Cytosolic ARFs are Required for Vesicle Formation but not for Cell-free Intra-Golgi Transport: Evidence for Coated Vesicle-independent Transport

Timothy C. Taylor,*‡ Martina Kanstein,* Peggy Weidman,† and Paul Melançon*

*Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80303-0215; and †Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104

Submitted December 6, 1993; Accepted January 21, 1994
Monitoring Editor: Randy W. Schekman

We investigated the role of ADP-ribosylation factors (ARFs) in Golgi function using biochemical and morphological cell-free assays. An ARF-free cytosol produced from soluble Chinese hamster ovary (CHO) extracts supports intra-Golgi transport by a mechanism that is biochemically indistinguishable from control transport reactions: ARF-free transport reactions are NSF-dependent, remain sensitive to the donor Golgi-specific inhibitor ilimaquinone, and exhibit kinetics that are identical to that of control reactions containing ARFs. In contrast, ARF-free cytosol does not support the formation of coated vesicles on Golgi cisternae. However, vesicle formation is reconstituted upon the addition of ARF1. These data suggest that neither soluble ARFs nor coated vesicle formation are essential for transport. We conclude that cell-free intra-Golgi transport proceeds via a coated vesicle-independent mechanism regardless of vesicle formation on Golgi cisternae.

INTRODUCTION

Vesicular transport between organelles is a central paradigm of modern cell biology (Palade, 1975). Vesicles mediate ER-to-Golgi transport in both mammalian cells (Jamieson and Palade, 1967) and yeast (Groesch *et al.*, 1990; Rexach and Schekman, 1991), and their involvement in later stages of the secretory/transcytotic pathways (Walworth and Novick, 1987; de Curtis and Simons, 1989; Sztul *et al.*, 1991) and in endocytosis (Gruenberg and Howell, 1989; Woodman and Warren, 1991) is well established. Vesicles have also been found in proximity to the Golgi complex (Farquhar and Palade, 1981). Although it is reasonable to assume that transport between cisternae of the Golgi complex proceeds by a mechanism analogous to that observed between other organelles, the importance of vesicles in this process has not been unequivocally established (Mellman and Simons, 1992). Indirect evidence for vesicular transport

within Golgi stacks comes from experiments on protein transfer between Golgi stacks in fused cells (Rothman *et al.*, 1984a,b). Here, glycosylation of a viral protein was found to occur at the same rate when transported either within a single Golgi stack, or between stacks originating from each partner of the heterokaryon. Pulse-chase studies in these fused cells further indicated that lateral and retrograde transfers were highly improbable *in vivo*. These observations were taken as evidence that transport between Golgi cisternae is a dissociative process, presumably involving vesicle carriers.

Are vesicles the only mechanism by which intracellular protein transport occurs? The dynamic formation and fusion of tubular processes, which allow diffusion of lipids between joined *trans*-Golgi elements, have been observed in living cells using fluorescently labeled precursors of *trans*-Golgi network lipid markers (Cooper *et al.*, 1990). In addition, studies with the drug Brefeldin A (BFA) suggest that the formation of transient membrane tubule connections can provide a mechanism for transfer of proteins between organelles (Orci *et al.*, 1991; Klausner *et al.*, 1992). An alternate mechanism of transfer has also been suggested from studies on the inter-

‡ Present address: Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06536

mediate compartment (IC), which functions between the ER and Golgi, and forms in part by the fusion of transport vesicles derived from the ER (Saraste and Kuismanen, 1992; Lippincott-Schwartz, 1993). Several observations indicate that transfer out of the IC may not involve vesicular intermediates, but rather may result from direct fusion of mature forms of these pleomorphic transport intermediates with the *cis*-Golgi network (CGN).

The GTP-dependent assembly of specific protein coats on membranes may regulate the formation and function of vesicles and membrane tubules (Klausner *et al.*, 1992; Rothman and Orci, 1992). Most studies have focused on the assembly of a novel type of nonclathrin coat on Golgi membranes (Orci *et al.*, 1986). This nonclathrin coat is assembled from a large soluble complex of proteins, called coatomer (Waters *et al.*, 1991). Analysis of the Golgi nonclathrin coated vesicles (GCVs) that accumulate in the presence of GTP γ S reveals the presence of the soluble GTPase ARF in amounts stoichiometric with those of coatomer proteins (Serafini *et al.*, 1991). Indeed, ARFs and coatomers appear sufficient to promote formation of coated vesicles on Golgi membranes in the presence of GTP γ S (Orci *et al.*, 1993b). Since recruitment of ARF to membranes depends on GTP-binding (Kahn, 1991; Walker *et al.*, 1992), the assembly of the nonclathrin coat may be regulated by nucleotide exchange factors that promote exchange of bound GDP for GTP on ARF. For example, inhibition of nucleotide exchange on ARFs by BFA (Donaldson *et al.*, 1992b; Helms and Rothman, 1992; Randazzo *et al.*, 1993) causes a reduction in coatomer binding, which is then thought to prevent the formation of vesicles at the ends of buds and tubules, thus allowing their elongation and fusion with other membranes (Duden *et al.*, 1991; Klausner *et al.*, 1992). In vivo, coatomers and ARFs may therefore function to specify either vesicular or non-vesicular (e.g., tubular) mechanisms of transport within the Golgi and possibly between other organelles as well.

