Effects of Activated ADP-ribosylation Factors on Golgi Morphology Require neither Activation of Phospholipase D1 nor Recruitment of Coatomer*

(Received for publication, August 27, 1999, and in revised form, November 4, 1999)

Jun Kuai, Annette L. Boman‡, Rebecca S. Arnold, Xinjun Zhu, and Richard A. Kahn§

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

Nine mutations in the switch I and switch II regions of human ADP-ribosylation factor 3 (ARF3) were isolated from loss-of-interaction screens, using two-hybrid assays with three different effectors. We then analyzed the ability of the recombinant proteins to (i) bind guanine nucleotides, (ii) activate phospholipase D1 (PLD1), (iii) recruit coatomer (COP-I) to Golgi-enriched membranes, and (iv) expand and vesiculate Golgi in intact cells. Correlations of activities in these assays were used as a means of testing specific hypotheses of ARF action, including the role of PLD1 activation in COP-I recruitment, the role of COP-I in Golgi vesiculation caused by expression of the dominant activating mutant [Q71L]ARF3, and the need for PLD1 activation in Golgi vesiculation. Because we were able to find at least one example of a protein that has lost each of these activities with retention of the others, we conclude that activation of PLD1, recruitment of COP-I to Golgi, and vesiculation of Golgi in cells are functionally separable processes. The ability of certain mutants of ARF3 to alter Golgi morphology without changes in PLD1 activity or COP-I binding is interpreted as evidence for at least one additional, currently unidentified, effector for ARF action at the Golgi.

ADP-ribosylation factors (ARFs)

ADP-ribosylation factors (ARFs) contribute to vesicle formation. ARFs are a family of 20-kDa GTPases first identified as the guanine nucleotide cofactor required for the ADP-ribosylation of the G_s protein, catalyzed by the ADP ribosylation factor (ARF) subunits of the bacterial toxins, cholera toxin or Escherichia coli heat-labile toxin (LT_A1) (1). The high degree of structural and functional conservation of ARFs cloned from a wide variety of eukaryotic organisms has allowed a variety of experimental approaches addressing the mechanism(s) and role(s) of ARFs in cell regulation. For example, in the yeast Saccharomyces cerevisiae, ARFs are required for protein secretion (2), sporulation (3), mitotic growth (4), and respiration (4); in flies, deletion of the arl gene (Arl1) causes embryonic lethality (5). The complexity of ARF signaling in any organism or cell is incompletely understood, and the number of immediate downstream effectors continues to expand (6). The two best characterized effectors are the heptameric coat complex, termed coatomer or COP-I, and phospholipase D1 (PLD1). Indeed, the recruitment of COP-I by ARF has been proposed as the nucleating step in budding and subsequent formation of coated vesicles in transport from Golgi stacks (7, 8) and the activation of PLD1 by ARF has similarly been proposed as an alternative initiating event in vesicle formation (9).

Vesicular transport is important not only in the cargo that is selectively shuttled between compartments but also in the maintenance of gradients or differences in the luminal contents or lipid composition of the donor and acceptor membranes. The presence of protein coats on buds, and later vesicles, emanating from a membrane site is proposed to assist in the formation of the vesicle, in the selection of cargo destined to enter the nascent vesicle, in the targeting to the appropriate docking site, and in the regulation of the fusion by regulating the timing and site of uncoating. There are currently five different, multimeric, protein complexes designated as coats: COP-I, COP-II, AP-1, AP-2, and AP-3. Each of these has been reported to be recruited to membranes by a member of the ARF family: COP-I (10), AP-1 (11), AP-2 (12), and AP-3 (13) by an ARF itself and COP-II by the more divergent SAR1 protein (14). The most extensively characterized interaction is that between COP-I and ARF proteins as they were first found to copurify on COP-I-coated vesicles and later purified ARF was found to be necessary and sufficient to support the binding of purified coatomer to Golgi membranes upon activation with the nonhydrolyzable analog GTP_yS (7, 10). Thus, the activation of ARF is proposed as the initiating event, leading directly to recruitment of coats and later steps that result in the formation of coated vesicles.

With the discovery that ARF proteins are direct activators of PLD1 came the realization that another model for ARF action in the regulation of coat protein recruitment is also plausible. PLD catalyzes the conversion of phosphatidylcholine to phosphatidic acid, and phosphatidic acid has been shown to increase membrane fluidity; the lack of the head group is ideal for the changes involved in membrane reorganization coincident with budding or fission. The model that ARFs could promote coatomer recruitment and coated vesicle formation through the activation of PLD was tested and evidence to support it was provided by Kistakis et al. (9). The localization of PLD1 to Golgi membranes (15) further strengthens this model. However, not all ARF-promoted coat assembly is sensitive to inhibition.
bition of PLD (12), and the membrane phosphatidic acid level was not found to increase during the formation of coatomer-coated vesicles (16).

The functions of ARF proteins have been studied in vivo by expressing the dominant, activating mutant, Q71L, because this renders the protein refractory to the hydrolysis of bound GTP which normally accompanies interaction with ARF GTPase-activating proteins (GAPs). Induction of the expression of [Q71L]ARF1 (17, 18), [Q71L]ARF3, or [Q71L]ARF4 in stably transfected NRK cells caused the vesiculation of Golgi stacks and expansion of the ER lumen (17). The effects on Golgi morphology are so dramatic as to be readily seen using light microscopy. These effects on Golgi and ER structures were assumed to be mediated by the increased stabilization of COP-I binding at the target membranes but could result from sustained activation of PLD1. To distinguish between and test each of the two prevailing models for ARF action in vesicular transport and to tie the results with in vivo effects of ARF, we sought second site mutations in [Q71L]ARF3 which would specifically ablate the activation of PLD1, or the recruitment of COP-I to Golgi membranes, or the ability to alter the morphology of the Golgi in intact cells. If such a mutant could be found to lose one but not other activities we would be able to resolve one or more activities cleanly and test specific interactions and their consequences in mammalian cells.

Regulatory GTPases have at least two switch regions whose conformation is sensitive to the bound guanine nucleotide. First described in Ras proteins (19, 20), these switch I and switch II regions have been described in G protein α subunits (21) and, more recently, in ARFs (22, 23). Changes in these two switches account in large part for the change in affinity of GTPases for their effectors and modulatory proteins, exchange factors, and GAPs. Thus, targeting these switches for site-directed mutagenesis would likely identify mutants defective in one or more ARF activities. However, the reverse two-hybrid assay offers a powerful means of screening thousands of mutants for loss of specific interactions. Unfortunately, neither PLD1, presumably because of its hydrophobicity, nor COP-I, because of its heptameric nature, is amenable to two-hybrid analyses. Instead, we took advantage of recently described ARF-binding partners from two-hybrid screening to identify loss-of-interaction mutants with those partners and counter-screened them for PLD1 activation, COP-I recruitment, and Golgi vesiculation. Each of the four ARF-binding proteins, LTA4 (6), POR1/ARFAPTIN2 (24, 25), MKLP1 (6), and GGA13 interacts preferentially with human [Q71L]ARF3 over wild type ARF3. The mutants identified in this study have allowed clear separation of PLD1 activation from COP-I recruitment, indicating that activation of PLD1 cannot be the sole means of promoting COP-I recruitment to Golgi membranes. Surprisingly, neither PLD1 activation nor COP-I recruitment correlates well with the ability to vesiculate Golgi in live cells, leading us to propose the presence of another, as yet unidentified, effector for this action of ARF.

