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ARTICLE *in* CELL · NOVEMBER 1991

Impact Factor: 32.24 · DOI: 10.1016/0092-8674(91)90176-Y · Source: PubMed

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Cell, Vol. 67, 239–253, October 18, 1991, Copyright © 1991 by Cell Press

ADP-Ribosylation Factor Is a Subunit of the Coat of Golgi-Derived COP-Coated Vesicles: A Novel Role for a GTP-Binding Protein

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Summary

ADP-ribosylation factor (ARF) is an abundant and highly conserved low molecular weight GTP-binding protein that was originally identified as a key element required for the action of cholera toxin in mammalian cells, but whose physiological role is unknown. We report that ARF family proteins are highly concentrated in non-clathrin-coated transport vesicles and are coat proteins. About three copies of ARF are present on the outside of coated vesicles per α-COP (and thus per coatomer). ARF is highly enriched in coated vesicles as compared with parental Golgi cisternae, as shown both by biochemical and morphological methods, and ARF is removed from transport vesicles through uncoating during transport. Furthermore, ARF binds to Golgi cisternae in a GTP-dependent manner independently of coated vesicle budding. These observations strongly suggest a new role for GTP-binding proteins: ARF proteins may modulate vesicle budding and uncoating through controlled GTP hydrolysis.

Introduction

A role for GTP hydrolysis in constitutive secretion was first proposed on the basis of the Saccharomyces cerevisiae *SEC4* gene sequence, which predicted a 23.5 kd GTP-binding protein (Salminen and Novick, 1987). Sec4p function is needed for the attachment of post-Golgi vesicles to the cell surface, and it is localized to post-Golgi secretory vesicles and the cytoplasmic face of the plasma membrane (Goud et al., 1988). The 21 kd product of the *YPT1* gene of S. cerevisiae (Gallwitz et al., 1983) is also a GTP-binding protein (Schmitt et al., 1986), but has a role early in the secretory pathway (i.e., between the endoplasmic reticulum and Golgi) in vivo (Segev et al., 1988; Schmitt et al., 1988) and in vitro (Bacon et al., 1989; Baker et al., 1990). Genes encoding mammalian *YPT1* homologs, the *rab* genes, have been cloned (Touchot et al., 1987; Haubruck et al., 1987; Bucci et al., 1988; Zahraoui et al., 1989; Chavrier et al., 1990a, 1990b), one of which (rab1) can replace the *YPT1* gene in yeast (Haubruck et al., 1989). The *rab* gene products, like Sec4p and Ypt1p, are specifically localized to particular intracellular membrane compartments (Fischer von Mollard et al., 1990; Mizoguchi et al., 1990; Darchen et al., 1990; Goud et al., 1990; Chavrier et al., 1990a, 1990b); therefore, it has been suggested that these proteins function in vesicle targeting, perhaps serving to confer specificity and directionality on vesicular transport (Segev et al., 1988; Bourne, 1988; Walworth et al., 1989).

Another family of GTP-binding proteins is the ADP-ribosylation factor (ARF) family. Vibrio cholerae secretes a toxin that catalyzes the ADP-ribosylation of the α subunit of the trimeric G protein G_s, thus leading to activation of adenylate cyclase (Cassel and Pfeuffer, 1978). ARF was originally identified as a protein cofactor required in this ribosylation reaction (Schleifer et al., 1982; Kahn and Gilman, 1984), and was subsequently shown to be a GTP-binding protein in its own right (Kahn and Gilman, 1986). It is an abundant eukaryotic protein, representing 0.3%–1% of bovine brain protein, and is predominantly cytosolic (at least 50% and often 90%, depending upon the source [Kahn et al., 1988]). The first clue to its physiological role in the cell was immunofluorescence and immunocytochemistry data showing that ARF is concentrated in the Golgi apparatus (Stearns et al., 1990). Two ARF genes exist in S. cerevisiae (one of which is minimally required for viability) whose predicted protein products are roughly 80% identical in amino acid sequence to the predicted product of the mammalian ARF1 gene (Sewell and Kahn, 1988; Stearns et al., 1990). The mammalian ARF1 gene can substitute for the two yeast genes (Kahn et al., 1991), and yeast strains exhibit a complex secretion-defective phenotype in the absence of the yeast ARF1 protein (Stearns et al., 1990).

Other evidence for the role of GTP hydrolysis in secretion comes from studies in cell-free systems. The slowly hydrolyzable GTP analog GTPγS and the trimeric G protein–activating species AlF_{3-5} (added as AlF_4^-; Sternweis and Gilman, 1982; Bigay et al., 1987; Higashijima et al., 1991) inhibit transport (Melançon et al., 1987) in an in vitro system that reconstitutes vesicular transport between the cis and medial cisternae of the Golgi (Balch et al., 1984; Balch and Rothman, 1985). GTPγS also inhibits transport in several other in vitro systems (Baker et al., 1988; Ruohola et al., 1988; Beckers and Balch, 1989; Mayorga et al.,

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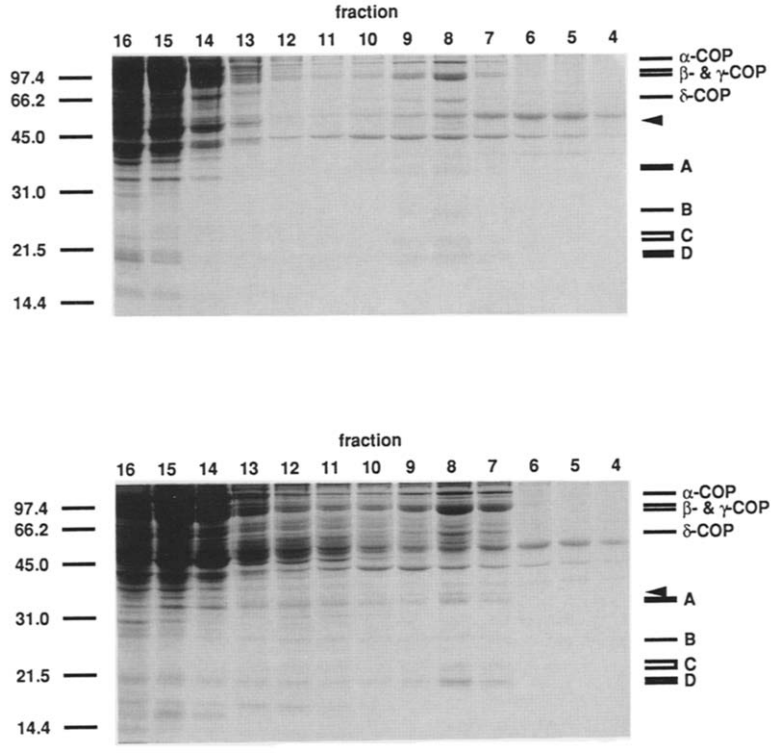

Figure 1. Low Molecular Weight COP-Coated Vesicle Proteins

COP-coated vesicles were prepared from either CHO Golgi membranes (from clone 15B cells that were VSV infected, i.e., donor membranes) or rabbit liver Golgi membranes, and proteins in fractions 4 through 16 of the isopycnic gradients were trichloroacetic acid precipitated and subjected to SDS–PAGE in 15% acrylamide gels. Fraction sucrose density increases toward the right. ▾

(Top) CHO Golgi-derived COP-coated vesicles peak in fractions 7–9 (COPs indicated on the right); cofractionating bands are labeled (on the right) as A (doublet of 35 and 36 kd), B (major band of 27 kd plus several minor species), C (triplet of bands at 22–23 kd), and D (doublet at ~20 kd).

(Bottom) Rabbit liver Golgi-derived COP-coated vesicles also peak in fractions 7–9 (COPs again indicated on the right); the pattern of protein bands present in CHO Golgi-derived coated vesicles is also seen in these COP-coated vesicles (again labeled A through D). Note, however, that not all bands in this region are identical between vesicle preps; the band marked with the arrowhead in the top panel is diminished in intensity or absent from the protein profile in the bottom panel, and likewise for the protein marked with the arrowhead in the bottom panel. Half of each fraction was loaded in the bottom panel. Molecular weights are indicated on the left (standards, from top to bottom: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme).

1989; Bomsel et al., 1990; Tooze et al., 1990; Miller and Moore, 1991; Goda and Pfeffer, 1991). Under conditions of either GTPγS or AlF_4^- inhibition in the cis to medial Golgi transport system, large numbers of non–clathrin-coated transport vesicles (Griffiths et al., 1985; Orci et al., 1985, 1986) accumulate (Melançon et al., 1987) and can be purified from salt washes of inhibited Golgi membranes (Malhotra et al., 1989; Serafini et al., 1991). Four high molecular weight coat proteins (COPs: α-COP, 160 kd; β-COP, 110 kd; γ-COP, 98 kd; and δ-COP, 61 kd) have been identified as major constituents of the non-clathrin coat (Serafini et al., 1991). The four COPs exist in the cytosol with three other lower molecular weight proteins in a complex termed the coatomer (Waters et al., 1991), so named as it is presumably the protomeric unit of the non-clathrin coat. Indeed, brefeldin A, a drug that blocks intracellular transport, blocks binding of coatomer to Golgi membranes and coated vesicle production (Orci et al., 1991).

Because we expect the COP-coated transport vesicles to be enriched in the protein machinery mediating vesicular transport (such as the COPs), we have begun the task of identifying other vesicle proteins that may be transport components. We report here that in addition to the four high molecular weight COPs, several low molecular weight proteins are also present in COP-coated vesicles. Several are GTP-binding proteins, and two of these are members of the ARF family.

