arrow in the upper region of the main panel*). An untransfected cell is seen in the upper right region of the main panel, and it is devoid of labeling. *n*, nucleus; *m*, mitochondria; *p*, plasma membrane. Bar, 1 μm; bar in inset, 200 nm.

HA-ARF1 is predominantly localized to the Golgi and associated structures over a wide range of expression levels. Second, the overexpression of the tagged mutants affect the Golgi structure, association of coat proteins, and intracellular traffic, as predicted from in vitro biochemical and transport assays, as well as from the studies on the cellular effects of BFA. Therefore, overexpression of ARF1 and the presence of the COOH-terminal epitope tag do not result in nonphysiological localization, nor do they inhibit the predicted effector functions of the protein. Furthermore, the effects and localizations of ARF1 have no overlap with those of ARF6 for both wild-type and mutant versions of the proteins. The distinct effects of equivalently expressed epitope-tagged ARF1 serve as an internal specificity control for the novel but distinct effects of ARF6.

ARF1, but Not ARF6, Regulates the Assembly of Coat Proteins onto the Golgi Complex In Vitro

The localization of ARF1 to the Golgi complex and associated structures (Stearns et al., 1990) in intact cells is

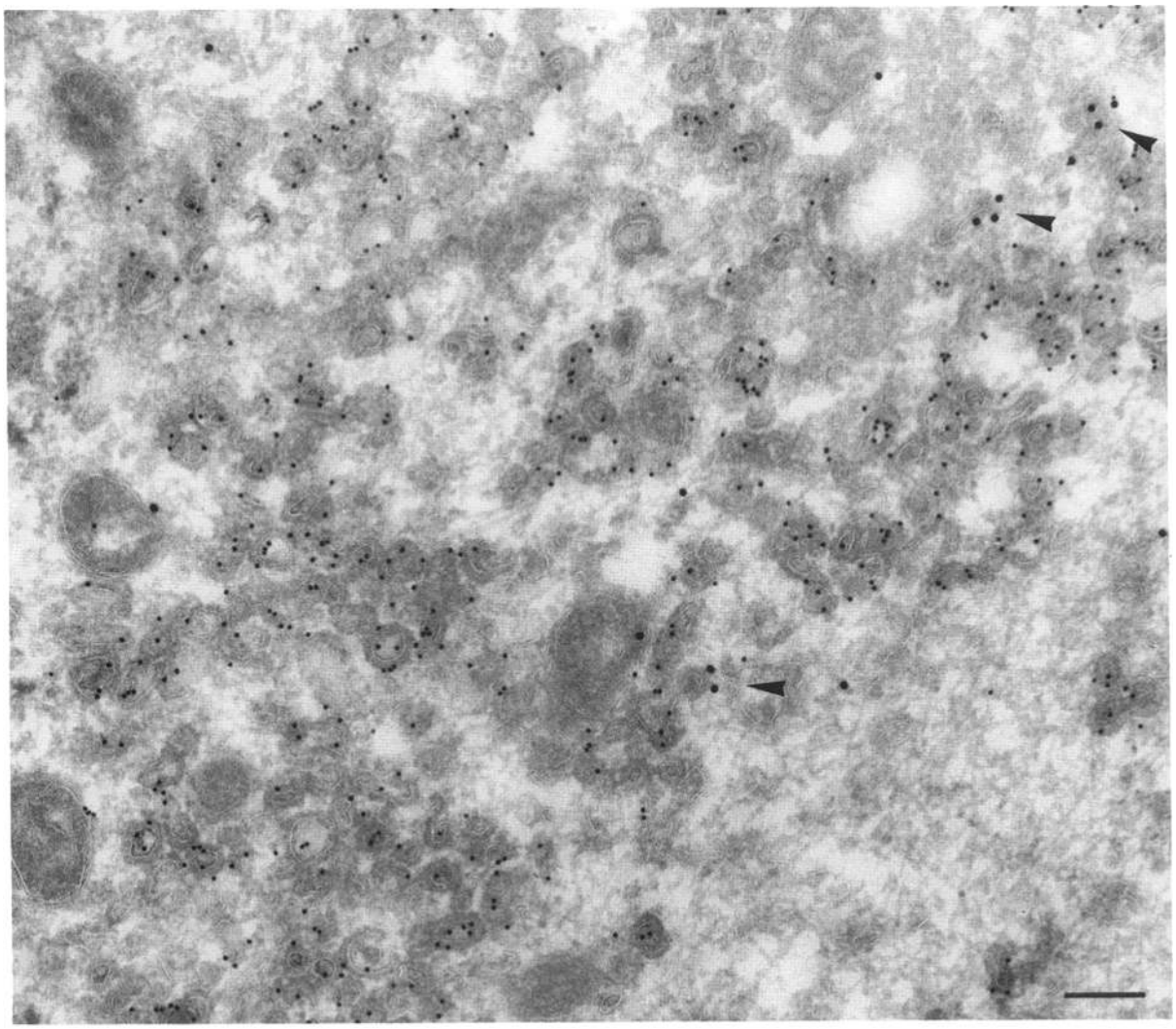

Figure 9. A comparison of the endosomal coat accumulated by the T27N mutant versus the clathrin coat. 293 cells were transiently transfected with the T27N mutant, and they were then processed for ultrathin cryosections. Samples were stained first with a rabbit anticlathrin antibody followed by protein A–conjugated gold particles (15 nm), and then the mouse monoclonal anti-HA antibody (12CA5) followed by protein A–conjugated gold particles (10 nm). Vesicular profiles demonstrating immunolabeling with clathrin antibodies (15 nm gold) are shown with arrowheads. Bar, 200 nm.

consistent with a growing body of experimental evidence that this protein can support the GTP-dependent assembly of at least two sets of coat proteins; the Golgi-associated COP1 (Donaldson et al., 1992*a*; Palmer et al., 1993) and the TGN-associated AP1/clathrin complexes (Stamnes and Rothman, 1993; Traub et al., 1993). A number of observations support that the ARF1 GTP cycle can determine the assembly and disassembly of these coat proteins within the intact cell. First, as previously shown, expression of a GTPase-defective mutant of ARF1 protects the membrane association of β-COP from the effects of either the addition of BFA or of energy depletion (Tanigawa et al., 1993; Teal et al., 1994; Zhang et al., 1994). In this study, we introduce a further observation that ties wild-type ARF1 expression to the distribution of these coats in vivo. Overexpression of wild-type ARF1 has no obvious effect on Golgi structure. It does, however, protect both β-COP and γ-adaptin from the effects of energy or GTP depletion. The protection afforded by the overexpression of