**Experimental Procedures**

**Materials**—Unless otherwise specified, chemicals and reagents were purchased from Sigma. Yeast and bacterial media reagents were purchased from Difco. Media and reagents for NRK and Chinese hamster ovary cells were purchased from Life Technologies, Inc. All lipids were purchased from Avanti Polar Lipids.

**Reverse Two-hybrid Assay**—Human ARF3 and the [Q71L]ARF3 mutant were engineered for expression in yeast as fusion proteins with the addition of the GAL4 binding domain at the COOH terminus, followed by the epitope from hemagglutinin recognized by the 12Ca5 antibody. Each of four ARF-binding proteins (LTA4, full-length (6, 26); partner of RAC1 (POR1, the NH2-terminal truncation (70–313)) POR1 = POR1AN (24, 25); mitotic kinesin-like protein 1 (MKLP1), the NH2-terminal truncation mutant (669–960)MKLP1 (6); and the ARF binder (GGA1, the truncation mutant (145–551)GGA1)3 was expressed in yeast as a NH2-terminal fusion protein with the GAL4 activation domain fused to a BamHI site at the NH2 terminus. The original expression vectors (pHSI-CYH2 and pACT2) and yeast strains (Y190 and Y187) were the generous gift of Dr. Stephen Elledge (26). A plasmid, pBG4D, which allowed expression of the GAL4 binding domain fusions at the COOH terminus of test proteins was the generous gift of Rob Brazas. These cells allow read-out of two-hybrid interactions as either β-galactosidase activity or histidine prototrophy, resulting from expression of the HIS3 gene product in these two assays were performed as described (26) and give identical qualitative results, but the β-galactosidase assay is easier to score so that is the one used most often and here reported.

For the reverse two-hybrid screen, random mutations were introduced into the open reading frame of [Q71L]ARF3 performing a PCR under conditions designed to reduce the fidelity of the polymerase (1/5 normal dATP concentration and addition of 50 μM MnCl2) (27). A gap was introduced into the plasmid by restriction enzyme digestion to remove only the ARF coding region while the PCR product extended an additional 130 base pairs on each end. The mutagenized PCR product and gapped plasmid were used to transform Y190 cells that already carried plasmids directing the expression of the ARF-binding partner (Y187) with Y191AN, YAB453 (MKLP1), or YAB466 (GGA1) (6).3 Transforms were replicated on nitrocellulose membranes and assayed for β-galactosidase activity directly. Colonies giving white or light blue colors were picked and checked for the expression of full-length ARF3 by detection on immunoblots of the hemagglutinin tag at the COOH terminus. For each of the three screens (POR1, MKLP1, and GGA1) about 7,000 transformants were screened with an average of about 350 white or light blue colonies picked. 25–50% of these were found to be full-length. Plasmids expressing a full-length ARF3 were then rescued from yeast and transformed into Y187 to allow mating with each of the other ARF-binding partners in Y190 cells. The diploid strains harboring both plasmids were selected and assayed for protein interactions by the nitrocellulose filter bound, β-galactosidase assay. ARF3 mutants that retained interaction with at least one of the four binding partners were sequenced to identify the mutation.

**Expression and Purification of Nonmyristoylated and Myristoylated ARF Proteins**—One set of primers from the ends of the open reading frame, which incorporate an NdeI site at the initiating methionine and a stop codon followed by a BamHI site at the 3′-end, were used to amplify the ARF3 mutants from the two-hybrid vectors to allow subsequent expression in Escherichia coli. The resulting plasmids were transformed into BL21(DE3) cells for protein expression at 37 °C, as described previously (28). For myristoylated ARF proteins, the BL21 cells were cotransformed with a second plasmid, directing expression of human N-myristoyltransferase (29). In this case, cells were induced at room temperature and after the addition of 200 μM myristic acid (30). The purification of ARF proteins was performed as described previously (28) except that N-myristoylation of each ARF preparation was determined by reverse phase HPLC analysis on a C4 column loaded in 0.1% trifluoroacetic acid and eluted with a linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid. Base-line resolution of the two peaks was readily achieved with retention times for nonacylated and acylated proteins of typically 46 and 52 min, respectively.

**GTPγS Binding Assay**—The binding of [35S]GTPγS to ARF proteins was determined using the nitrocellulose filter trapping method (1, 36) to separate bound and free ligand, under two different conditions: the “standard” conditions and those used in the assay for PLD1 activity. The main differences between these conditions are temperature, the concentrations of free magnesium, and the identity and form of lipids/detergents. The standard conditions included incubation at 30 °C of 1 μg ARF, 10 μg [35S]GTPγS, 20 μM Tris-Cl, pH 7.4, 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM MgCl2, 3 mM sonicated α-dimyristoylphosphatidylcholine, and 0.1% (2.5 mM) sodium cholate. Binding under PLD1 assay conditions (31) was performed at 37 °C with 4 μM ARF, 30 μg [35S]GTPγS, 2.5 mM MgCl2, 1.7 mM CaCl2, 3.5 mM EGTA, 40 μM EDTA, 80 mM KCl, 1.2 mM NaCl, 20 mM HEPES, pH 7.5, and 690 μM lipid vesicles (10 mol % dipalmitoylphosphatidylcholine, 56 mol % dioleoylphosphatidylethanolamine, and 4 mol % phosphatidylinositol, 4,5-bisphosphate.)

**PLDI Assay**—Hexahistidine-tagged human PLD1 was expressed in HEK cells using a baculovirus construct, the generous gift of Sung Ho Ryu, Pohang University, Pohang, Korea. Membranes from these cells were prepared according to a procedure described by Brown et al. (37). The PLD1 was then extracted from the membrane by resuspension of

4 C. Zhang and R. A. Kahn, unpublished observation.
the membrane in 20 mM HEPES, pH 7.5, 500 mM KCl, 1% n-octyl \(\beta\)-D-galactopyranoside, and protease inhibitors. After 1 h at 4 °C, the resuspension was cleared by centrifugation at 100,000 \(\times\) g for 1 h. The supernatant was diluted with buffer A (20 mM HEPES, pH 7.5, containing 1 mM dithiothreitol, 1 mM EDTA, and 1% n-octyl \(\beta\)-D-galactopyranoside) to a final concentration of KCl of 100 mM before application to a 5-ml HiTrap SP column (Amersham Pharmacia Biotech). Proteins were eluted with a 100–500 mM KCl gradient in buffer A over a 25-min period at 1 ml/min. The fractions containing PLD activity (fractions 35–43) were pooled to give a final protein concentration of 50 \(\mu\)g/ml and frozen in aliquots in liquid nitrogen before storage at −80 °C.