Results

Identification of Low Molecular Weight Vesicle Proteins

We have previously reported the identification of four coat proteins (α-, β-, γ-, and δ-COP) and several minor proteins of relatively high molecular weight (>40 kd) present in COP-coated vesicles (Serafini et al., 1991). However, several lower molecular weight (<40 kd) proteins are also prominent in COP-coated vesicle preparations. When COP-coated vesicles are produced using donor membranes (a Golgi-enriched fraction from vesicular stomatitis virus [VSV]-infected CHO 15B cells), several low molecular weight proteins copurify with α–δ-COP and the coated vesicles (in fractions 7–9, Figure 1, top panel) at a sucrose density of 1.18 g/ml. These vesicle proteins are also present in vesicles derived from a different membrane source, namely, rabbit liver Golgi membranes (fractions 7–9, Figure 1, bottom panel). Therefore, these proteins are unlikely to be transported cargo molecules and are most likely part of the transport machinery. The vesicle proteins of 14–40 kd common to COP-coated vesicles prepared from both membrane sources include a doublet of bands at 35 kd and 36 kd (labeled A in Figure 1, top and bottom), one major and possibly several minor species at 27 kd (B), a triplet of bands at 22–23 kd (C), and a major and minor species in a doublet at ~20 kd (D). The two protein bands

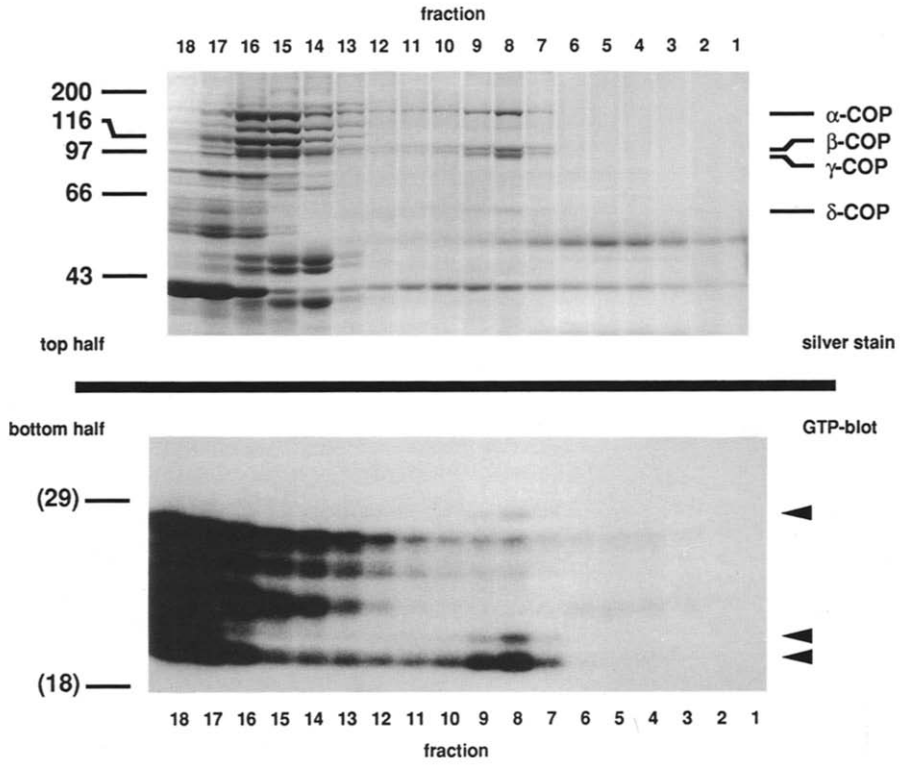

Figure 2. Several Low Molecular Weight GTP-Binding Proteins Are Present in COP-Coated Vesicles

Proteins in fractions 1–18 of the isopycnic gradient of a CHO Golgi-derived COP-coated vesicle preparation were subjected to SDS–PAGE in a 15% acrylamide gel as described in Figure 1; the proteins in the top half of the gel were silver stained (top panel) while lower molecular weight proteins in the bottom half were transferred to nitrocellulose and GTP-blotted (bottom panel) (see Experimental Procedures). (Top panel) The four major COPs (indicated on the right) peak again in fractions 7–9. (Bottom panel) At least three GTP-blotting proteins also peak in these three fractions (arrowheads): one protein at ~29 kd and a doublet at ~20 kd. Molecular weights are indicated on the left; those indicated within parentheses are approximate values determined using prestained markers (standards, from top to bottom: myosin heavy chain, β-galactosidase, phosphorylase B, bovine serum albumin, and ovalbumin [Top panel]; prestained carbonic anhydrase and β-lactoglobulin [Bottom panel]). To effect the separation observed, voltage was applied until the ~18 kd prestained marker had nearly run off the gel.

at 36 and 35 kd comigrate during SDS–PAGE with the p36 and p35 proteins of the coatomer, and their abundance in the vesicles relative to the identified COPs is similar to that observed in the coatomer (Waters et al., 1991). Therefore, these two proteins likely represent two additional COPs (i.e., ε- and ζ-COP), but a firm conclusion awaits sequence comparison of p35 and p36 in coated vesicles and coatomer. The major low molecular weight vesicle protein (as determined by Coomassie staining) is the major band of the doublet D.

Certain minor proteins are significantly more prominent in vesicles derived from one membrane source than the other (for example, the bands marked by the arrowheads in Figure 1, top and bottom). This might suggest that these proteins are tissue-specific cargo molecules or perhaps reflect tissue or species specificities in transport components. However, given the differences in purity between the two coated vesicle preparations in Figure 1, top and bottom, it is certainly possible that these differences reflect different contaminants from the two sources.

GTP-Binding Proteins in Coated Vesicles

Because GTP-binding proteins (of 20–29 kd) have been shown to be associated with transport vesicles (Sec4p and the rab3A protein [Goud et al., 1988; and Fischer von Mollard et al., 1990; Mizoguchi et al., 1990; and Darchen et al., 1990, respectively]), we were interested in whether any such proteins were present in COP-coated vesicles. As a first step, we decided to employ GTP blotting (McGrath et al., 1984) to detect such proteins. This method takes advantage of the remarkable fact that many of the known monomeric (20–30 kd) GTP-binding proteins, after SDS–PAGE and transfer to nitrocellulose, will refold and bind GTP in situ. At least three small GTP-binding proteins (arrowheads in Figure 2, bottom panel) copurify in fractions 7–9 with the coated vesicles (as marked by the COPs in fractions 7–9, Figure 2, top), with their relative abundances mirroring that of the COPs in these fractions. Other GTP-binding proteins are found in large amounts near the top of the gradient (where the gradient was loaded) and must represent either soluble forms of these proteins or those associated with membranes of a very low density. This implies a specificity in the association of the former group of GTP-binding proteins with the COP-coated vesicles. The GTP-binding protein of approximately 29 kd (top arrowhead in Figure 2, bottom) is noteworthy since it appears only in the vesicles and virtually nowhere else in the gradient. The most abundant GTP-binding protein in the

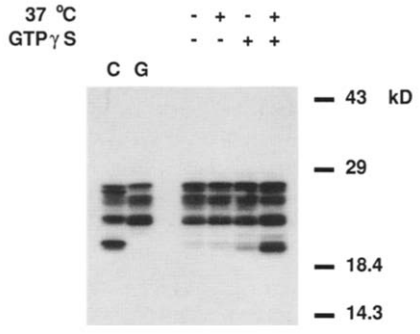

Figure 3. GTP-Binding Proteins of ~20 kd Accumulate on Golgi Membranes in the Presence of GTPγS at 37°C

CHO Golgi membranes (donor and acceptor membranes mixed 1:1) were incubated in reactions either kept on ice or at 37°C (– or + 37°C) for 15 min in the absence or presence of 20 μM GTPγS (– or + GTPγS). Membranes were reisolated, and the proteins present subjected to SDS–PAGE and GTP blotting. Lane C, 50 μg of bovine brain cytosol; lane G, 50 μg of CHO Golgi membranes (from a TCA precipitation of 80 μl of membranes). Molecular weights are indicated on the right (standards, from top to bottom: ovalbumin, carbonic anhydrase, β-lactoglobulin, and lysozyme). Transport (measured as the incorporation of [^{3}H]GlcNAc into VSV-G protein) was measured in 75 μl duplicate aliquots of the binding reactions, confirming that transport does not occur to any significant extent on ice or in the presence of GTPγS: 84 cpm (–/–), 3123 cpm (+/–), 98 cpm (–/+), and 677 cpm (+/+).

vesicles revealed by GTP blotting is approximately 20 kd, as estimated by SDS–PAGE.

ARF Is Present in COP-Coated Vesicles

A clue to the identity of this major 20 kd coated vesicle GTP-binding protein came from studying the distribution of small GTP-binding proteins observed in the starting materials for the in vitro reaction used to produce the vesicles (Figure 3). The bovine brain cytosol used in our system has major GTP-binding proteins near 20 kd (lane C in Figure 3), whereas the CHO membranes we use do not have any GTP-binding proteins of this size (lane G). Incubation of the membranes with cytosol at 37°C leads to membrane association of at least some of these ~20-kd GTP-binding proteins, an effect that is dramatically enhanced by GTPγS (rightmost two lanes, Figure 3), the same conditions used to accumulate COP-coated vesicles. Of the known small GTP-binding proteins, only the 21 kd ARF is predominantly cytosolic in subcellular fractionation. As previously mentioned, ARF has been shown by immunofluorescence to be concentrated in the Golgi apparatus when bound to membranes, and has been shown to have a probable role in intracellular protein transport in S. cerevisiae. In light of this, we tested the hypothesis that the 20 kd GTP-binding protein of the coated vesicles is ARF.