Transport of proteins through the Golgi complex in mammalian cells has been studied intensively through the use of an assay developed by Rothman and his collaborators (Balch *et al.*, 1984a,b; Braell *et al.*, 1984). This assay, which reconstitutes transport between Golgi stacks in a cell-free system, is based on the observation made in fused cells that proximity of cisternae within a single Golgi stack is not required to observe protein transfer (Rothman *et al.*, 1984a,b). Briefly, the assay measures the transport-coupled glycosylation of the VSV-G protein upon transfer from a donor Golgi compartment lacking *N*-acetylglucosamine transferase I to an acceptor Golgi compartment containing this enzyme (Balch *et al.*, 1984a). Kinetic experiments reveal a characteristic lag period before any transport-coupled glycosylation is detected, a lag which can be largely eliminated by preincubation (i.e., activation) of donor but not acceptor Golgi membranes (Balch *et al.*, 1984b).

Since GCVs appear on Golgi membranes upon incubation with cytosol and ATP, and their formation coincides with the activation of donor membranes, this lag has been attributed to the time required for formation of transport vesicles (Balch *et al.*, 1984b; Orci *et al.*, 1986). Furthermore, addition of GTP γ S to transport reactions permanently activates ARFs (Taylor *et al.*, 1992) leading to an irreversible assembly of protein coats, the accumulation of GCVs and a block of transport (Melançon *et al.*, 1987; Orci *et al.*, 1993a; Weidman *et al.*, 1993a). The formation of functional coated vesicles using Golgi membranes and a minimal set of cytosolic proteins has been recently achieved (Osterman *et al.*, 1993). Although these GCVs can fuse with and deliver cargo to acceptor Golgi membranes, their mode of preparation and assay precludes any estimate of the extent to which they contribute to transport measured in the standard assay. Whether or not GCVs formed on Golgi membranes actually contribute significantly to the Golgi transport process in vitro therefore remains to be proven.

Several proteins of essential function have been identified through use of the intra-Golgi transport assay (Rothman and Orci, 1992). These include a 115 kDa peripheral membrane protein (Waters *et al.*, 1992), an *N*-ethylmaleimide sensitive fusion protein (NSF; Block *et al.*, 1988) and soluble NSF-attachment proteins, (SNAPS; Clary *et al.*, 1990) which recruit NSF to specific receptors on Golgi membranes (Weidman *et al.*, 1989). Recent studies have established that short peptides derived from the N-terminus of ARFs block the intra-Golgi transport assay (Kahn *et al.*, 1992). This led to the conclusion that ARFs are also essential for intra-Golgi transport, in agreement with the observation that deletion of the ARF1 and ARF2 genes in *S. cerevisiae* is lethal (Stearns *et al.*, 1990). Nevertheless, ARFs (and vesicles) may not be required for intra-Golgi transport since cell-free transport between Golgi stacks occurs normally in the presence of BFA (Orci *et al.*, 1991). In addition, we have established that cell-free transport between Golgi stacks proceeds with normal kinetics and extent even at reduced concentrations of ARFs and coatomers (Taylor *et al.*, 1992). These latter studies were not conclusive, however, since in the first case BFA may have perturbed the system in unknown ways, and removal of ARFs in the second case was only partial.

Using biochemical fractionation methods, we have produced a pool of soluble CHO proteins lacking >98% of soluble ARFs. This ARF-free cytosol is active in cell-free intra-Golgi transport reactions. Furthermore, several biochemical characteristics of transport, including kinetics, are indistinguishable from those observed with a control cytosol containing ARFs. Nevertheless, using a novel method for obtaining high resolution 3-D images of Golgi membranes (Weidman *et al.*, 1993a), we demonstrate that ARF-free cytosol does not support coated vesicle formation and that addition of pure bovine ARF1

to this cytosol reconstitutes vesicle formation. We conclude that ARF is required for vesicle formation but not for cell-free transport. Since the kinetics of intra-Golgi transport are independent of the density of coated vesicles formed on Golgi cisternae, we further suggest that cell-free intra-Golgi transport reactions proceed via a coated vesicle-independent mechanism regardless of vesicle formation and present an alternate model.