The PLD assay was performed as described by Lopez et al. (31) with slight modifications. Briefly, the substrate was prepared in the form of lipid vesicles composed of 10 mol % \([3^H\text{-methylcholine}]\) dipalmitoylphosphatidylcholine, 86 mol % dioleoylphosphatidylethanolamine, and 4 mol % phosphotidylinositol, 4.5-bis-phosphate at a final concentration of 690 \(\mu\)M. Myristoylated ARF and GTP\(_S\) were preincubated in the presence of lipid vesicles at 37 °C for 40 min prior to the addition of PLD1. The reaction was stopped by the addition of 1 ml of chloroform:methanol (50:50:0.3), followed by adding 350

RESULTS

To test the relationships between PLD1 activation and COP-I recruitment, as well as each of these activities of ARFs to other cellular responses, we sought point mutations in an ARF which would lose one or more of these functions to allow the molecular signaling of ARF signaling both in vitro and in intact cells. The size and hydrophobicity of PLD1 and heptameric nature of COP-I make them unusable as targets in yeast two-hybrid assays as a means of monitoring interactions with ARFs. Thus, we screened for loss-of-interaction mutations with a recently identified set of ARF effectors and then looked among them for specific defects in PLD1 activation and COP-I recruitment.

Generation of Human ARF3 Mutants—This approach was made possible by the recent identification of at least four different binding partners that bind ARF3 preferentially in the activated GTP-bound state and demonstrate activity in the yeast two-hybrid assay. Reverse two-hybrid screens were performed to identify mutants of human ARF3 which specifically lost the interaction with one effector but retained the interaction with others. Three separate reverse two-hybrid screens were performed with POR1, MKLP1, and GGA1, as described under “Experimental Procedures.” In each of the reverse two-hybrid screens the effector was expressed as a fusion protein with the GAL4 activation domain. The ARF3 was also expressed as a fusion protein, with the GAL4 binding domain at the COOH terminus, followed by a hemagglutinin epitope at the COOH terminus. In addition, the ARF3 fusion protein in each case contained the GTPase-deficient, activating, Q71L mutation because this leads to higher levels of GTP-bound ARF in cells and promotes interactions with effectors in the two-hybrid assay. Random mutagenesis of Q71LARF3 was achieved by PCR under conditions of reduced polymerase fidelity. Loss-of-interaction mutants were identified as white or pale blue colonies in the \(\beta\)-galactosidase assay, performed on nitrocellulose filters soaked in 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside. The presence of a full-length ARF3 fusion protein in each of these colonies was confirmed by immunoblotting, using the EAGE anti-\(\beta\)-COP (33) or R-1023, anti-ARF3/3 polyclonal rabbit antiserum (34). Fractions found to be free of ARF and enriched in cofactor were pooled and snap frozen in aliquots for use in the yeast two-hybrid assay.

GoGol-enriched membranes were prepared from Chinese hamster ovary cells as described in Beckers and Rothman (35). Membranes were washed with 0.5 \(\times\) KCl on ice before use in the recruitment assay.

ARFs and the enriched cofactor preparation were cleared by centrifugation at 100,000 rpm for 30 min in a TLA 100.1 rotor (Beckman) before use in the assay. All reactions were performed in siliconized microcentrifuge tubes. GoGol-enriched membranes (35–43 \(\mu\)g of protein) were incubated with 4 \(\mu\)M myristoylated ARF proteins and 0.6 mg/ml cofactor-enriched cytosol at 37 °C for 20 min in a final volume of 100 \(\mu\)l in the presence of 25 \(\mu\)M of GTP\(_S\) or GDP\(_S\), 2.5 mM MgCl\(_2\), 1 mM dithiothreitol, 0.2 \(\times\) sucrose, 15 \(\mu\)g/ml bovine serum albumin, 1 mM ATP, 2 mM creatine phosphate, and 8 IU/ml creatine phosphokinase. The reaction was terminated by centrifugation of the membranes through an 80% cushion of 20 mM HEPES, pH 7.4, 0.5 \(\times\) sucrose at 100,000 \(\times\) g for 15 min at 4 °C. The pellet was washed with 20 mM HEPES, pH 7.4, and dissolved in 15 \(\mu\)l of SDS sample buffer. The proteins were resolved in gradient (8–16%) polyacrylamide gels and transferred to nitrocellulose membranes to allow immunoblotting using the rabbit polyclonal antibody to \(\beta\)-COP (R-23200; 33). Immunoreactivity was visualized with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham Pharmacia Biotech) and the enhanced chemiluminescence sub- stray from Amersham. [Q71L]ARfS was included as a positive control on each gel to help control for variability seen in the electrophoretic transfer to nitrocellulose. Nevertheless, some variation in background (that seen with no nucleotide or GDP\(_S\) added) recruitment of \(\beta\)-COP was observed. For this reason the amount of \(\beta\)-COP recruited to membranes with GTP\(_S\) was compared with that seen with GDP\(_S\). These experiments were repeated at least three times, and the ability of each mutant to recruit \(\beta\)-COP in a GTP\(_S\) dependent manner was qualitatively the same in each case.

Transient Expression of ARF3 Mutants in NRK Cells—The coding regions of each of the ARF3 mutants were subcloned into the mammalian expression vector pCDNA3 (Invitrogen) at the NdeI and XhoI sites to allow constitutive expression under control of the cytomegalovirus promoter. The resulting plasmids (125 ng/ml) and the enhanced chemiluminescence substrate from Amersham. [Q71L]ARfS was included as a positive control on each gel to help control for variability seen in the electrophoretic transfer to nitrocellulose. Nevertheless, some variation in background (that seen with no nucleotide or GDP\(_S\) added) recruitment of \(\beta\)-COP was observed. For this reason the amount of \(\beta\)-COP recruited to membranes with GTP\(_S\) was compared with that seen with GDP\(_S\). These experiments were repeated at least three times, and the ability of each mutant to recruit \(\beta\)-COP in a GTP\(_S\) dependent manner was qualitatively the same in each case.