Using an affinity-purified anti-peptide antiserum directed against residues 23–36 of bovine ARF (antiserum R5; Kahn et al., 1988), we detected ARF in the COP-coated vesicles by Western immunoblotting (Figure 4). ARF cofractionates with α–δ-COP (compare Figure 4, first and third panels) in fractions 7–9 (1.18 g/ml, 40.4% [w/w] sucrose, Figure 4, fourth panel) of the final isopycnic gradient of a COP-coated vesicle preparation, appearing as a doublet of 21 kd (minor band) and 20 kd (major band). ARF is found in the gradient both in the coated vesicle–containing

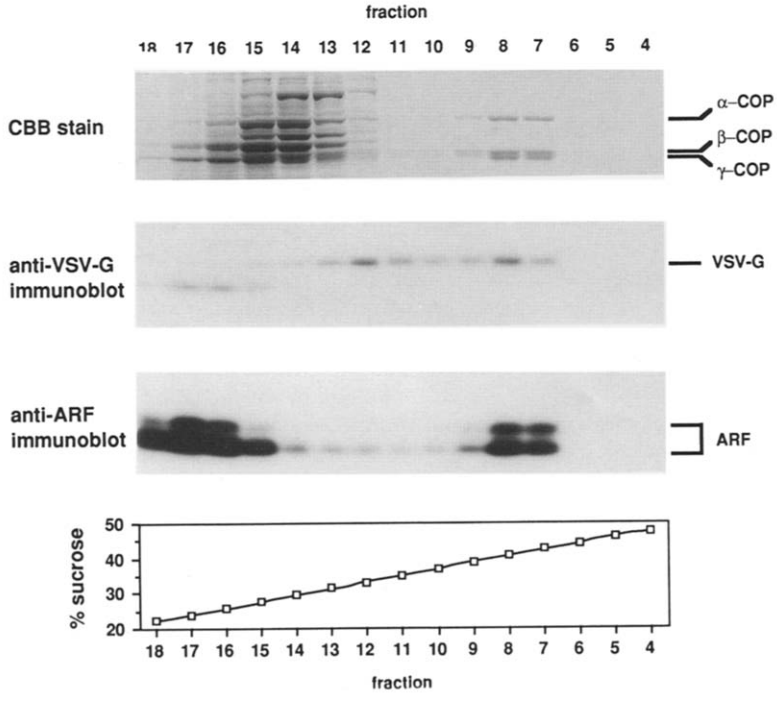

Figure 4. ARF Is Present in COP-Coated Vesicles

Proteins in fractions 4–18 of the isopycnic gradient of a CHO Golgi (donor)-derived vesicle preparation were subjected to SDS–PAGE; the top third of the gel was removed, and the proteins within were stained with Coomassie brilliant blue (CBB) (first panel), while the proteins in the bottom two-thirds of the gel were transferred to nitrocellulose. The nitrocellulose filter was cut into a top and bottom half; the top half (i.e., middle portion of the original gel) was immunoblotted to detect VSV-G protein using a monoclonal antibody (second panel), and the bottom half (i.e., bottom portion of the original gel) was immunoblotted to detect ARF proteins using an affinity-purified anti-peptide antiserum (third panel).

(First panel) The COP-coated vesicles (as delineated again by the COPs, the three largest of which are indicated on the right) peak in fractions 7–9.

(Second panel) VSV-G protein (indicated on the right) peaks in two places in the gradient: in fractions 7–9 (with the coated vesicles) and in fractions 11–13 (with Golgi membranes). The lower molecular weight band of lesser immunoreactivity near the top of the gradient (fractions 16 and 17) is either a soluble "clipped" form of VSV-G (Little and Huang, 1977) or a cross-reacting, soluble contaminant.

(Third panel) ARF (appearing as a doublet) cofractionates in the gradient with the coated vesicles, with its abundance mirroring that of the COPs in fractions 7–9. No enrichment of ARF is seen in fractions 11–13; rather, the remaining ARF in the gradient is near the top of the gradient.

(Fourth panel) Percent sucrose (w/w) composition of the gradient fractions as determined by refractometry.

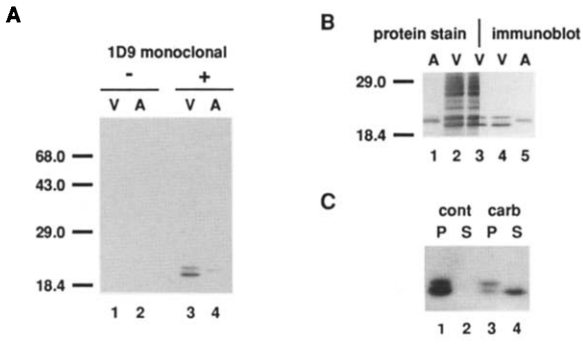

Figure 5. The Two Vesicle-Localized ARF Proteins Are Distinct, yet Immunologically Related Species, One of Which Is the Major Low Molecular Weight Protein of the COP-Coated Vesicles

(A) COP-coated vesicle proteins (V, one-tenth of the protein in pooled peak fractions [7–9] from a rabbit liver Golgi-derived preparation) and purified, recombinant mammalian ARF1 protein (A, 67 ng) were subjected to SDS–PAGE in a 12.5% minigel, transferred to nitrocellulose, and immunoblotted with (+) a monoclonal antibody (1D9) raised against ARF1. The ARF doublet in the vesicles appears also with this antibody. Detection was with a rabbit secondary antibody and [^{125}I]protein A. Omission of this monoclonal antibody (−) evinces the specificity of the detection. Identical specificity was observed with the affinity-purified R5 anti-peptide antiserum, and the relative reactivities of the three bands as observed here are roughly the same as compared with those observed when using whole R5 antiserum (data not shown).

(B) Vesicles (V, as in [A]) and ARF1 protein (A, as in [A]) were subjected to SDS–PAGE and transferred to nitrocellulose. The filter was sliced down the center of lane 3; the left half was stained with colloidal gold to detect total proteins, and the right half was immunoblotted with the 1D9 monoclonal to detect ARF proteins (using a horseradish peroxidase–conjugated goat secondary antibody and DAB/metal). The two halves were then realigned. The faster migrating ARF band aligns with the major low molecular weight vesicle protein of 20 kd; this alignment and the abundance (by staining and immunoblotting) of this band relative to the ARF1 protein sample lead to the tentative assignment of this protein as the faster migrating ARF band. (This protein stains much more intensely with either Coomassie brilliant blue or Ponceau S relative to the other vesicle proteins than it does with either silver or colloidal gold.) The more slowly migrating ARF band does not align with any one protein (it aligns in the middle of a doublet of proteins more abundant relative to the ARF1 sample than it is).

(C) Most of the faster migrating ARF band extracts with carbonate into the supernatant (S), while all of the more slowly migrating ARF band remains with the membranes in the pellet (P) under these conditions (cont, control dilution; carb, carbonate extraction; see Experimental Procedures). Under these extraction conditions, several of the other low molecular weight vesicle proteins also behave as integral membrane proteins (data not shown).

fractions (7–9) and at the top of the gradient (fractions 15–18) where soluble proteins sediment. It was conceivable that the presence of ARF in the coated vesicles is due to a nonspecific association. However, ARF is highly concentrated in coated vesicles as compared with bulk Golgi membranes, based on the following: We have shown previously (Serafini et al., 1991) that when COP-coated vesicles are produced using Golgi membranes from VSV-infected cells, the viral G protein, as expected, is present in the coated vesicles (Figure 4, second panel, fractions 7–9). VSV-G protein is also present in the gradient in fractions corresponding to Golgi membrane contaminants (fractions 11–13, approximately 35% [w/w] sucrose; the CHO Golgi membranes added to the reaction, and from which the vesicles are derived, were originally isolated after a floatup to a 35%/29% [w/w] sucrose interface). The concentration of VSV-G protein is the same in coated vesicles as in parental Golgi cisternae (Orci et al., 1986), and can thus serve to quantify secretory pathway membrane throughout the gradient. ARF is clearly enriched in the COP-coated vesicles, with a distribution in the three peak fractions (7–9) that mirrors that of the COPs, but very little ARF is present in fractions 11–13, even though the VSV-G protein content of fractions 11–13 and 7–9 is very similar. Thus, ARF is specifically associated with the COP-coated vesicles, and not bulk secretory membranes.

Immunoblotting of COP-coated vesicles using a monoclonal antibody (1D9) raised against the recombinant mammalian ARF1 protein (R. A. K., unpublished data) confirmed that both the 20 kd and 21 kd species identified with the ARF-reactive anti-peptide antiserum will also react with an ARF-specific monoclonal antibody (compare lane 3 and lane 1, Figure 5A). Note that we have not conclusively shown that the GTP-binding proteins of ~20 kd identified by GTP blotting are these two ARF proteins, although the correspondences in molecular weights and relative abundances strongly suggest this; however, none of our conclusions concerning ARF would be invalidated were these GTP-binding proteins shown not to be ARF proteins.