MATERIALS AND METHODS

General Methods

All reagents were analytical grade except where indicated. UMP-kinase (NMK) was purchased from Sigma (St. Louis, MO). Purified NSF (10 ng/ μ l working dilution, a generous gift of Dr. D. Clary) was purified according to Block *et al.* (1988). A 13-residue peptide derived from the N-terminus of ARF1 (peptide 13; Kahn *et al.*, 1992) was a generous gift of Dr. R. Kahn. Ilimaquinone was obtained from Dr. V. Malhotra and kept as a 12.5 mM stock in dimethyl sulfoxide (DMSO) at -20°C . This stock was diluted prior to use such that DMSO concentration in transport assays was $\leq 0.2\%$. Most protein assays were performed with the Bradford method (Bio-Rad, Richmond, CA) using γ -globulin standards. For samples containing membranes, protein concentrations were measured with the BCA method (Pierce, Rockford, IL) in the presence of 0.1% sodium dodecyl sulfate (SDS) using BSA standards. SDS-polyacrylamide gel electrophoresis was performed on 15% acrylamide mini-gels (Hoefer, San Francisco, CA) as described (Laemmli, 1970).

All chromatographic steps were performed on a Pharmacia FPLC System. Columns and resins were purchased from Pharmacia (Piscataway, NJ) except where specified otherwise. Buffers were filtered through a 0.22 μm membrane (COSTAR, Cambridge, MA) prior to chromatography. Tritiated UDP-N-acetylglucosamine (UDP- ^3H -GlcNAc) was synthesized essentially as described (Lang and Kornfeld, 1984) using D-[6- ^3H]-glucosamine-HCl (NEN) as starting material.

ARF(−) and Control CHO Cytosols

CHO cells (pro $^{-5}$) were grown in spin culture to a density of $\sim 1 \times 10^6$ cells/ml. Four liters of cells were harvested, homogenized and a crude soluble extract prepared as described (Taylor *et al.*, 1992; Balch *et al.*, 1984a). A crude supernatant was obtained by centrifugation of the extract at $100,000 \times g$ for 1 hr at 4°C (45Ti rotor, Beckman, Fullerton, CA), which was brought to a total salt concentration of 250 mM with KCl.

The size-exclusion ARF(−) pool and Control cytosol were prepared as follows. The supernatant mentioned above (4.2 ml) was loaded directly onto a Superdex-75 column equilibrated with 10 mM Tris (hydroxymethyl) aminomethane (Tris), 100 mM KCl, 1 mM MgCl_2 , 5% wt/vol glycerol, pH 7.9 (4°C), plus 1 mM dithiothreitol (DTT). Since crude cytosol is diluted 1.4-fold during desalting over P6-DG resin (Bio-Rad), this load is equivalent to 5.9 ml of desalted CHO cytosol (see below). Fractions (4 ml) of the Superdex-75 effluent were collected at 0.8 ml/min and then pooled as follows: set 1—from the excluded volume (~ 37 ml effluent) to 64 ml, inclusive; set 2—65 to 81 ml, inclusive; set 3—82 to 91 ml, inclusive. The boundaries of the ARF(−) and ARF(+) pools were determined in a preliminary experiment using GTP-ligand blot analysis of 21 kDa GTPases. Sets 1 and 3 were combined and concentrated by ultrafiltration (Amicon [Dancers, MA] 50 ml unit, YM10 membrane) to a volume of 14.5 ml and then centrifuged at $10,000 \times g$ at 4°C for 5 min to remove any insoluble material. A volume of 2.5 μl (i.e., 14.5 ml/5.9 ml) of this pool is equivalent to 1 μl of desalted cytosol. This concentrated pool constituted the size-exclusion ARF(−) pool. Set 2 (17 ml) was considered ARF(+). Control CHO cytosol was prepared by combining the ARF(−) and ARF(+) pools in volumes equivalent to that of the column load (i.e., 2.5 μl ARF(−) and 2.9 μl ARF(+)) combined gave 5.4 μl ,

which was equivalent to 1 μl of the desalted cytosol). For routine assay purposes, equivalent volumes of 2.5 μl for this ARF(−) pool and 5.4 μl for the Control cytosol were used. A second preparation (with a different load volume and dilutions) yielded a size-exclusion ARF(−) pool and a Control cytosol with equivalent volumes of 1.53 and 2.19 μl , respectively.