The transient expression of ARF3 mutants in NRK cells was achieved by transfection using DEAE-dextran (1.2 mg/ml; Molecular Probes) followed by injection into NRK cells, grown on coverslips, using an Eppendorf automatic microinjector system. Injections were targeted to the nucleus with the aid of a 400 x light microscope. After injection the cells were incubated at 37 °C for 7 h before indirect immunofluorescence staining was performed using the polyclonal antibody to mannosidase II as primary and Texas red-conjugated anti-rabbit IgG antibody as secondary antibody.
The interactions of mutants of ARF3 (listed in the leftmost column) with different partners were tested. Diploid yeast strains, each harboring a set of ARF3 binding domain and effector activating domain plasmids, were assayed for β-galactosidase activity after lysis on nitrocellulose filters, as described under "Experimental Procedures."  Minus sign (−) represents white color indicating the loss of interaction; +/+ represents barely detectable blue color development; +/+ represents pale blue color indicating the retention of some binding; ++/+ represents more binding; ++/+ represents full interactions, as seen with the [Q71L]ARF3 control. Color development was detected by eye after 30 min.

| Mutants       | POR1 | MKLP1 | GGA1 | LTA1 |
|---------------|------|-------|------|------|
| ARF3          | −    | −     | +    | +    |
| [Q71L]ARF3    | +++ | +++   | +++  | +++  |
| [S62G,Q71L]ARF3 | +++ | +++   | +++  | +++  |
| [I49T,Q71L]ARF3 | ++  | −     | −    | −    |
| [F51Y,Q71L]ARF3 | ++  | −     | −    | −    |
| [I74T,Q71L]ARF3 | −   | −     | −    | −    |
| [R79C,Q71L]ARF3 | −/+ | ++    | +++  | +++  |
| [Y81C,Q71L]ARF3 | −   | −     | −    | −    |
| [Y81H,Q71L]ARF3 | −   | −     | −    | −    |
| [Q53R,Q71L]ARF3 | −   | ++    | +++  | +++  |
| [T55A,Q71L]ARF3 | −/+ | +++   | +++  | +++  |

T85A, were each identified originally by the loss of interaction with POR1. Two switch II, Y81C, I74T, and one switch I mutant, I49T, were from the MKLP1 screen, with two of these three mutants independently isolated twice. The switch I mutant F51Y was found among those mutants that lost interaction with GGA1. The S62G mutation was a PCR-generated mutation that was isolated by chance during the construction of the [Q71L]ARF3 plasmid and was found to lie between switch I and II and so served as an additional control in several studies. Residues 49 and 51 are each in switch I, whereas residues 74–85 are in switch II.

Although mutagenesis and reverse two-hybrid screening were not saturated, the data in Table I offer an initial, low resolution map of the sites of interactions between multiple ARF effectors and the activated GTPase. For example, switch I and specifically residues 49 and 51 are likely important to the binding of MKLP1 and GGA1 but perhaps not to that of POR1 or LTA1. POR1 binding to ARF3 appears to be very sensitive to any changes in the switch II domain as all six changes described lead to loss of POR1 interaction in two-hybrid assays. LTA1 might bind to ARF3 in a fashion quite distinct from these other effectors as only one mutant caused the loss of LTA1 interaction. The reverse two-hybrid screening and identification of residues involved in the binding of LTA1 to ARF3 are the subject of another study so will not be discussed further here. By scanning through the results in Table I it appears clear that none of these four effectors shares identical binding sites with ARF3. This serves to highlight the diversity in effector-GTPase interactions and also supports the utility of this approach in identifying specific loss-of-interaction mutations.

It is evident from the data in Table I that ARF effectors bind to distinct but overlapping residues in switch II and probably switch I. It should also be clear that mutations, such as Y81C, in which the ARF3 has lost interactions with multiple effectors but retains full binding to one, GGA1 in this case, will make potentially very powerful probes for the in vivo roles of ARFs and their different effectors in cell regulation of multiple pathways. A cautionary note is also provided by the two mutations at residue 81. Changing this tyrosine to cysteine or histidine yielded quite different spectra of binding partners (see Table I), suggesting that not only the absence of the wild type residue may be important but also the residue to which it is being changed can have important consequences.

Purification and Characterization of Recombinant Human [Q71L]ARF3 Second Site Mutants—Each of the switch I and II mutants was expressed in bacteria and purified to allow biochemical analyses. The proteins were also coexpressed in bacteria with an N-myristoyltransferase to allow the production of myristoylated ARF3 proteins. As described previously (28, 30), this results in only partial N-myristoylation, and resolution of the acylated and nonacylated forms cannot be achieved readily. We typically obtain ARFs that are about 30% acylated, with a range of 20–50% myristoylated proteins for the mutants (see Table II), as determined by reverse phase HPLC (see "Experimental Procedures").

The rate and extent of binding of [35S]GTPγS were determined under our standard conditions (see Ref. 1 and "Experimental Procedures") which include 3 mM 1,2-dimyristoylphosphatidylcholine and 2.5 mM cholate. Also, the free magnesium concentration was held constant, in the low micromolar range, by the inclusion of 1 mM EDTA and 0.5 mM MgCl2. As seen in Table II, all of the mutants retained the ability to bind GTPγS, though to somewhat different extents. Although the rates of nucleotide exchange were similar, reaching half-maximal binding in less than 5 min (see Fig. 1A), the binding stoichiometries under our standard conditions varied from 49 to 140% of the control, [Q71L]ARF3, which bound about 0.3 mol of GTPγS/mol of protein when N-myristoylated and about 0.05–0.1 mol of GTPγS/mol of protein when nonacylated.

Very similar relative binding levels were observed when the conditions in the nucleotide binding assay were changed to those used in the assay for PLD. This assay requires a different lipid mixture and higher concentrations of free magnesium, resulting in slower off-rates for GDP, and consequently on-rates for GTPγS. Under these conditions, the binding of GTPγS was fairly linear for at least 1 h in each case (Fig. 1B). Thus, all of the recombinant proteins retained binding characteristics similar to those of the wild type and control, [Q71L]ARF3, proteins with the main difference noted in the extent of binding, ranging between 30 and 300% that of the [Q71L]ARF3 control. The only mutant that behaved qualitatively differently in the two nucleotide binding assays was Y81H, which was slightly lower than controls in the standard assay (85%) but just over twice the level of binding was observed in the PLD assay conditions (204%).

Identification of ARF3 Mutants That Lose the Ability to Activate PLD1—The purified, recombinant, N-myristoylated ARF3 mutant proteins were next assayed for the ability to activate recombinant PLD1, using a modification of the method of Lopez et al. (31). This assay uses exogenous 3H-labeled phosphatidylcholine as substrate and measures the release of the water-soluble [3H]choline. Human PLD1 was expressed in insect cells as an NH2-terminal hexahistidine-tagged form and partially purified by solubilization of Hi5 membranes in 1% n-octyl-β-D-galactopyranoside and passing the extract over a HiTrap column to enrich for ARF-stimulated PLD activity, as described by Singer et al. (40).

As seen in Fig. 2, activation of PLD activity in this assay is dose-dependent for added ARF and GTPγS. The activity of both ARF3 and [Q71L]ARF3 was increased by N-myristoylation. Whereas the increased activity with acylation shown is about 3–5-fold for the wild type protein and about 10-fold for the Q71L mutant, the specific activities are even greater because the acylated proteins are only about 30% myristoylated. The concentrations of ARF proteins needed to achieve half-maximal activities were also different, with the nonmyristoylated forms requiring around 2 μM ARF and the myristoylated proteins less
than 1 μM. The $K_{1/2}$ for N-myristoylated ARF as activator of PLD1 is seen more clearly in Fig. 3 and estimated at between 50 and 100 nM. Another difference seen between these preparations is that the nonmyristoylated proteins reached a plateau at around 2 μM ARF, and this held constant to at least 16 μM ARF (data not shown). In contrast, the acylated ARF preparations that stimulated PLD1 activity peaked with around 2 μM ARF, and activities then decreased. This effect is not totally understood but has been described previously and was worse when we tried performing the assays with the PLD on Hi5 membranes. Solubilization in octyl glucoside and partial purification made the PLD activity less sensitive to inhibition at high ARF concentrations. We focused our analyses on the modified proteins as N-myristoylation is a cotranslational event so only the myristoylated forms are thought to be present in cells. However, as the nonacylated proteins are more homogeneous preparations and assist in the interpretation of results from preparations that include 30% acylated and 70% nonacylated proteins, results from analyses with nonmyristoylated protein preparations are also included. Typically, no qualitative differences were noted when nonacylated ARF proteins were compared.