To determine if any of the observed low molecular weight vesicle proteins (see Figure 1) are these ARF proteins, purified recombinant mammalian ARF1 and purified COP-coated vesicles were subjected to SDS–PAGE, electrophoretically transferred to nitrocellulose, and either stained to identify protein bands or immunoblotted with the 1D9 antibody (Figure 5B). The major low molecular weight (20 kd) protein band in the COP-coated vesicles aligns precisely with the faster migrating ARF species present in the vesicles (in lane 3 of Figure 5B, a split lane, the left half of which was stained to identify protein and the right half of which was immunoblotted to identify the ARFs present); moreover, the immunoreactivity of the purified ARF1 standard relative to this faster migrating ARF species is that expected from the protein-staining intensities of the ARF1 standard and the 20 kd vesicle band (compare lanes 5 and 4 with lanes 1 and 2, respectively). Thus, this major 20 kd vesicle band is composed for the most part of the faster migrating ARF protein. The more slowly migrating ARF band in the vesicles does not align exactly with any one vesicle protein band (compare the right and left halves of lane 3), and this fact, together with a comparison of protein-staining intensities and immunoreactivities, precludes an assignment of any observed vesicle protein as this more slowly migrating ARF species.

That ARF should appear as a doublet (with neither band comigrating with recombinant mammalian ARF1) is not surprising, as two distinct ARF proteins have been purified from bovine brain membranes (Kahn and Gilman, 1984) and cytosol (Tsai et al., 1988), and several mammalian cDNAs have been cloned (see Discussion). Furthermore, since some but not all ARF proteins are myristylated (Kahn et al., 1988; Weiss et al., 1989), this or other posttransla-

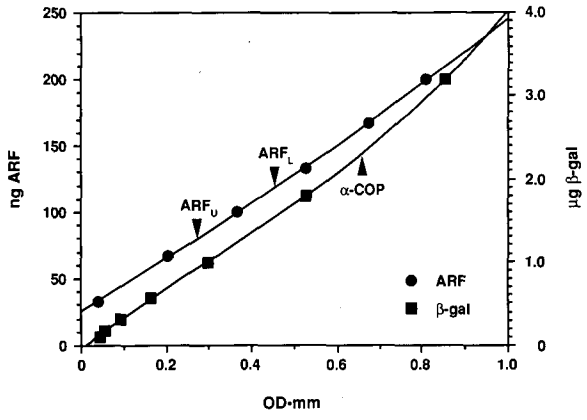

Figure 6. Quantitation of ARF in the COP-Coated Vesicles

See Experimental Procedures for details. The integrated optical densities (OD·mm) of the standards are plotted against the quantities loaded (μg for β-galactosidase, ng for ARF1). The lines plotted for the data are as follows: ng of ARF = 25.825 + 190.46(OD·mm) + 29.317(OD·mm)^2, R^2 = 1.000; μg of β-gal = −0.041360 + 3.8657(OD·mm) − 1.7527(OD·mm)^2 + 1.9571(OD·mm)^3, R^2 = 1.000. The positions of the unknowns along the curves are indicated by arrowheads (ARF$_U$, upper ARF vesicle band; ARF$_L$, lower ARF band; α-COP). The calculated values for the vesicle proteins are as follows: 200 ng (119 ng + 80 ng) of ARF in one-fourth the amount of coated vesicles loaded to yield 2.3 μg of α-COP. Assuming a molecular weight of 20 kd for both ARF bands and 160 kd for α-COP, the measured values yield a molar ratio of approximately three ARF molecules per α-COP molecule in the coated vesicles.

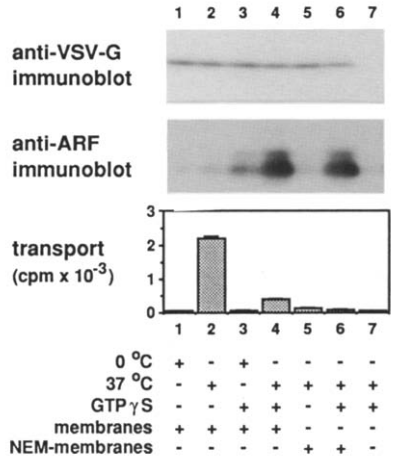

Figure 7. ARF Binding to Golgi Membranes Correlates with the Accumulation of Coated, but Not Uncoated, Vesicles

Various binding reactions were constructed with CHO Golgi membranes (1:1 mix of donor and acceptor membranes) that were untreated (+ membranes) or had been NEM treated to eliminate NEM-sensitive fusion protein activity (+ NEM membranes). Reactions were either held on ice (+ 0°C) or were incubated 15 min at 37°C (+ 37°C); several contained 20 μM GTPγS (+ GTPγS). After incubation, the membranes were recovered by centrifugation, and the proteins present were subjected to SDS–PAGE and transferred to nitrocellulose. The top portion of the filter was immunoblotted to detect VSV-G protein (top panel), while the bottom portion was immunoblotted with affinity-purified antipeptide antiserum (R5) to detect the ARF proteins present (middle panel). The VSV-G signal served as a control for membrane recovery (the reaction of lane 7 lacked any membranes). Under conditions that accumulate coated vesicles (lane 4), much ARF binding to the membranes is observed, while those conditions promoting the accumulation of uncoated vesicles (lane 5) reveal little or no ARF binding to the membranes. NEM treatment does not, however, eliminate the ability of the membranes to bind ARF (lane 6; the slightly lower ARF signal is due to the slightly lower level of membrane recovery). (Bottom panel) Transport was measured as described in the legend to Figure 3.

tional modifications could also account for the multiple species observed with SDS–PAGE. The two ARF bands are clearly distinct proteins, however, even if they are immunologically very similar, since carbonate extraction of vesicles releases most of the faster migrating but not the more slowly migrating ARF species into a soluble form (Figure 5C); whether or not this reflects a difference in myristylation or some other posttranslational modification remains to be determined.

ARF Is a COP-Coated Vesicle Coat Protein

We quantitated the amount of ARF present in the vesicles by comparing its abundance with that of α-COP (Figure 6). Approximately three ARF molecules are present for every α-COP molecule in the COP coat of a coated vesicle (assuming ARF is distributed equally among all vesicles, and summing contributions from both the upper and lower ARF species). Thus, ARF is present in comparable amounts to coat subunits, approximately three ARFs per coatomer, implying that ARF also is a coat protein.

If so, ARF should be present in transport vesicles before but not after uncoating. To address this question, Golgi membranes were incubated under conditions that lead to the accumulation of either coated or uncoated vesicles and assayed for an accumulation of ARF on the membranes. Golgi membranes incubated in vitro in the presence of GTPγS accumulate the COP-coated vesicles (Melançon et al., 1987), while those incubated after pretreatment with N-ethylmaleimide (NEM) accumulate vesicles that are uncoated (Malhotra et al., 1988). This accumulation of uncoated vesicles occurs because NEM treatment inactivates the NEM-sensitive fusion protein present on the membrane (Glick and Rothman, 1987), blocking the fusion of transport vesicles with their target membranes, but not their prior uncoating. As expected if ARF is in coated vesicles, when Golgi membranes are incubated at 37°C in a reaction containing GTPγS (to accumulate coated vesicles), ARF accumulates on the membranes (compare lane 4 with lane 2, Figure 7). Moreover, NEM-treated Golgi membranes, which accumulate uncoated vesicles, show no such accumulation of ARF (compare lane 5 with lane 4). (In fact, the amount of ARF present on NEM-treated membranes after incubation is actually less than that found on untreated, control membranes after a similar incubation [compare lane 5 with lane 2]; these latter membranes are known to have a population of coated buds and vesicles, but much less than with GTPγS.) NEM, however, does not abolish the ability of membranes to bind ARF per se, since incubation of NEM-treated membranes in the presence of GTPγS leads to a large accumulation of ARF on the membranes (compare lane 6 with lane 5). Under these same conditions, it has been shown previously that coated vesicles accumulate

(Orci et al., 1989). These data support the interpretation that ARF is present in the vesicles as a coat component, and also suggest that most of the ARF bound to Golgi complexes is in coated vesicles and not in Golgi cisternae at large, consistent with the enrichment of ARF in coated vesicles relative to coated vesicle-depleted Golgi membranes revealed by density gradients (see Figure 4).

ARF was localized in these same Golgi membrane fractions by immunoelectron microscopy to test independently the idea that ARF is a coat protein. ARF-specific immunolabeling is present over the profiles of the COP-coated vesicles accumulated from Golgi membranes incubated in GTPγS-containing reactions (Figure 8A), while virtually no label is present over the uncoated vesicles accumulated from NEM-treated membranes (Figure 8B). Quantitatively, the surface and linear densities of ARF in coated vesicles are at least 10-fold higher than in uncoated vesicles accumulated in incubations lacking NEM-sensitive fusion protein activity (Table 1, lines 1–3 versus lines 4–6).

However, there is a caveat to this experiment that needs to be ruled out. Because ARF is itself a GTP-binding protein, it is conceivable that GTPγS induces ARF to bind in a nonspecific fashion to already formed coated vesicles, which have independently accumulated with GTPγS. To rule this out we have used AlF_4^- to accumulate coated vesicles (Melançon et al., 1987; Rothman and Orci, 1990), because ARF is not activated by $AlF_{3\text{-}5}$ (R. A. K., submitted).