ARF1 is actually a shift in the sensitivity to energy depletion. Thus, it requires more severe regimens (higher 2-deoxyglucose and/or lower glucose concentrations) to observe the redistribution of either β-COP or γ-adaptin in ARF1–overexpressing cells than in untransfected cells. One possible explanation for this effect is that it is the product of the concentrations for ARF1 and GTP that determine the ability of these proteins to bind to their target membranes. Thus, a drop in GTP levels can be compensated for by the increase in ARF1. These observations link the expression of ARF1 to the membrane association of these proteins within the cell and show that, at limiting GTP levels, ARF1 becomes limiting for coat protein assembly onto membranes.

As discussed above, the specificity of the distinct ARF family members is clearly illustrated by the complete lack of effect of ARF6 and ARF6 mutants on any aspect of Golgi structure and function. This specificity suggests that even in cells perturbed by massive overexpression of ARF proteins,

the effects reflect defined targets of each member of the ARF family.

The ARF6 GTP Cycle and Its Localization in the Recycling Endocytic Pathway

Both the intracellular localization and the functional effects of ARF6 are nonoverlapping with ARF1. At all levels of expression observed, the distribution of wild-type ARF6 was mainly between the plasma membrane and TfR-positive recycling endosomes. There was no apparent fine structure to the plasma membrane distribution, and no obvious accumulation at coated pits was detected. We did however, have the impression that the endosomal wild-type ARF6 was preferentially found on the tubulovesicular extensions of the recycling endosomes, suggesting that it may be recycled, via those structures, to the plasma membrane (Geuze et al., 1983, 1984). While wild-type ARF6 is capable of associating with both the plasma membrane and recycling endosomes, the GTP cycle mutants reside either on the plasma membrane (Q67L) or in internal structures (T27N). Despite the fact that overexpression of either of these mutants profoundly perturbs the structure of the organelles on which each is found, their distribution and effects are distinct and essentially nonoverlapping. We can interpret the restricted localization as reflecting the GTP cycle requirements for ARF6 to exit from each location. Thus, the fact that the Q67L hydrolysis-defective mutant is restricted to the cell surface suggests that GTP hydrolysis is a prerequisite for ARF6 to be internalized. Conversely, the restriction of T27N to endosomes suggests that GTP loading is a prerequisite for its recycling from that structure to the cell surface. The fact that the wild-type protein can be found in both locations is consistent with its being able to both bind and hydrolyze GTP. While the precise membrane localization of ARF6 appears to depend on its GTP state, association with membrane requires myristoylation, as demonstrated by the G2A mutation.