Each of the switch I mutants (I49T and F51Y) had lost the ability to activate PLD1, whereas only one of the switch II mutants (Y81C) had compromised PLD-stimulating activity (see Table II or Fig. 3). Differences between these three mutants and the other proteins were even more dramatic when the nonacylated proteins were assayed (data not shown). In that case, there was no PLD-stimulating activity for any of these three proteins. Because the PLD assay appears most sensitive when acylated ARFs are used, we focused our analysis on these preparations (see Table II). Although the data shown in Fig. 3 suggest a dose-dependent increase in PLD activity at the highest concentrations of the I49T mutants, this was not always the case. The lack of a clear dose dependence to the small activities seen and the lack of statistical difference between stimulation by the I49T mutant and that seen with the parental protein in the presence of GDPγS is highly suggestive of an inactive protein in our assay. The F51Y mutant consistently lacked any discernible activity in this assay.

The other control, [S62G]ARF3, was equally active with controls, as expected for this positive control. Although some differences in the binding of GDPγS were noted (see Fig. 1), these did not appear to be a primary determinant of PLD1-stimulating activity. For example, the F51Y and Y81C mutants each bound nucleotides to higher stoichiometries than the others yet had among the lowest activities in the PLD assay.

**Recruitment of COP-I to Golgi Membranes Is Also Impaired in Several Mutants of ARF3**—The direct recruitment of COP-I has been proposed as the mechanism of ARF action as regulator of vesicular traffic in eukaryotes, and an in vitro assay has been developed to monitor this activity. We used this assay to determine if any of the switch mutants has lost the ability to recruit COP-I to Golgi membranes.

Bovine brain cytosol was resolved on a gel filtration column to separate the high molecular weight, coatomer complex and the monomeric ARF proteins to provide a source of COP-I that was free of ARFs. Partially purified Golgi membranes from Chinese hamster ovary cells were prepared as described (35) and further stripped of extrinsic proteins by washing in 0.5 M KCl. The coatomer fraction, Golgi membranes, and myristoylated ARF proteins were incubated with either GDPγS or GDPβS, as described under “Experimental Procedures.” The reaction was stopped by collecting the membranes by centrifugation, and COP-I recruitment was detected by immunoblot analysis using the β-COP antiserum (33).

As reported previously (10) and shown in Fig. 4A, the binding of coatomer in this assay was dependent on the addition of ARFs and the activating guanine nucleotide. The nonmyristoylated ARF proteins did not promote the recruitment of coatomer to membranes (data not shown). Most of the myristoylated mutants retained the ability to recruit coatomer onto Golgi membranes in a GDPγS-dependent fashion (see Fig. 4B). However, the I74T, Y81C, and Y81H mutations resulted in the loss of coatomer recruitment activity. The data in Fig. 4B were generated using the same amounts of each ARF3 protein but do not take into account differences in GTP binding. When this experiment was repeated after adjusting for steady-state binding of GDPγS so that equal amounts of activated ARF3 proteins were being compared, the results were qualitatively the same as those shown in Fig. 4B (data not shown). Thus, isoleucine 74 and tyrosine 81 are required for the recruitment of coatomer onto Golgi membranes by ARF3. The two mutations in switch I, I49T and F51Y, also lead to a substantial decrease in COP-I recruitment, although they consistently promoted more GDPγS-dependent COP-I binding than the mutations in residue 74 or 81.

Note that the ability of ARF mutants to recruit COP-I did not correlate with their ability to activate PLD1. For example, I74T retained almost 80% of the stimulatory activity for PLD1 but could not recruit any coatomer. In contrast, I49T and F51Y lost PLD activation but still recruited coatomer to Golgi, although

### Table II

| Mutants        | N-Myristoylation | Relative GDPγS binding | Relative PLD activity |
|----------------|------------------|------------------------|-----------------------|
|                | %                | Standard | PLD |
| [Q71L]ARF3     | 31               | 100      | 100 |
| [Q71L]ARF3 (GDPγS) | 49            | 109      | 110.0 ± 10.8 |
| [I49T,Q71L]ARF3| 37               | 74       | 8.2 ± 3.2 |
| [F51Y,Q71L]ARF3| 30               | 140      | 3.1 ± 1.9 |
| [I74T,Q71L]ARF3| 28               | 49       | 63.9 ± 14.4 |
| [R79G,Q71L]ARF3| 55               | 78       | 121.7 ± 12.9 |
| [Y81C,Q71L]ARF3| 21               | 119      | 90.2 ± 6.0 |
| [Y81H,Q71L]ARF3| 33               | 85       | 74.4 ± 13.6 |
| [Q83R,Q71L]ARF3| 36               | 99       | 85.4 ± 10.1 |
| [T85A,Q71L]ARF3| 44               | 82       | 82.0 ± 11.0 |
less well than ARF3 (data not shown) or [Q71L]ARF3. Thus, these data reveal a clear biochemical dissociation between the activation of PLD1 and recruitment of COP-I to Golgi membranes.

Some Switch Mutants Have Lost the Ability to Vesiculate Golgi When Expressed in NRK Cells—Expression of the dominant activating mutant, [Q71L]ARF1 (17) or [Q71L]ARF3, in cultured mammalian cells leads to engorgement of the ER lumen and gross enlargement and vesiculation of the Golgi stacks. These observations are in contrast to predictions from the simple model for ARF proteins as mediators of coatomer recruitment to the Golgi membranes yet remain among the few phenotypes associated with the expression of activated ARF in mammalian cells. Such studies have added value in not relying on the use of nonspecific activators of GTPases, i.e. nonhydrolyzable guanine nucleotide triphosphates.