We find that ARF is also present on vesicles accumulated in the presence of AlF_4^- (Figure 8C), at a similar (at least 50%) level to that found in GTPγS-accumulated coated vesicles (Table 1, lines 1–3 versus lines 7–9). Furthermore, as predicted from the ARF-binding and fractionation studies, ARF is concentrated in coated vesicles as compared with bulk cisternal membranes. Coated vesicles accumulated with AlF_4^- have at least 4-fold higher surface and linear densities of ARF than does surrounding cisternal membrane (Table 1). It is noteworthy that at least twice as much ARF is bound to bulk cisternae in the presence of GTPγS than in the presence of AlF_4^-, implying a GTP-dependent association of ARF with cisternae that is independent of coated vesicle budding. Nevertheless, the actual concentration of ARF in coated vesicles is relatively independent of whether the coated vesicles are accumulated with GTPγS or AlF_4^-.

ARF Binding to Membranes Requires a Membrane-Associated Protein

A membrane-associated protein component (e.g., a receptor) is required for GTPγS-dependent ARF binding to Golgi membranes. Pretreatment of Golgi membranes for 10 min at 55°C or 100°C (Figure 9, lanes 2 and 3, respectively) progressively abolishes the ability of the membranes to bind ARF in the presence of GTPγS. Furthermore, trypsin treatment of the membranes completely eliminates the ability of the membranes to support ARF binding in a subsequent in vitro reaction (compare mock trypsin treatment, lane 4, and trypsin treatment, lane 5). With one-half the previously added amount of mock trypsin–treated membranes present in the reaction, roughly one-half of the full level of ARF binding is observed (lane 6), and as expected, no ARF binding is observed when one-half of the previously added amount of trypsin-treated membranes is added (lane 7). More importantly, trypsin treatment does not create an inhibitor of ARF binding, since roughly one-half of the normal amount of ARF binds when equal amounts of trypsin- and mock trypsin–treated membranes are mixed in a reaction (lane 8). In this case, the amount of ARF binding is precisely that expected if ARF binds only to mock trypsin–treated membranes (lane A) and not to all membranes present in the reaction (lane B). Thus, the membrane-associated protein that is required for ARF binding most likely does not convert ARF into a soluble "activated" form capable of binding to membranes in general; i.e., ARF binding occurs only to those membranes that contain a specific protein component.

Discussion

We find that ARF is a structural component of coated vesicles, a coat protein. This suggests a precise role for ARF in cell biology—in the formation and uncoating of coated transport vesicles. The essential problem involved in employing a coat to bud vesicles from one membrane in order to deliver a piece of that membrane (and its lumenal contents) to another membrane is one of vectoral and spatial regulation: how, in the very same cytosolic compartment, to assemble coats at the donor membrane and disassemble coats at the target membrane. One possible solution to this problem would be to control coat assembly and disassembly according to the types of nucleotides bound to a coat substituent such as ARF, with GTP entering at the donor and GDP exiting at the acceptor. Like the GTP of tubulin in a microtubule, nucleotide triphosphate binding to a coat substituent could be the process permitting stable coat assembly, while hydrolysis of that triphosphate could be the process promoting coat disassembly.

The biophysical properties of ARF make it well suited for such a role. ARF, as purified from cytosol, is accompanied by a bound GDP molecule and has no measurable GTPase activity (upper estimate of 0.0015/min), nor will it readily exchange GTP for endogenous GDP (Kahn and Gilman, 1986). (However, exchange can be accomplished under conditions of high ionic strength or denaturation/renaturation [Kahn and Gilman, 1986; Weiss et al., 1989].) Thus, in the absence of presumptive external protein effectors (such as nucleotide exchange proteins or GTPase-activating proteins [GAPs; Trahey and McCormick, 1987]), the nucleotide state of ARF will be maintained indefinitely. Interestingly, ARF[GTP] binds to phospholipid vesicles (a property requiring N-terminal myristate), but ARF[GDP] does not bind to such vesicles (R. A. K., unpublished data). How, then, can ARF bind specifically to Golgi membranes (e.g., Figure 9) when ARF[GTP] has a nonspecific affinity for bilayers? And how can Golgi binding be stimulated by GTPγS when ARF[GDP] is stable?

We favor a model (diagrammed in Figure 10A) in which the Golgi membrane contains an ARF-specific nucleotide

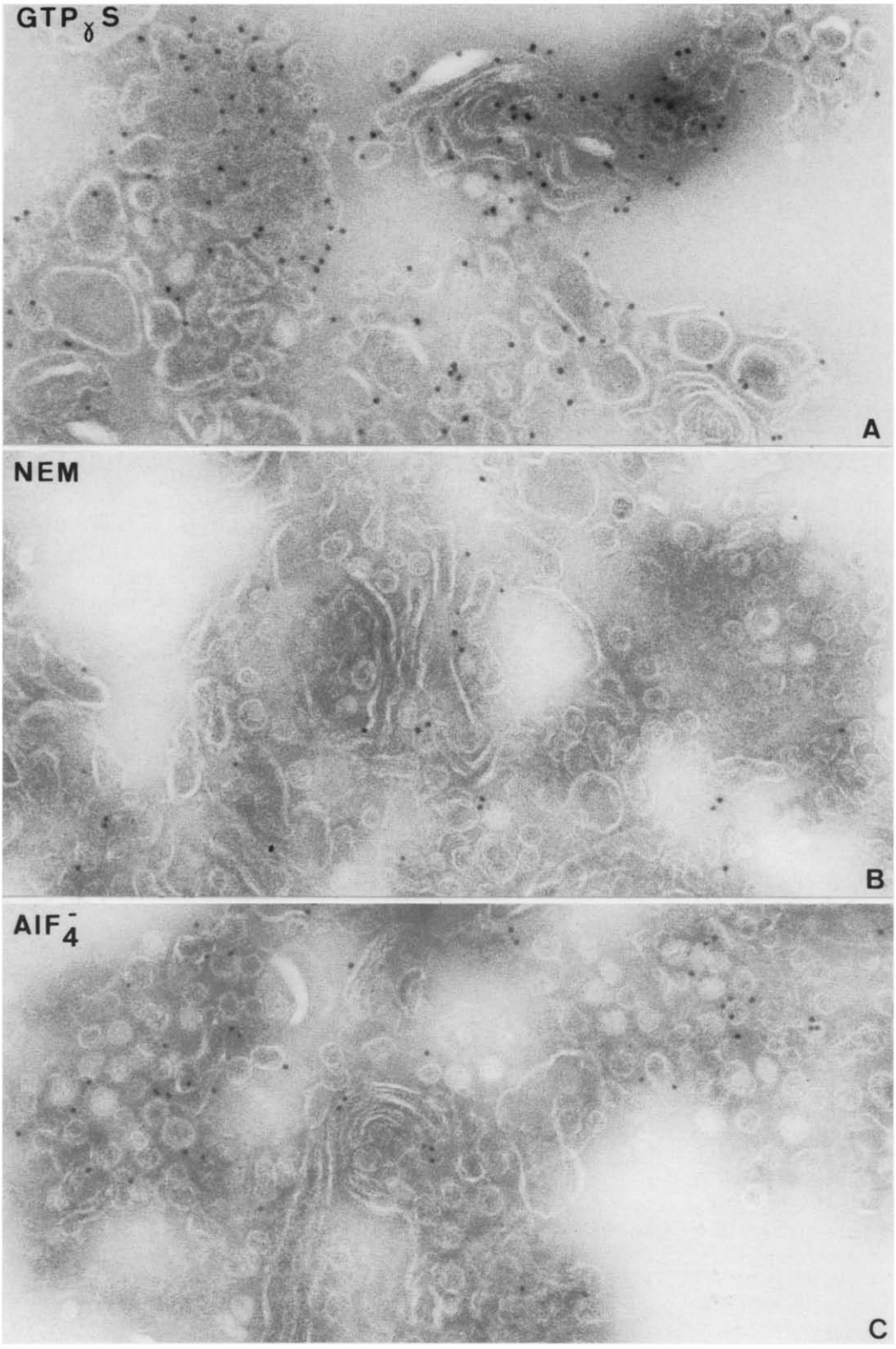

Figure 8. Immunolocalization of ARF in Golgi Membrane Fractions Incubated to Accumulate COP-Coated or Uncoated Vesicles

Golgi membranes were recovered from in vitro incubations, fixed, and cryosectioned. Sections were immunostained with the affinity-purified ARF anti-peptide antiserum (R5) and labeled by the protein A–gold method. In fractions incubated in the presence of GTPγS (A), numerous immunogold particles are associated with the periphery of vesicular profiles and the Golgi cisternae themselves. However, gold labeling is reduced to a very low level on the Golgi cisternae and the uncoated vesicles accumulated using NEM-treated membranes (B). During incubations with AlF_4^-, while

Table 1. Relative Densities of ARF in Golgi Cisternal Membranes and Coated or Uncoated Vesicles

Condition	Experiment	Surface Density of ARF over Cisternal Membranes[d]	Linear Density of ARF over Cisternal Membranes[e]	Surface Density of ARF over Bud and Vesicle Membranes[f]	Linear Density of ARF over Bud and Vesicle Membranes[e]
1.	I[a]	44 ± 6	1.1 ± 0.2	118 ± 5	2.06 ± 0.09
2. +GTPγS	II[b]	36 ± 4	0.99 ± 0.07	116 ± 8	2.0 ± 0.2
3.	III[c]	43 ± 5	1.2 ± 0.1	157 ± 9	2.7 ± 0.2
4.	I	8 ± 2	0.14 ± 0.03	10 ± 2	0.17 ± 0.04
5 NEM-Golgi	II	7 ± 2	0.19 ± 0.05	2 ± 2	0.04 ± 0.02
6.	III	9 ± 2	0.25 ± 0.06	16 ± 6	0.3 ± 0.1
7.	I	24 ± 4	0.54 ± 0.07	110 ± 10	1.8 ± 0.2
8. +AlF_4^-	II	9 ± 2	0.29 ± 0.06	60 ± 10	1.1 ± 0.2
9.	III	17 ± 4	0.5 ± 0.1	120 ± 10	2.2 ± 0.2

Incubations, membrane recovery, EM processing, and quantitation were performed as described in Experimental Procedures. Ten Golgi areas were analyzed in each case. Shown is the mean value followed by the standard error of the mean.