The Mono Q ARF(−) pool was prepared as follows. Desalted CHO cytosol was prepared as described (Taylor *et al.*, 1992). One milliliter of desalted CHO cytosol, containing 50 mM KCl, was loaded onto a Mono Q (HR 5/5) column equilibrated with 10 mM Tris, 50 mM KCl, 1 mM MgCl_2 , pH 8.0 (4°C), with 1 mM DTT. A flow-through volume of 2.9 ml was collected, followed by a 500 mM KCl eluate of 1.85 ml. The 500 mM eluate constituted the Mono Q ARF(−) pool and was dialyzed in column equilibration buffer to a final salt concentration of ~ 50 mM, and centrifuged to remove any insoluble material. A volume of 1.85 μl of this ARF(−) pool was equivalent to 1 μl of the desalted cytosol load. A second Mono Q ARF(−) pool of 1.7 ml was prepared using 1.1 ml of desalted cytosol, yielding an equivalent volume of 1.55 μl (1.7 ml/1.1 ml).

Complete ARF(−) cytosol was prepared by combining a 1 μl equivalent volume of the size-exclusion ARF(−) pool with either 0.5 μl or 1 μl equivalent volume of the Mono Q ARF(−) pool (e.g., 2.5 μl with either 0.92 or 1.85 μl , respectively). This constituted "ARF(−) cytosol." The protein concentration of the ARF(−) cytosol was 3.8 mg/ml and that for the Control cytosol was 1.9 mg/ml (from the first preparations described above).

Intra-Golgi Transport Assay and Inhibition Assay

Acceptor Golgi membranes fractions were prepared from wild-type CHO cells (pro $^{-5}$, ATCC) as described (Balch *et al.*, 1984a). For donor membranes, a CHO mutant cell line (lec1, Stanley *et al.*, 1975) lacking GlcNAc transferase I activity, was infected with VSV and Golgi-enriched fractions isolated following homogenization (Colombo *et al.*, 1991). For the production of "wt donor" membranes used in the glycosylation assay (Figure 9), wild-type CHO cells were infected with VSV and processed as described for the production of standard donor membranes. Membrane protein content was typically 400 $\mu\text{g}/\text{ml}$. All tests for the requirement of the transport factor NSF were carried out with Golgi-enriched membrane fractions treated as follows. Membranes were thawed and incubated with NEM (0.25 mM) for 15 min on ice, at which time DTT was added to 0.5 mM to quench any remaining NEM. Treated membranes were stored at -70°C for later use.

Unless otherwise indicated, single time point transport assays were performed. Each assay (50 μl) contained 1.5 μl of donor, 1.5 μl of acceptor Golgi membranes and the cytosol sample to be tested in a standard buffer mixture containing 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.3, 2.5 mM MgCl_2 , 2 $\mu\text{Ci}/\text{ml}$ [^3H]-UDP-GlcNAc, 100 μM ATP, 250 μM UTP, 5 mM creatine phosphate, 8 I.U./ml creatine phosphokinase, 0.5 mM DTT, 4 μM palmitoyl-CoA and 0.2 M sucrose. Assays were incubated at 30°C for 2 h. Assays to measure sensitivity of transport to GTP γ S (Figure 2) were performed as described (Taylor *et al.*, 1992) using 1 μl donor and 0.5 μl acceptor Golgi membranes and 1.5 μl of a CHO cytosol lacking sensitivity to GTP γ S ("GTP γ S-CHO cytosol", 7 $\mu\text{g}/\mu\text{l}$; Taylor *et al.*, 1992) in assays containing 0.16 M sucrose. Duplicate assays containing unknown protein samples were carried out in the absence and presence of 4 μM GTP γ S. Percent inhibition by GTP γ S was calculated by taking the difference between transport measured in the absence and presence of GTP γ S, and dividing this difference by transport measured in the absence of GTP γ S, times 100%.

GTP-Ligand Blot

Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were electrophoretically transferred (Idea Scientific Genie, Corvallis, OR) from gels to nitrocellulose (MSI, NitroBind, 0.22 μm) in the absence of SDS. Small GTPases were visualized by

Figure 1. Chromatographic preparation of ARF(-) and Control cytosols. Open and closed arrows indicate ARF(+) and ARF(-) pools, respectively. (A) Effluent collected from size-exclusion chromatography (Superdex-75) of crude CHO cell extract. The ARF(-) and ARF(+) fractions were concentrated via ultrafiltration. Molecular weight standards indicated by arrowheads are BSA (68 kDa) and chymotrypsinogen (25 kDa). (B) Desalted CHO cytosol (eluate from P6-DG chromatography of crude extract, not shown) was bound at low salt concentration and eluted with 500 mM KCl from an anion-exchange column (Mono Q). "ARF(-) cytosol" was prepared by combining the ARF(-) fractions from both columns. "Control cytosol" was prepared by recombining the ARF(-) and ARF(+) fractions from the column in A. Details of the chromatography are given in MATERIALS AND METHODS.