The ability of the induced expression of [Q71L]ARFs in stably transfected cell lines to alter Golgi morphology dramatically was used and modified to develop a more rapid assay for ARF functions. Wild type, [Q71L]ARF3, and the second site mutants were each subcloned into pcDNA3, which uses the strong, constitutive cytomegalovirus promoter, to direct expression in mammalian cells. NRK cells were microinjected with the pcDNA3-derived plasmids, and Golgi morphology was monitored by indirect immunofluorescence using antisera directed against mannosidase II, a protein that localizes to the lumen of predominantly medial Golgi. Injected cells were identified by the coinjection of fluorescein isothiocyanate-dextran (molecular weight \(10,000\)). Uninjected cells, or those injected with the empty vector, or with pcDNA3 directing expression of wild type ARF3, reveal a tight perinuclear staining of mannosidase II (Fig. 5, panel B) in NRK cells. The appearance of the Golgi is altered dramatically after expression of [Q71L]ARF3 (see Fig. 5, panel D) as seen by the larger area and more diffuse images of mannosidase II staining around the entire nucleus. Changes in Golgi structure can be seen within 1–2 h after injection, and the number of cells displaying

---

**Fig. 1. Time course of the binding of GTP\(^{\gamma}\)S to partially myristoylated ARF proteins.** Panel A, GTP\(^{\gamma}\)S binding was determined at 30 °C under standard conditions, as described under “Experimental Procedures,” with 1 μM ARF and 10 μM \([^{35}S]\)GTP\(^{\gamma}\)S in a total volume of 100 μl. Panel B, GTP\(^{\gamma}\)S binding was performed at 37 °C with 4 μM ARF and 30 μM GTP\(^{\gamma}\)S using conditions optimal for assay of PLD activity, as described under “Experimental Procedures,” in a total volume of 150 μl. In each case, duplicate samples (10 μl) were taken at each time point. The key shows the proteins assayed, with ARF3Q the [Q71L]ARF3 protein; each of the indicated mutations represents second site mutations present with the Q71L change.
the altered morphology usually peaks between 5 and 7 h and then decreases. This decrease may result from cell lysis, as described for the inducible expression of activating ARF mutants (17), but this question was not pursued further in these studies. The percentage of injected cells that display vesiculated Golgi varies between experiments and was between 20 and 60% when assayed 7 h postinjection with the [Q71L]ARF3 plasmid. To allow comparisons between experiments the [Q71L]ARF3 positive control was always included and the percentage of injected cells with vesiculated Golgi set to 100% when comparing other mutants.

Each of the switch mutants of ARF3 was then tested in the Golgi vesiculation assay. Because each of the expressed proteins contains the Q71L mutation they should induce morphological changes unless the second site mutation interferes with this activity. The results are shown in Table III. Three different types of responses were noted; wild type and the F51Y mutant completely lacked the ability to induce changes in Golgi structure, I49T and R79G had undiminished capacity for vesiculation, and the others (I74T and Y81C) had diminished but detectable activity in this assay. In other experiments (not shown) the time after injection and the amount of plasmid injected were varied, but the results were qualitatively the same as those reported in Table III.

DISCUSSION

We describe here the identification and characterization of nine mutants in the switch I and II regions of human ARF3. Five different assays were employed in the characterization of these proteins, including reverse two-hybrid screens in which...
they were first identified, the ability to bind guanine nucleotides with high affinity, activation of PLD1, recruitment to Golgi membranes of COP-I, and the ability to vesiculate the Golgi and enlarge the area occupied by this organelle in intact cells. When each of these activities was assessed, a range of deficiencies was noted among the mutants that allowed tests of two current models for ARF action: (i) that activation of PLD1 by Arf is a required step in the recruitment of coatamer and (ii) that recruitment of coatamer, without the means to release it, results in enlarged ER and Golgi lumen with concomitant vesiculation of Golgi in intact cells expressing the dominant activating mutant, [Q71L]ARF. Surprisingly, our data support neither of these models. Rather, on the basis of these analyses we conclude that there exists a currently unidentified effector for ARF action at the Golgi which is required for the vesiculation and enlargement of Golgi structures in the presence of an excess of activated ARF.

Screens for loss-of-interaction mutants allowed the unbiased identification of specific residues critical to specific effector interactions with activated ARF. Not surprisingly, mutations in the mobile and nucleotide-sensitive switch regions were prominent among the mutations found. From the results shown above and by homology to previous results with other GTPases, it is evident that different effectors bind to distinct but overlapping regions of ARF3. It appears from this limited sampling of mutants that specificity for effectors was more likely derived from the interaction with the switch II region. The ability to screen thousands of mutants for the loss and retention of different combinations of effector interactions offers a low resolution map of protein binding sites which is very useful in testing models for integration of ARF signaling and should also aid parallel efforts aimed at solving the more detailed structures by both NMR and x-ray crystallography.

The three ARF-dependent activities used in these studies each offer information that is crucial to the testing of current models of ARF action at the Golgi. The COP-I recruitment assay was thought to measure the rate-limiting step in the budding of nascent vesicles that mediate intra-Golgi transport (7, 8). The activation of PLD1 by ARF is clearly established as a direct G protein-effector system that has been offered as an alternative hypothesis (15) to the COP-I recruitment hypothesis; and the vesiculation of Golgi was described as the consequence of the expression of the dominant activated mutant, [Q71L], of ARF1 (17) or ARF3. This assay is particularly important as it examines the consequences of ARF mutations in live cells and thus is not dependent on the nonspecific GTPase activator, GTPγS. It is worth noting that the regulated expression of [Q71L]ARF1 was first used in stably transformed cells to test the COP-I recruitment hypothesis, and the observation of large, heterogeneously sized vesicles runs counter to the prediction made by that hypothesis (17). The cell-based assay employed in these studies used the microinjection of plasmids and represents a modification of the original assay that allowed more rapid testing of ARF mutants and in an assay more easily controlled.

PLD1 has a number of features that make it a very attractive candidate effector for the action(s) of ARF on membrane traffic at the Golgi. It was purified as an ARF-sensitive activity that localizes to the Golgi (15), and phospholipase activity was found to mimic several of the previously described actions of ARFs in *in vitro* Golgi transport assays (15, 38). Characterization of the PLD1 assay revealed an activity that is highly dependent on ARF and GTP or GTPγS. Other protein activators of PLD1 (e.g. members of the RAC/RHO family or PKCα) were not tested in our assay but have been described elsewhere (39–41). N-Myristoylation lowers the $K_a$ for ARF and gives

---

**Fig. 4. ARF-promoted binding of COP-I to Golgi membranes.** The amount of COP-I that bound to enriched Golgi membranes (10 μg) in the presence of different combinations of N-myristoylated ARF proteins (4 μM; as labeled in the figure) and GTPγS or GDPγS (25 μM) was detected by immunoblotting with antibody to the β-COP subunit. Panel A, the binding of β-COP to the Golgi membrane is dependent on the addition of myristoylated ARF3 and GTPγS (compare lanes 3 with 6, and 5 with 6, respectively) but not on GDPγS (lane 5). As controls, Golgi membranes did not contain coatamer (lane 4), and no coatamer was precipitated without membrane (lane 1). Panel B, binding of β-COP to the Golgi membrane with different mutants of ARF3 (as labeled in the figure) in the presence of GDPγS (odd numbered lanes) and GTPγS (even numbered lanes). Each reaction contained enriched Golgi membranes and ARF-free coatamer, prepared as described under “Experimental Procedures.” The mutations labeled in the figure were secondary mutations on top of the [Q71L]ARF3. As seen in the control (lane 2), the binding of β-COP to Golgi membranes seen with [Q71L]ARF3 (ARF3Q) is still dependent on the addition of GTPγS.