[a] Thin cryosections were processed for immunocytochemistry using affinity-purified R5 antiserum (~200 μg/ml) followed by protein A–gold (15 nm).

[b] Thin sections of Lowicryl K_4M-embedded pellets were processed for immunocytochemistry using affinity-purified R5 antiserum (~200 μg/ml) followed by protein A–gold (15 nm).

[c] Thin sections of Lowicryl K_4M-embedded pellets were processed for immunocytochemistry using 1D9 monoclonal antibody (~70 μg/ml), rabbit anti-mouse IgG (1:400), and protein A–gold (15 nm).

[d] Gold particles per μm^2 of cisternal surface.

[e] Gold particles per μm of membrane contour length.

[f] Gold particles per μm^2 of bud and vesicle surface.

exchange protein. Nucleotide exchange would thus occur only at the Golgi membrane, and the ARF[GTP] thus generated would bind (in a myristate-dependent manner) to the only nearby bilayer, that of a Golgi cisterna. This would give rise to the "receptor"- and GTP-dependent binding of ARF to Golgi cisternae that we observe (Figures 7 and 9), and this pool of ARF would presumably serve as the precursor for coat assembly. (Of course, Golgi-bound ARF[GTP], like myristylated *src* [Resh, 1989], might also be bound by a protein receptor, but the affinity of ARF[GTP] for bilayers and the overabundance of ARF in the coated vesicles relative to other vesicle proteins suggest that this is not likely.) Thus, in this hypothesis, a localized nucleotide exchange protein would catalyze membrane insertion of ARF, triggering coat assembly for budding.

The coat structure of the resulting vesicle would be stable until such time as the vesicle encountered an ARF-specific GAP, presumably at the target membrane. Hydrolysis of the bound GTP could destabilize the coat, as ARF[GDP] has no affinity for lipid bilayers. With ARFs present throughout the coat structure, the conformational change in ARF during GTP hydrolysis (Kahn and Gilman, 1986) could have a profound effect. When ARF is loaded with GTPγS during budding, coats would be permanently stable and coated vesicles would accumulate, as observed. Upon GTP hydrolysis, ARF[GDP] would be released into the cytosol and thus recycled. As the cytosolic coatomer does not contain ARF (data not shown), it is likely that ARF and other coat proteins recycle separately. This speculative model is summarized in Figure 10B.

It is not inconceivable that ARFs carry targeting information as well as control coat assembly, as a growing number of ARF family members are being identified (Kahn et al., 1991; R. A. K., unpublished data). This, of course, would require a set of specifically localized ARF-specific nucleotide exchange proteins and a complementary set of specifically localized ARF-specific GAPs. Thus, targeting information would be assigned to vesicles as they bud, using recycling ARF proteins from a cytosolic pool. There would be no need to recover from the acceptor any presumptive, transported integral membrane proteins serving as targeting components, as the exchange enzymes would remain behind as permanent residents of the donor membrane.

Of course, ARF should be a necessary component of the budding reaction if it is an integral part of the coat, no matter what its exact role. Unfortunately, at this time there are no antibodies that recognize the ARF proteins in their native state, so this expectation is not easily tested. However, we note that a distant member of the ARF family (~35% identity with yeast ARFs), the product of the *SAR1* gene of S. cerevisiae, is required for endoplasmic reticulum to Golgi transport (Nakano and Muramatsu, 1989). *SAR1* was recovered as a multicopy suppressor of *sec12-4* (Nakano et al., 1988), the wild-type allele of which is needed for vesicle budding (Kaiser and Schekman, 1990). This genetic interaction is consistent with the proposal that ARFs are required during the budding reaction.

Recent results indicate that ARF is identical to inhibitory factor (Melançon et al., 1987), the cytosolic factor required at a relatively high concentration to cause the inhibition of

the cisternae remain relatively unlabeled, the vesicles that accumulate show a distinct labeling (C). See Table 1 for the quantitative evaluation of the labeling. Magnifications: (A) 82,800×; (B) 82,100×; and (C) 82,100×.

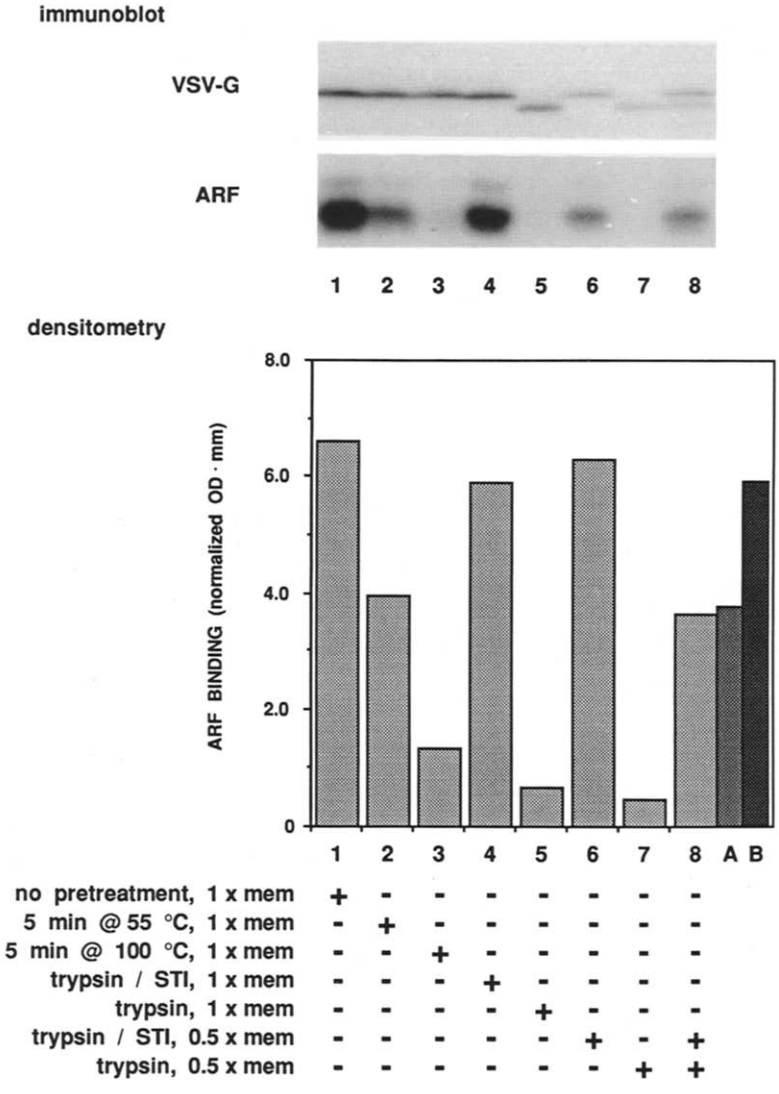

Figure 9. A Membrane-Associated Protein Is Required for ARF Binding to Membranes

CHO Golgi membranes were pretreated as indicated (trypsin/STI [soybean trypsin inhibitor], mock trypsin treatment; trypsin, proteolysis with trypsin; see Experimental Procedures). 1× mem indicates the standard amount of membranes present, while 0.5× mem indicates half this amount present during the incubation. Membranes were recovered and processed as in Figure 7. Integrated optical densities were normalized to the recovery of membranes (as assayed by recovery of VSV-G protein) in lane 1. The ability of the membranes to support subsequent ARF binding is progressively decreased by preincubation at 55°C and 100°C (lanes 2 and 3, respectively), as is ARF-binding ability after protease treatment (lanes 5 and 7). Proteolysis does not produce an inhibitor of binding, however, since mixing mock-treated and protease-treated membranes still allows ARF binding (lane 8). Furthermore, the amount of ARF binding is that expected if binding is only to the mock-treated membranes present in the mixture (lane A), not that expected if binding is to all membranes present (lane B). (The values of lanes A and B could be calculated since the amounts of mock- or trypsin-treated membranes recovered could be determined by quantitating the two different bands of VSV-G protein observed.)

in vitro transport and vesicle accumulation observed in the presence of GTPγS (P. Melançon, personal communication). Inhibitory factor is known to act at the acceptor compartment to cause this inhibition and to bind to membranes. That ARF binding to the acceptor membrane should cause an inhibition of transport is not inconsistent with its role in coat formation, as we have postulated that transport machinery at the acceptor membrane must recognize ARF in the coated vesicle to initiate vesicle uncoating. It is entirely plausible that overloading the acceptor membranes with free ARF (as distinct from ARF incorporated in coated vesicles) would result in accumulation of coated vesicles and a cessation of net transport.