the GTP-ligand blotting technique, essentially as described (Lapetina and Reep, 1987; Schmitt *et al.*, 1986). Ligand binding was carried out by incubating blots with 2 μ Ci/ml [α^{32} P]-GTP (NEN, Wilmington, DE) in the presence of 100 μ M ATP. Blots were analyzed by autoradiography and/or quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Electron Microscopy

CHO Golgi membranes were incubated *in vitro* under transport reaction conditions and visualized by the platinum replica technique following the procedure of Weidman *et al.* (1993a). Briefly, freshly isolated membranes were diluted 15-fold into 0.6 M sucrose, and spun down onto 3 mm² glass coverslips that had been pretreated with 5 mg/ml polylysine and 1% glutaraldehyde. The coverslips were blocked and washed with a solution of 10 mM Tris pH 7.4, 0.6 M sucrose, 5 mg/ml soybean trypsin inhibitor and transferred to humidified petri dishes on ice. The wash buffer on the coverslips was replaced with 8 μ l of the standard transport assay reaction mixture, described above, except that membranes were omitted. Four coverslips were prepared for each incubation condition. The petri dishes were incubated at 37°C for 10 min and the reactions terminated by addition of buffer [25 mM HEPES, pH 7.0, 20 mM KCl, 1 mM EDTA, 3.5 mM Mg(OAc)₂] containing 1% glutaraldehyde and 0.3 M sucrose pre-equilibrated at 37°C. After 10 min at room temperature, the fixative was replaced with buffer by a series of dilution steps.

A minimum of two coverslips from each experimental sample were quick frozen according to the method of Heuser and Evans (1980), vacuum dried, and coated with platinum and carbon as previously described (Heuser *et al.*, 1979). Replicas were mounted on formvar coated grids and viewed in a JEOL (100 CX or 200 CX) electron microscope. Twenty to twenty-five micrographs were taken of representative fields from two grids for each experimental sample. Negatives from all the experimental samples were coded and randomized for data analysis. The negatives were projected at 2.5 $\times$ magnification onto a 15 $\times$ 15 mm grid using a Beseler 45 M enlarger, and the Golgi membranes scored for the number of coated buds/vesicles, the predominant length of cisternal tubules, and area encompassing the membranes relative to the grid, as previously described (Heuser and Evans, 1980). The few uncoated buds present at equally low density in all samples were not counted in this analysis.

RESULTS

21 kDa GTPases Can Be Removed from Cytosol by Chromatography

Transport within the Golgi complex *in vitro* has an absolute requirement for exogenous cytosolic proteins (Balch *et al.*, 1984a). This probably reflects a requirement for many soluble transport factors, which may include one or more ADP-ribosylation factors. To test the requirement for soluble ARFs in intra-Golgi transport, cytosol lacking ARFs was obtained. Immunodepletion of ARFs from soluble extracts is not possible at present since none of the available antibodies to ARFs neutralize or immunoprecipitate ARF proteins under native conditions. As an alternative, we employed chromatographic methods to produce a pool of soluble proteins lacking ARFs.

The approach used to produce an "ARF(-) cytosol" from extracts of CHO cells is illustrated in Figure 1. First, crude CHO cytosol was fractionated on either size-exclusion or anion exchange columns. Fractions lacking ARFs in each column were then pooled to generate two ARF(-) pools. "ARF(-) cytosol" was then produced by combining these ARF(-) pools.

Soluble CHO proteins were first fractionated on a size-exclusion column. The ARF(-) pool A consisted of fractions of apparent molecular weight greater than ~35 kDa and smaller than ~15 kDa (Figure 1A). The ARF(-) pool B was a 500 mM KCl eluate of CHO cytosol from a Mono Q anion-exchange column, loaded under conditions such that all ARFs were recovered in the flow-through fraction. The intermediate molecular weight fractions of column A and the flow-through from column B constituted two distinct ARF(+) pools. Finally,