|  | myr-Arf3 | GTPγS | GDPγS | Golgi membrane | coatamer |
|---|---------|-------|-------|----------------|---------|
| A |   +     |   +   |   +   |     +          |    +    |
|   |   +     |   -   |   +   |     +          |    +    |
|   |   -     |   -   |   -   |     -          |    -    |
|   |   +     |   +   |   +   |     +          |    +    |

---

|  | myr-Ars | Arf3Q | I49T | F51Y | I74T | R79G | Y81H | Y81C | Q83R | T85A |
|---|---------|-------|------|------|------|------|------|------|------|------|
| B |   +     |   +   |   +  |   +  |   +  |   +  |   +  |   +  |   +  |   +  |
|   |   +     |   -   |   +  |   +  |   +  |   +  |   +  |   +  |   +  |   +  |
|   |   +     |   +   |   +  |   +  |   +  |   +  |   +  |   +  |   +  |   +  |
Activated ARF Effects on Golgi Morphology

NRK cells were injected with pcDNA3-derived plasmids containing ARF3 or [Q71L]ARF3 or double mutants. The changes in Golgi morphology of injected cells were visualized by indirect immunofluorescence staining, using antibodies to mannosidase II 7 h after injection with plasmids, as described under “Experimental Procedures.” The number of cells that showed the expanded, diffuse, perinuclear staining indicative of Golgi vesiculation was scored. In different experiments, the percentage of parental [Q71L]ARF3 injected cells exhibiting the Golgi vesiculation varied from 20 to 60%. The relative percentage of cells injected with different double mutants which exhibited the Golgi vesiculation was normalized to the parent [Q71L]ARF3. Data in the table are the average of three different experiments with at least 200 cells injected for each experiment.

| Mutants                        | Relative percentage of injected cells showing Golgi vesiculation |
|--------------------------------|-----------------------------------------------------------------|
| ARF3                          | 0                                                               |
| [Q71L]ARF3                    | 100                                                             |
| [I49T,Q71L]ARF3               | 98                                                              |
| [F51Y,Q71L]ARF3               | 0                                                               |
| [I74T,Q71L]ARF3               | 34                                                              |
| [R79G,Q71L]ARF3               | 105                                                             |
| [Y81C,Q71L]ARF3               | 23                                                              |

TABLE III

Relative abilities of mutants of ARF3 to promote expansion and vesiculation of Golgi in intact mammalian cells

Fig. 5. Expression of ARF3 mutants in NRK cells as an in vivo assay for function at the Golgi. The pcDNA3-derived plasmids that direct expression of the different mutants of ARF3 (as labeled in the figure) were coinjected with fluorescein isothiocyanate-dextran into NRK cells grown on coverslips. The left panels show the injected cells, visualized by green fluorescence of the fluorescein isothiocyanate-dextran. The right panels show the immunofluorescence staining with mannosidase II (Man II) antibody, visualized by Texas red-conjugated secondary antibody. The expanded, diffuse, and perinuclear staining with mannosidase II antibody is indicative of Golgi vesiculation (panels D, F, J, L, and N). As control, the mannosidase II staining showed tight perinuclear staining that did not change with overexpression of wild type (WT) ARF3 or when buffer alone was injected into cells. The mutations indicated at the left of each set of panels are the secondary mutations on top of [Q71L]ARF3 (ARF3Q).

about a doubling in the maximal activity, compared with nonacylated ARF preparations (see Fig. 2). The partially (~30%) myristoylated ARF3 protein had a $K_m$ of 50–100 nM in our assay. This is very close to that reported for a purified bovine brain ARF preparation (20–30 nM; (42) and is indistinguishable if the incompleteness of the acylation in our preparations is taken into account. The presence of the Q71L mutation had a small effect but was not required for activity (see Fig. 2). This is important as all other mutations are being viewed within the context of this Q71L, activating mutation.

Each of the mutations in switch I, I49T or F51Y, resulted in the loss of the ability to activate PLD1 in our assay (see Fig. 3 and Table II). This increase in the $K_m$ for PLD1 activation by each of these mutants was at least 20-fold, and no activity was observed at the highest concentrations of ARF used in this assay (16 μM). The Y81C mutant was found to be completely inactive when assayed as the nonacylated protein but had up to 30% control levels of PLD stimulating activity after N-myristoylation. That interactions with at least one other partner in two-hybrid assays and the ability to bind guanine nucleotides were conserved in each case is evidence for a level of specificity in the loss of PLD1-stimulating activity. We interpret these results as a reflection of the lowering of the $K_m$ from N-myristoylation and a more dramatic increase in $K_m$ resulting from the mutation. It is possible that each of these residues makes direct contact with PLD1 in the activated heterodimer, and the mutations disrupt the high affinity binding. For example, each of these residues may be involved in hydrophobic interactions of the kind described by Goldberg (22) between switch I of ARF1 and the Sec7 exchange factor domain. If so, there must be specificity to this hydrophobic pocket being involved with PLD1 as the binding to other effectors, e.g. POR1 or LTA1, was not lost by the mutation of either Ile-49 or Phe-51.

The presence of ARF on purified COP-I-coated vesicles and the in vitro assay of the recruitment of coatomer to Golgi membranes in an ARF-dependent manner are central to one model for ARF in vesicular traffic. The COP-I recruitment assay is thought to monitor the critical nucleating step in bud formation. Other data support this model, e.g. electron microscopy data that correlate COP-I recruitment and bud emergence (7, 8) or genetic interactions between ARF and components of secretory machinery (2), but there are also a number of observations that appear inconsistent with the model. Most notable in this regard are the apparent lack of requirement for ARF (43–45) in any of the in vitro assays of membrane transport in which ARF was shown to be responsible for the inhibition of
transport in the presence of GTPγS (46–49) and the ability to form apparently normal COP-I-coated vesicles in the absence of ARF (9).

When each of the switch mutants was assayed for the ability to recruit coatomer to Golgi-enriched membranes, several were found to have lower specific activities than the control, [Q71L]ARF3. The switch II mutations I74T and each of the Y81 mutants appeared devoid of recruitment activity in our assay. Like the activation of PLD1, we assume that binding to COP-I involves multiple residues on the surface of each protein, so the loss of any one results only in a decreased affinity, but in this case it was decreased to the point that our assay could no longer detect the interaction. That the most dramatic effects on COP-I recruitment were found in switch II is surprising in that the results reported by Goldberg (23) implicated switch I as the site of COP-I binding. The binding of ARF GAP to switch II was shown by crystallographic studies, and the enhancement of ARF GAP activity by added COP-I was interpreted as evidence for its binding to switch I. Our findings that switch I mutants have decreased COP-I recruitment and some switch II mutants are even more affected in this activity are not necessarily at odds with the conclusions of Goldberg. It is possible that COP-I is recruited to membranes by ARFs as a consequence of the binding to both switch regions but that the binding of COP-I to only switch I may be sufficient to activate ARF GAP activity. It need not even be the same domain or protein in the heptameric COP-I complex which is responsible for these two activities. This speculation, and our results implicating both switch regions in COP-I binding, are consistent with the finding of cross-linking between switch I residues (Ile-46 and Ile-49) to γ-COP (49) and a switch II residue (Phe-82) to β-COP (51).