It should be noted that ARF, based on its role in the cholera toxin–catalyzed ADP-ribosylation of G_s, very likely interacts with the trimeric class of GTP-binding proteins. Because AlF_{3-5} does not activate any known monomeric GTP-binding proteins (R. A. K., submitted), the effect of AlF_4^- in our system suggests, as noted earlier (Melançon et al., 1987), that a trimeric GTP-binding protein is involved in Golgi transport. One wonders whether ARF may serve as a link between these two different classes of GTP-binding proteins.

Experimental Procedures

Materials

GTPγS was purchased from Boehringer Mannheim. Aluminum ammonium sulfate ($AlNH_4(SO_4)_2 \cdot 12H_2O$) and potassium fluoride were purchased from J. T. Baker. NEM and Na_2CO_3 were purchased from Sigma, as were trypsin (type XIII) and soybean trypsin inhibitor (type I-S). [α-^{32}P]GTP tetra(triethylammonium) salt (3000 Ci/mmol) was purchased from NEN. Colloidal gold stain (blotting grade) was obtained from Bio-Rad.

Affinity-purified rabbit anti-goat IgG (H+L) was obtained from Vector Laboratories; affinity-purified rabbit anti-mouse IgG (H+L) and affinity-purified goat anti-rabbit IgG (H+L) were purchased from Organon Teknika-Cappel. Horseradish peroxidase–conjugated, affinity-purified goat anti-mouse IgG (H+L) (blotting grade) was purchased from Bio-Rad. 3,3′-Diaminobenzidine (DAB) and nickel chloride (as hexahydrate) were both purchased from Sigma. ^{125}I-labeled protein A (>30 μCi/μg) was obtained from ICN. Gelatin was EIA grade from Bio-Rad. β-Galactosidase (from Escherichia coli, EIA grade) was purchased from Calbiochem, and glutaraldehyde was obtained from Poly-

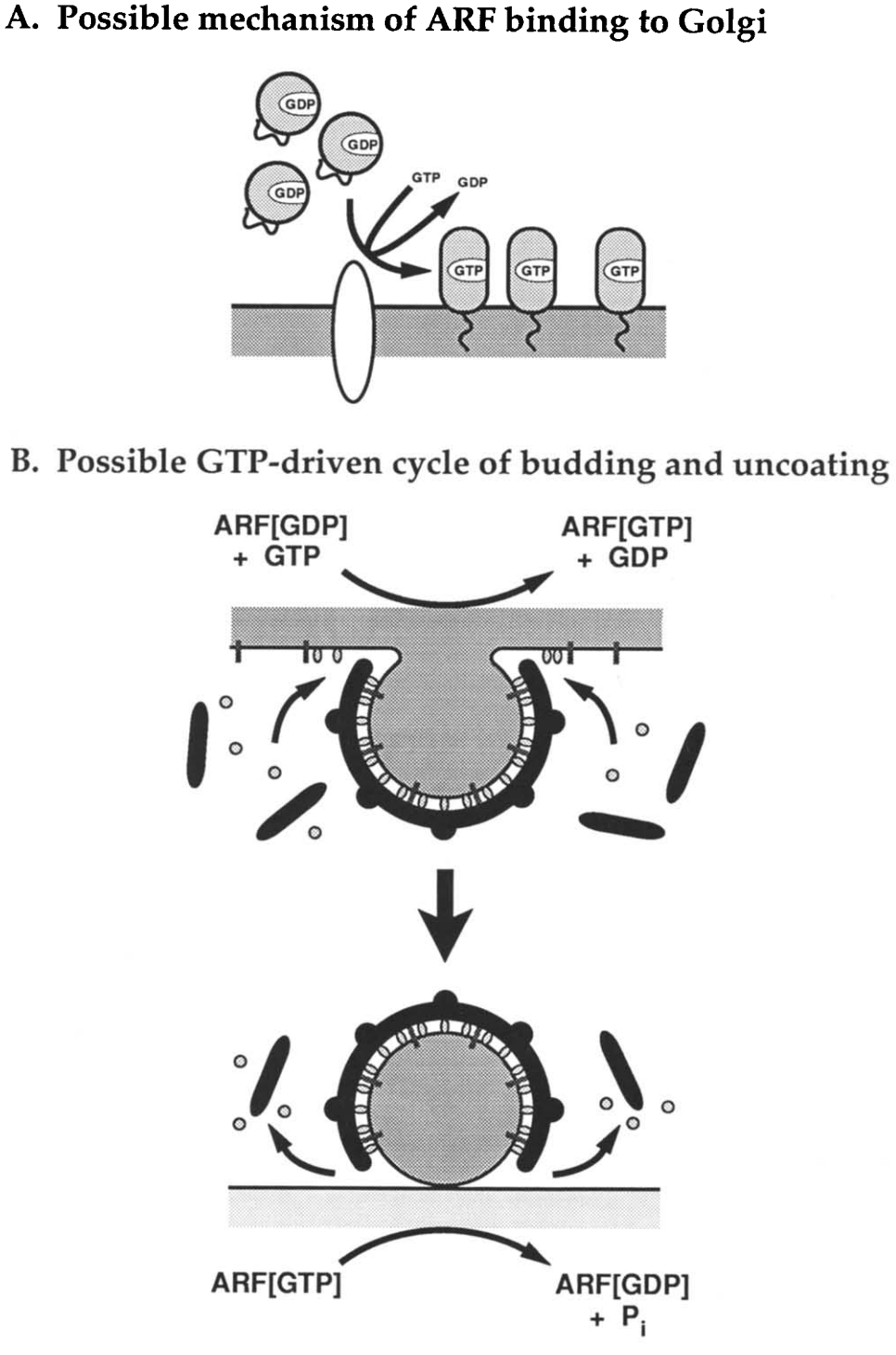

Figure 10. Models for the Mechanisms of ARF Action in Vesicular Transport

(A) Possible mechanism for ARF binding to membranes. As is known for the ARF1 protein (R. A. K., submitted), myristylated ARF proteins can bind directly to phospholipid bilayers when they have bound GTP (or GTPγS). Because, under the conditions of our in vitro system, ARF nucleotide exchange would otherwise be slow (Kahn and Gilman, 1986), a nucleotide exchange protein (open oval) catalyzes the exchange, allowing ARF to bind to the membranes.

(B) Possible GTP-driven cycle of budding and uncoating. Soluble ARF (small circles) would bind to the membranes (as small ovals) after the exchange protein–catalyzed nucleotide exchange of (A), and act, presumably together with other membrane-associated proteins (small rectangles), to cause COP coat assembly, presumably from coatomers (large ovals), during vesicle budding. After transfer of the coated vesicle to the target membrane, ARF would be induced to hydrolyze its bound GTP (perhaps by a localized GAP molecule), causing release of soluble ARF and free coatomers and an uncoating of the coated vesicle. These ARF and coatomer molecules would then be available for another round of coat assembly.

sciences. The mouse monoclonal antibody against VSV-G protein has been previously described (Orci et al., 1986), as have the R5 and affinity-purified R5 ARF anti-peptide antisera (Kahn et al., 1988); the 1D9 mouse monoclonal antibody raised against recombinant mammalian ARF1 will be described elsewhere (R. A. K., unpublished data). Purified recombinant mammalian ARF1 protein has been described previously (Weiss et al., 1989).

Golgi Membrane Fractions, Cytosol, and COP-Coated Vesicles

The preparation of donor and acceptor CHO Golgi fractions has been described previously (Balch et al., 1984; Balch and Rothman, 1985). Rabbit liver Golgi was prepared as previously described (Malhotra et al., 1989). Bovine brain cytosol was also prepared as previously described (Malhotra et al., 1989), except that two 500 g quantities of tissue were homogenized and taken through the preparation procedure together (with no changes in dialysis tank volumes) to yield approximately 250 ml of cytosol.

COP-coated vesicle preparations were performed using either CHO Golgi membranes or rabbit liver Golgi membranes as previously described (Serafini et al., 1991). In brief, for CHO Golgi-derived vesicles, reaction mixes (20 mM KCl, 25 mM HEPES–KOH [pH 7.0], 2.5 mM $Mg(OAc)_2$, 0.2 M sucrose, 125 μM DTT, 50 μM ATP, 250 μM UTP, 5 mM creatine phosphate, 8.0 IU/ml creatine kinase, 25 μg/ml RNAase A, 2.4 mg/ml bovine brain cytosol, 50–100 μg/ml CHO Golgi membranes, 20 μM GTPγS) were incubated in 30-ml Corex tubes for 15 min at 37°C and chilled on ice; membranes were collected by centrifugation at ~11,000 × g for 30 min. The membranes were resuspended in low salt stripping buffer (50 mM KCl, 25 mM HEPES–KOH [pH 7.2], 2.5 mM $Mg(OAc)_2$, 0.2 M sucrose; 0.6 ml of buffer per 60 ml of reaction mix), incubated on ice for 15 min, and then collected by microcentrifugation for 10 min. Vesicles were then stripped by resuspending the membranes as before, but in high salt stripping buffer (as low salt buffer, but 250 mM KCl). After 15 min on ice, the larger contaminating membranes were removed by microcentrifugation for 10 min. The supernatant (600 μl per 60 ml of reaction mix) was recovered and microcentrifuged for 15 min. The top 545 μl of supernatant was recovered and adjusted to 20% (w/w) sucrose by the addition of 55% stripping buffer (same as high salt buffer, but at the indicated sucrose concentration [w/w]). These crude vesicles were layered onto a discontinuous sucrose gradient (one gradient per 60 ml of reaction mix) formed by layering 714 μl of 55%, 50%, 45%, 40%, 35%, 30%, and 25% stripping buffer into an SW55 tube. The gradient was centrifuged for 18 hr at 100,000 × g and fractionated from the bottom using a peristaltic pump into ~20 fractions of 260 μl each. The protocol was the same for rabbit liver Golgi-derived vesicle production, except the reaction mix had a slightly different composition (DTT was used at 250 μM, bovine brain cytosol was at 4.8 mg/ml, and rabbit liver Golgi membranes were at 50–120 μg/ml), and 30 ml of reaction mix (instead of 60 ml) was treated as indicated.