Figure 2. Intra-Golgi assays containing ARF(-) cytosol are not sensitive to GTP γ S. (A) Control and ARF(-) cytosols were assayed for GTP γ S-dependent inhibition of a modified intra-Golgi transport assay as described (Taylor *et al.*, 1992). Transport factors were provided by the addition of 1.5 μ l of an ARF-deficient cytosol which is insensitive to GTP γ S (see Taylor *et al.*, 1992). Additionally, each assay contained 1.0 μ l of donor and 0.5 μ l of acceptor Golgi membranes and 4 μ l of either Control cytosol or ARF(-) cytosol. Assays were performed in the presence or absence of 4 μ M GTP γ S, and the results are expressed as the percentage of inhibition of transport in the presence of GTP γ S (see MATERIALS AND METHODS). The data are averages of 4 experiments. (B) ARF(-) cytosol is sensitive to GTP γ S when pure bARF1 is included in the assay. The IC₅₀ for bARF1 is $\sim$ 160 ng, or 0.15 μ M.

a "Control cytosol", was made by repooling all fractions from the size-exclusion column [ARF(-) pool A and ARF(+) pool A]. Details of these methods are described in MATERIALS AND METHODS.

ARFs are the only small soluble GTPases involved in the inhibition of intra-Golgi transport by GTP γ S (Taylor *et al.*, 1992). We analyzed both the ARF(-) and Control cytosols for their ARF content using a specifically modified transport assay that contains transport factors provided by low levels of a GTP γ S-insensitive cytosol. Addition of cytosol fractions containing ARFs to these reactions restores sensitivity to GTP γ S (Taylor *et al.*, 1992). Figure 2A shows that while reactions that receive the control cytosol are inhibited by GTP γ S, those receiving ARF(-) cytosol are completely insensitive. This suggests that the level of ARFs in ARF(-) cytosol is indeed low. Inhibition is restored when pure bovine ARF1 (bARF1) is added back to reactions containing the ARF(-) cytosol, and $\sim$ 160 ng (0.15 μ M) is sufficient to cause half maximal inhibition (Figure 2B). This demonstrates that the lack of sensitivity to GTP γ S is not due to the presence of an antagonist of ARF function.

To more precisely quantitate the removal of ARFs, ARF(-) cytosol and the Control cytosol were analyzed for their content of $\sim$ 21 kDa GTPases by GTP-ligand blot (Figure 3A). GTP-ligand blots are significantly more sensitive than ARF immunoblots for the detection of ARFs and can be readily quantitated using a PhosphorImager. Since the GTP-binding domains of all known ARFs are identical (Tsuchiya *et al.*, 1991), all ARFs should exhibit similar binding in this assay. Indeed, we established that bARFs 1 and 3 bind GTP to an equivalent extent by this method. A direct comparison of the amounts of ARFs in the ARF(-) and Control cytosols indicates that ARF(-) cytosol con-

Figure 3. ARF(-) cytosol contains <2% of $\sim$ 21 kDa GTPases relative to the control cytosol. (A) ARF(-) and Control cytosols (4 μ l, or $\sim$ 25 μ g of protein) were subjected to SDS-PAGE and analyzed by a GTP-ligand blot (Schmitt *et al.*, 1986; Lapetina and Reep, 1987). A sample of donor/acceptor Golgi membranes from CHO cells is also shown (3 μ l total, the volume in one transport assay). An autoradiogram of the blot is shown. Molecular weight markers: carbonic anhydrase and soybean trypsin inhibitor, 31 and 21.5 kDa. (B) Quantitation of $\sim$ 21 kDa GTPases in the cytosols in A was accomplished using a PhosphorImager. ARF(-) cytosol contained <2% of $\sim$ 21 kDa GTPases (i.e., ARFs) relative to Control cytosol in each of 2 independent experiments. The signals for the ARF(-) cytosol and Golgi samples were near background and therefore represent top limits of ARF abundance. (C) Standard curve of $\sim$ 21 kDa GTPases in the Control cytosol and of the pure GTPase bARF3 as detected by PhosphorImager analysis of GTP-ligand blots (curves are from the same experiment). Comparison of the two curves indicated that Control cytosol contained $\sim$ 40 ng of ARFs per microliter. Golgi membranes contained <1.5 ng of $\sim$ 21 kDa GTPases per microliter of Golgi relative to the Control cytosol (from data in A).

Figure 4. ARF(-) cytosol supports intra-Golgi transport. Titrations of ARF(-) and Control cytosols in single time point transport assays are shown (50 μ l assays; no GTP γ S was present). Assays were incubated at 30°C for 2 h, and the amount of 3 H-GlcNAc incorporated into VSV-G protein (i.e., "Transport") was assessed by specific immunoprecipitation of VSV-G. Transport at each experimental point is represented as the percentage of the maximal transport observed with the Control cytosol (achieved at 1.5 μ l of cytosol). To compensate for dilution of the cytosols during their preparation by chromatography, equivalent volumes of each cytosol were used. One volume equivalent is that volume of cytosol, which is equivalent to 1 μ l of unfractionated, desalted CHO cytosol (see MATERIALS AND METHODS). The data points are averages of two independent experiments and are representative of points obtained from a minimum of four independent experiments.