The distinct distribution patterns for the two mutant proteins demonstrate an additional difference between ARF6 and ARF1. ARF1 rapidly cycles on and off the membrane in a manner that is coupled to its GTP binding and hydrolysis cycle, consistent with in vitro binding data (Regazzi et al., 1991). In contrast, to date, we have no evidence that ARF6 ever dissociates from the membrane, regardless of its nucleotide status. Furthermore, neither ATP depletion nor BFA appears to affect the membrane association or distribution of ARF6. The latter may reflect the possibility that ARF6 activation is insensitive to BFA.

Effects of ARF6 on the Structure of the Peripheral Membrane System

Overexpression of mutant ARF6 proteins has profound effects on the structure of the plasma and endosomal membrane system. The function of this protein in the control of membrane dynamics and membrane–coat protein interactions may be elucidated by considering the effects of the two mutants. As with all such GTP-binding proteins, the function(s) of each protein is tied to its GTP binding and hydrolysis cycle. In all known cases, it is the GTP form of the protein that possesses active effector function. Thus, we may begin by asking where ARF6-GTP normally functions.

The T27N mutant, as predicted, appears to be defective in binding of GTP (D'Souza-Schorey, C., and P. Stahl, personal communication). If it acts as a competitive antagonist of the endogenous wild-type ARF6, the site where this "dominant negative" mutant accumulates should give us a clue as to where the activation of ARF6 is required. The most striking consequence of the overexpression of the T27N mutant is the accumulation of large aggregates of coated tubulovesicular structures. These structures could represent either unfused endocytic structures that have acquired a new coat or recycling endosomal remnants, because of the partial colocalization with the transferrin receptor, the lack of localization with LAMP1 and M6PR, and the loss of undisturbed endosomal profiles. However, the exact identity of the coated structures and their biogenesis will require alternative approaches, such as the microinjection of ARF6 mutant proteins and the evaluation of additional endosomal markers such as rab4 or rab5. If we assume that the overexpression of the T27N mutant is acting via the inhibition of production of ARF6-GTP, we must conclude that one of the functions of activated ARF6 would be to either inhibit the assembly or to stimulate the disassembly of the accumulated coat. The endosomal coat appears clearly different from classical clathrin coats by EM. Immunologically, this coat does not appear to have significant staining for clathrin (Fig. 9), β-COP (Fig. 4), and α- and γ-adaptins (not shown). Identification of this novel endosomal coat material remains to be determined.

The expression of the predicted hydrolysis-resistant mutant (Q67L) results in the elaboration of plasma membrane leading to extensive invaginations and sheet-like extensions. The restriction of the ARF6/Q67L to the cell surface suggests that hydrolysis of ARF6-GTP occurs at that site. This raises the possibility that an additional function of active ARF6 is to effect the structure of the cell surface. It remains to be determined how these morphological changes of ARF6-Q67L relate to those observed with T27N.

Regulators of Surface Membrane Dynamics

Despite the relatively long history of the study of receptor-mediated endocytosis, the detailed biochemistry of this process is only beginning to be unraveled (reviewed by Schmid, 1993). As with all dynamic processes within the cell, the internalization and recycling of molecules to and from the cell surface is likely to be subject to a complex array of switches and points of regulation. Throughout the exocytic and endocytic pathways, GTP-binding proteins appear to provide a prominent class of such switches. Two rab proteins, rab4 and rab5, have been reported to have significant effects on the dynamics of membrane movement in the early endocytic pathway (van der Sluijs et al., 1992; Bucci et al., 1992). While overexpression of rab5 enhanced endocytosis and endosome fusion, overexpression of rab4 increased the rate of receptor recycling. A more direct demonstration of a regulator of endocytosis is dynamin, a distinct GTP-binding protein that is involved in the early steps of clathrin-mediated endocytosis (Shpetner and Vallee, 1989). First connected to endocytosis when it was recognized as the gene responsible for the *Drosophila shibire* mutation (van der Bliek and Meyerowitz, 1991), dynamin has been shown, via the effects of GTP-binding mutants, to be necessary for the endocytosis of surface transferrin receptor (van der Bliek et al., 1993; Herskovits et al., 1993). Specifically, dynamin is required for

the constriction of coated pits to form coated vesicles (Damke et al., 1994).

The results presented in this paper define ARF6 as a potential regulator of membrane traffic in the peripheral membrane system. The profound effects on the structure of the plasma membrane and endosomes observed with the ARF6 mutants provide compelling reasons to identify and understand the cellular effectors responsible for these changes.

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