Preparation of vesicle-containing fractions for SDS–PAGE was also as described previously (Serafini et al., 1991).

GTP Blotting and Immunoblotting

Electrophoretic transfer of proteins from SDS–PAGE gels to nitrocellulose was identical for both GTP blots and immunoblots; transfer was at 80 V for 4 hr in 20 mM Tris, 150 mM glycine, 20% (v/v) MeOH (Towbin et al., 1979) with no pretreatment of the gel.

The GTP blotting protocol was a derivation from various methods (McGrath et al., 1984; Schmitt et al., 1986; Lapetina and Reep, 1987) and was essentially as follows: After transfer, the filter (~8 × 12 cm) was immediately incubated for 1.5 hr in 200 ml of blocking buffer (Serafini et al., 1991). The filter was then incubated for 1 hr in 30 ml of binding buffer containing 2 μCi/ml [α-^{32}P]GTP (binding buffer: 20 mM Tris–HCl [pH 8.0], 10 mM $MgCl_2$, 2 mM DTT, 0.1% [w/w] Triton X-100, 0.5% [w/w] gelatin). The filter was then rinsed for 15 min with three changes of 200 ml of binding buffer, air dried, and exposed to autoradiography film with an intensifying screen at −80°C.

Immunodetection of bound proteins of interest using the various antibodies and antisera was performed essentially as described (Serafini et al., 1991); R5 antiserum was used diluted 1:1000 or 1:2000, affinity-purified R5 antiserum was used at ~5 μg/ml, the anti-VSV-G monoclonal was used at ~1–2 μg/ml, and the 1D9 anti-ARF1 monoclonal was used at ~3–7 μg/ml. For both the R5 and affinity-purified R5 antisera, the secondary antibody was affinity-purified goat anti-rabbit IgG (usually used at 0.2 μg/ml), followed by affinity-purified rabbit anti-goat IgG (used at 0.5 μg/ml) and 0.2 μCi/ml ^{125}I-labeled protein A. For the two monoclonals, the secondary was affinity-purified rabbit anti-mouse IgG (used at 0.3 μg/ml) or horseradish peroxidase–conjugated affinity-purified goat anti-mouse IgG (used at 1:3000), followed in the former case by [^{125}I]protein A and in the latter by incubation with DAB/metal (Harlow and Lane, 1988). Radiolabeled bands were visualized by autoradiography using preflashed film; autoradiographs used for densitometry were made without using an intensifying screen.

Colloidal gold staining of transferred proteins was performed according to the manufacturer's instructions.

Binding and Transport Assays

To assay GTP-binding protein or ARF binding to Golgi membranes, reaction mixes identical in composition to those used to produce COP-coated vesicles were utilized (except for the omission of 20 μM GTPγS where required), but contained membranes (or 0.2 M sucrose if omitted) that were a 1:1 mix of donor and acceptor membranes. Membranes were recovered from 800 μl of reaction mix in the case of GTP-binding protein-binding experiments (after chilling on ice) by centrifugation through a 600 μl 0.3 M sucrose/25 mM HEPES–KOH (pH 7.4) cushion as previously described (Orci et al., 1989) and in the case of ARF-binding experiments from 1 ml of reaction (after chilling) by centrifugation in a microfuge at ~15,000 × g for 30 min at 4°C. The supernatants were aspirated, and the membrane pellets solubilized in SDS–PAGE sample buffer. Transport assays were performed essentially as described (Balch et al., 1984), except that 75 μl aliquots of the binding reaction mixes were removed and transferred into tubes containing the [^{3}H]UDPGlcNAc.

NEM treatment of membranes was performed as described previously (Glick and Rothman, 1987). Trypsin treatment of CHO Golgi membranes was performed by incubating donor membranes with 100 μg/ml trypsin for 10 min at 37°C, followed by 1 mg/ml soybean trypsin inhibitor for 10 min at 37°C. Mock treatment was performed by adding trypsin and soybean trypsin inhibitor to the same concentrations from a mixture of the two that had been incubated for 10 min at 37°C and then incubating the membranes for 20 min at 37°C.

One-dimensional densitometry was performed on a Bio-Rad Model 620 Video Densitometer utilizing the 1-D Analyst software.

Carbonate Extraction of COP-Coated Vesicles

The three-peak vesicle fractions from a donor CHO Golgi-derived COP-coated vesicle preparation (fractions 7–9) were pooled, and two 375 μl aliquots were removed. One aliquot was diluted 6-fold with 250 mM KCl/2.5 mM $Mg(OAc)_2$ (control sample), while the other was diluted 6-fold with 230 mM Na_2CO_3/2.5 mM $Mg(OAc)_2$ (carbonate sample). After 15 min on ice, the membranes were pelleted by centrifugation in a TLA 100.3 rotor at 50,000 rpm (g_{av} = 100,000 × g) for 1 hr at 4°C. The supernatant was removed, and 30 μl of glacial acetic acid was added to the carbonate supernatant (30 μl of H_2O to the control sample). The proteins in the supernatants were then precipitated with trichloroacetic acid as previously described (Serafini et al., 1991), solubilized in sample buffer along with the pellets, and subjected to SDS–PAGE.

Quantitation of ARF in COP-Coated Vesicles

The three-peak vesicle-containing gradient fractions (fractions 7–9) were pooled from two rabbit liver Golgi-derived COP-coated vesicle preparations (a total of six fractions) into ~200 μl of sample buffer and used as the source of COP-coated vesicles for the quantitation experiments. For the α-COP quantitation: 50 μl of this vesicle sample was subjected to SDS–PAGE along with seven known amounts of β-galactosidase (ranging from 100 ng to 3.2 μg, see Figure 6) in a 6% acrylamide gel, and the gel was stained with Coomassie brilliant blue. Under these conditions, of the four high molecular weight COPs, only α-COP is a single band isolated from other bands or background staining. The stained gel was dried between cellophane sheets in an Easy Breeze gel dryer (Hoefer) and used for densitometry. For the ARF quantitation: 12.5 μl of the COP-coated vesicle preparation was subjected to SDS–PAGE along with six known amounts of purified recombinant mammalian ARF1 (ranging from 33 ng to 200 ng, see Figure 6) in a 12.5% acrylamide minigel, and the proteins were transferred to nitrocellulose for 1 hr at 100 V and immunoblotted with the

R5 antiserum as described above (only in a 12.5% gel do the two ARF bands separate sufficiently to allow an accurate analysis). The incubated filter was exposed to preflashed autoradiography film without an intensifying screen, and the resulting autoradiograph was used for densitometry. Protein determinations were performed according to Peterson (Peterson, 1977) using bovine serum albumin as a standard.

Densitometry

Because band width and shape varied owing to the wide range of protein masses loaded, two-dimensional densitometry was performed (using the Bio-Rad Model 620 Video Densitometer) both for α-COP and ARF quantitation. Using the 2-D Analyst software, the two-dimensional information for each lane was compressed into a 1-D tracing, which was then analyzed using the one-dimensional Analyst software to yield the standard curves seen in Figure 6. The lines fitted to the data were generated using Cricket Graph, v 1.3 (Cricket Software, Malvern, PA).

Electron Microscopy

GTPγS-containing and NEM-treated membrane-containing in vitro reactions were prepared as previously described (see above and Serafini et al., 1991); AlF_4^--containing reactions were prepared similarly, except that potassium fluoride was added to 5 mM and $AlNH_4(SO_4)_2$ was added to 50 μM. After incubation, the membranes were pelleted by centrifugation for 30 min in a horizontal microfuge, and the supernatants were aspirated. The membranes were fixed for 30 min on ice by overlaying the pellets with 1% glutaraldehyde in 0.1 M phosphate buffer [pH 7.4]. Ultrathin cryosections were prepared from part of the pellets according to Tokuyasu (1980). Embedding at low temperature in Lowicryl K_4M resin (Armbruster et al., 1982) was performed on the remaining portion of the pellet. Immunostaining protocols of cryo- or Lowicryl sections are indicated in Table 1. The surface and linear densities of the vesicular/cisternal Golgi elements and the number of gold particles per unit surface/unit length were recorded with the aid of an electronic pen and appropriate computer program. All morphological experiments were done in a double-blind fashion using a code that was not broken until the quantitation was completed.

Acknowledgments

We thank Barbara Devlin for preparing the CHO Golgi membranes and Mariella Ravazzola for immunoelectron microscopic procedures, and we are grateful to Paul Melançon for communicating unpublished results. We also thank members of the Rothman laboratory for helpful discussions, and Gregory Flynn, Marya Postner, and Duncan Wilson for comments on the manuscript. This work was supported by National Institutes of Health grant CA47767-01A1 (J. E. R.), grant 31.26625.89 from the Swiss National Science Foundation (L. O.), and a grant from the Human Frontier Science Program Organization (J. E. R., L. O., and F. T. Wieland).

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Received June 5, 1991; revised August 2, 1991.

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