tains <2% of the ARFs present in the Control cytosol (Figure 3B). The concentration of ARFs in the Control cytosol was determined by quantitating GTP-ligand blots of a titration of Control cytosol and comparing with a standard curve prepared with pure bARF3 (Figure 3C). Control cytosol contains ~40 ng of ARFs per microliter, or a concentration of ~1.9 μ M, whereas the ARF concentration in the ARF(-) cytosol is <0.04 μ M, or 50-fold lower. ARFs do not associate significantly with Golgi membranes during isolation from CHO cells (Taylor *et al.*, 1992; see Figure 3A). Quantitation indicates that <1.5 ng of ARFs are present per microliter of Golgi membranes. The final concentrations of ARFs from all sources in the transport assay (2 μ l cytosol and 3 μ l Golgi membranes per 50 μ l assay) are estimated as 90 nM for reactions containing Control cytosol, but <6 nM for reactions containing ARF(-) cytosol.

ARF(-) Cytosol Supports Transport in the Intra-Golgi Assay

To test the absolute requirement for ARFs in intra-Golgi transport, a series of transport assays were carried out in the presence of increasing volumes of either ARF(-) or Control cytosols (Figure 4). The amounts of cytosol required to achieve maximum transport are identical for ARF(-) and Control cytosol reactions, although the extent of transport at saturation is lower for ARF-free

cytosol. These results suggest that ARFs are not required to observe transport between Golgi stacks.

To demonstrate that the slightly lower transport levels observed with the ARF(-) reactions did not result from the absence of ARFs, pure ARFs from bovine brain were added to ARF(-) and Control cytosol transport reactions. These experiments were carried out using levels of cytosols that gave either sub-maximal (Figure 5A) or maximal (Figure 5B) levels of transport. Addition of either a two- or fivefold excess of pure bARFs to either ARF(-) or Control cytosol had little effect on transport, even at a subsaturating concentration of cytosol (Figure 5A). Similar results were obtained upon addition of either 1- or 10-fold excess of ARFs. Thus ARF proteins alone are not sufficient to stimulate transport in ARF(-) reactions to levels obtained with Control cytosol.

The data above imply that transport factors distinct from ARFs were inactivated or removed from ARF(-) cytosol during preparation by chromatography. To further explore the nature of this "stimulatory activity" in the Control cytosol, we first tested the effect of adding UMP-kinase, a 25 kDa protein required for intra-Golgi transport (Hiebsch and Wattenberg, 1992). Due to its size, this soluble protein may be present in the Control cytosol but absent from the ARF(-) cytosol (see Figure 1A). Addition of pure UMP kinase (0.002–0.2 I.U. in 50 μ l assays) to transport assays containing ARF(-) cytosol gave no stimulation of transport. We next frac-

Figure 5. Addition of pure ARFs does not stimulate ARF(-) transport. Assays were performed as in Figure 4 at two different cytosol concentrations: 1 which is sub-maximal for transport [(A) 0.75 μ l equivalents of cytosol per 50 μ l assay] or one at which transport is maximal [(B) 2 μ l equivalents of cytosol]. Purified bARFs (ARF1 and ARF3) were added to both ARF(-) and Control cytosols at concentrations 1-, 2-, 5-, and 10-fold that found in CHO cytosol (~40 ng/ μ l, see Figure 3C). Data for the 2- and 5-fold ARFs experiments are presented and are representative of all data. ARFs were added in excess to account for any loss of activity due to purification. ARFs were added in a weight ratio of 1:2 (ARF1:ARF3) which approximates their relative abundance during purification from bovine brain cytosol (see Taylor *et al.*, 1992). Data from one experiment are presented and are representative of three independent experiments.

tionated the ARF(+) size-exclusion pool A (see Figure 1A) by anion-exchange chromatography and found a fraction which stimulates transport of ARF(-) cytosol but does not contain ARFs (T. Taylor, unpublished observations). Thus the stimulatory activity and CHO ARFs are separable and distinct factors. This clearly demonstrates that ARF proteins are not responsible for the greater transport activity exhibited by the Control cytosol.

Previous studies have demonstrated that peptides derived from the N-terminus of ARFs inhibit transport, suggesting that ARFs play an essential role in intra-Golgi transport (Kahn *et al.*, 1992). We found that peptide 13 (the most potent inhibitory ARF peptide, a generous gift of R. Kahn) does indeed block transport in ARF(-) and Control cytosol reactions, but that contrary to prediction, the sensitivity to this peptide is independent of the level of ARFs in the assay. Identical IC_{50} values of $\sim 10 \mu M$ were measured whether reactions contained Control cytosol ([ARF] ~ 90 nM) or ARF(-) cytosol (≤ 6 nM). This indicates that the effect of the ARF peptide is unlikely due to competition with ARFs for some essential factor as previously postulated (Kahn *et al.*, 1992). Rather, inhibition may arise from some other nonspecific effects on membrane integrity (Weidman *et al.*, 1993b).