All three of the mutants that lost the ability to recruit coatomer in our assay (I74T, Y81I, and Y81C) had normal, or very nearly so, ability to activate PLD1, and both of the mutants that lost detectable PLD1 activation (I49T and F51Y) retained the ability, though diminished, to recruit coatomer. Comparison of other mutants in these two assays reveals a general lack of correlation. These results are inconsistent with the model for ARF action at Golgi in which the recruitment of COP-I is dependent on the activation of PLD1 (15). This conclusion is consistent with the data in Stamnes et al. (16) in which there was poor correlation between the production of phosphatidic acid (the product of PLD) and coatomer recruitment.

Engorgement of Golgi elements and their transformation into large vesicles, diverse in size, with concomitant enlargement of the ER lumen are phenotypes associated with the expression of the dominant activating mutant, Q71L, of ARF1 (17), ARF3, or ARF4.4 These changes in morphology were described originally in NRK cells stably transformed with ARF alleles under control of an inducible promoter and analyzed using electron microscopy. We have adapted these results into an assay that can be carried out over the course of a few hours by microinjecting cells with plasmids directing the expression of the ARFs and analyzing the morphology of the Golgi using specific markers of that compartment, e.g. mannosidase II for the lumen or β-COP for the periphery. Although the mechanism by which activated ARFs can cause the Golgi to change in this way is not clear, it is clearly the result of increased ARF activity and not simply an artifact from overexpression of the protein (17). The morphological changes accompanying expression of [Q71L]ARF3 include a large expansion in the area of mannosidase II staining around the nucleus (see Fig. 5, panels D, F, J, L, and N) and a more diffuse staining in the cytosol which is apparent by microscopy but not readily captured in pictures, such as those in Fig. 5.

Results from the cell-based Golgi expansion assay reveal a lack of correlation among the assays for ARF function employed in this study. When the mutant ARFs were tested for the ability to vesiculate Golgi, three different responses were observed: the control [Q71L]ARF3 and two of the switch domain mutants, I49T and R79G, had equivalent activities, some (I74T and Y81C) had decreased activity, and some (wild type ARF3 or F51Y) had no activity. The loss of activity of the F51Y mutant in this assay contrasts with its ability to bind GTPγS and retention of, though diminished, COP-I recruitment. Although this mutant has also lost the ability to stimulate PLD1 activity, a further comparison of activities of the I49T mutant, in which PLD1 activation is lost but Golgi expansion is retained, led us to conclude that neither PLD1 stimulation nor coatomer recruitment is required for Golgi expansion in NRK cells. Because partial activity in a cell-based assay is difficult to interpret, we focused our discussion on the F51Y mutant, but further comparisons of other mutants in the different assays revealed an overall poor correlation, consistent with some level of independence in mechanisms.

Finally, we address the possibility that two effectors, working together, may result in vesiculation of Golgi in the presence of [Q71L]ARF. We know of at least six different proteins, or complexes, that localize to the Golgi in an ARF-sensitive manner: PLD1, COP-I, AP-1, and three GGA proteins. We recently observed that the overexpression of GGA1 actually prevents the vesiculation that results from expression of [Q71L]ARF1, eliminating this group of proteins. Although we did not find a second site mutation in [Q71L]ARF3 which eliminated both PLD1 activation and COP-I recruitment with retention of Golgi vesiculation, we conclude that the magnitude of the loss of affinity of mutants for each of these effectors was sufficient to rule out the possibility that PLD1 activation and COP-I recruitment work cooperatively to nucleate vesicle budding and formation, leading to vesiculation in the presence of [Q71L]ARF3.

Recent results have turned the focus of COP-I action toward earlier steps in the secretory pathway, e.g. actions at the intermediate compartment (52) and its function primarily in retrograde transport (53). It thus seems more likely that if COP-I is involved in defects in the secretory pathway, resulting from expression of an activated ARF, it would be acting at an earlier step, e.g. expansion of the ER lumen, and not the changes in Golgi morphology. This leaves only AP-1, either acting alone or in concert with another effector, to explain the changes to Golgi morphology. A role for ARF in recruitment of AP-1 to the trans-Golgi network was first suggested by Stamnes and Rothman (11) and characterized in detail by Traub and colleagues (54). The recruitment of AP-1 by ARF to Golgi membranes appears to be indirect as it occurs after GTP hydrolysis on ARF is completed (55). Further, we found no evidence of AP-1 accumulation on vesiculated Golgi of NRK cells expressing [Q71L]ARF1.4 Although we cannot rigorously exclude the possibility that some combination of these previously identified effectors is sufficient to vesiculate Golgi membranes when ARF activity is in excess, we favor the simpler explanation that there exists at least one more effector, not yet identified, which is responsible for this activity.

Definitive conclusions regarding cellular mechanisms are difficult because of the problems inherent in translating from reconstitution assays in vitro to functions in live cells. How much residual PLD1 activity is enough to serve its role? How much COP-I recruitment activity will suffice in a cell? By comparing effects of specific mutations we have been able to uncouple clearly each of the three different ARF-dependent activities: stimulation of PLD1, COP-I recruitment, and Golgi vesiculation. We conclude that (i) activation of PLD1 is not a
required step in the recruitment of coatamer; (ii) activation of PLD1 is not required for vesiculation of Golgi in cells; and (iii) recruitment of COP-I by ARF is not required for vesiculation of Golgi. We further conclude that there must exist an effector for ARF action at the Golgi, responsible in whole or part for the vesiculation in the presence of excess activated ARF. Further refinement of specific loss-of-interaction mutants will likely be the best hope for further dissection of the complex signaling paths that seem to converge at or near the Golgi but can impinge upon a diverse array of cellular activities.

Acknowledgments—We thank Michael G. Roth and Nicholas T. Kistakis for reading the manuscript and providing valuable comments. We also thank J. David Lambeth, Haiyan Fu, John Hepler, and Harish Joshi for suggestions and comments. Valuable reagents were the generous gifts of Stephen J. Elledge (two-hybrid strains and plasmids), Rob Brazas (pBG4D plasmid expressing the GAL4 binding domain fusion protein at the COOH terminus of bait protein), Sung Ho Ryu (viral plasmid expressing the KEX2 signal peptide), and Aimee Anido helped with preparation of Chinese hamster ovary cells, and Mary-Ann Roelen (rabbit antibodies to mannosidase II). Chun-Jiang Zhang provided assistance with yeast genetics. Joseph L. Sztul, John H. Heal, and Robert A. Kahn thank Michael G. Roth and Nicholas T. Kistakis for reading the manuscript and providing valuable comments.