The Formation of Vesicles on Golgi Membranes Requires ARF

To investigate the mechanism of transport in incubations lacking cytosolic ARFs, we employed high resolution 3-dimensional transmission electron microscopy (Weidman *et al.*, 1993a). We analyzed the morphology of immobilized Golgi membranes after *in vitro* incubation with transport reaction mixtures containing Control or ARF(-) cytosols (Figure 6). Golgi membranes incubated in reactions containing Control cytosol were indistinguishable from membranes incubated with normal, unfractionated CHO cytosol, both in terms of morphology (compare Figure 6, A and B) and the density of coated buds and vesicles on Golgi cisternae (Table 1). In striking contrast, the densities of coated buds and vesicles on Golgi cisternae incubated with ARF(-) cytosol (Figure 6C; Table 1) were approximately 10-fold lower, and were comparable to densities found on membranes incubated on ice (Weidman *et al.*, 1993a) or in the absence of ATP or cytosol (Figure 6, E and F; Table 1). It is quite significant, however, that membranes incubated without cytosolic ARFs displayed no dramatic change in morphology: they retained the typical morphology of Golgi cisternae and exhibited no tendency to form long tubules (Figure 6C, Table 1). Immobilization of the membranes was not responsible for suppressing tubule formation during incubations without ARF, since long tubules did form on Golgi membranes incubated with ATP in the absence of cytosol (Figure

6F), as previously reported (Weidman *et al.*, 1993a). We conclude that ARF-free cytosol does not promote either the formation of coated vesicles or long tubules on Golgi cisternae.

Vesicle formation on Golgi cisternae was restored to normal levels by adding pure ARF to ARF(-) cytosol (Figure 6D; Table 1). Addition of pure bARF1 to $3 \mu g/ml$, a concentration commensurate with that normally present in transport incubations containing CHO cytosol ($4 \mu g/ml$), caused an almost 10-fold increase in coated bud and vesicle density (Figure 6D; Table 1). The minimum concentration of ARF required to effect vesicle formation ($2 \mu g/ml$) is about one-half that of Control reactions (Table 1). Cytosolic ARF is clearly required for coated vesicle formation on Golgi cisternae but not for cell-free intra-Golgi transport. Therefore, transport in the absence of ARFs must be occurring via a mechanism independent of coated vesicle formation. This mechanism does not appear to involve tubular extensions from the cisternae since removal of cytosolic ARF was insufficient to stimulate tubule growth.

Removal of ARFs from Transport Assays Has no Effect on the Rate of the Reaction

The results presented above demonstrate that removal of cytosolic ARFs effectively reduces formation of vesicles to background levels, and therefore suggest that a novel nonvesicular mechanism may be responsible for intra-Golgi transport measured under these conditions. To further characterize the effects of removing ARFs from transport reactions, we examined the kinetics of transport, as well as the dependence on ATP and the transport factor NSF. We found that omission of ATP from ARF(-) and Control assays reduced signal to $< 4\%$ of that observed with reactions containing ATP. In addition, transport observed with the ARF(-) cytosol exhibited the same requirements for the transport factor NSF as reactions containing Control cytosol (Figure 7). These data suggest that an aberrant pathway of unregulated membrane fusion was not activated by removal of cytosolic ARFs.

If removal of ARFs uncouples fusion from budding and leads to direct fusion of donor and acceptor membranes, then the lag in glycosylation-coupled transport thought to reflect the time required to form vesicles on the donor Golgi membranes (Balch *et al.*, 1984b; Rothman and Orci, 1990) should disappear. Assays containing saturating concentrations of cytosol were incubated for various intervals at $30^\circ C$ and then transferred to ice to arrest further transport (Balch *et al.*, 1984a). Transport was measured at each time point, and the results, expressed as a percentage of the maximal transport attained for either the ARF(-) or the Control cytosol, are shown in Figure 8. Although the Control cytosol exhibited $\sim 25\text{--}35\%$ greater transport at each time point (as in Figure 4A), the relative kinetics of transport in

Figure 6. The formation of Golgi-coated vesicles is dependent on the presence of ARF1. Freshly isolated CHO Golgi-enriched membranes were immobilized and incubated with *in vitro* transport reaction mixtures containing different cytosols for 10 min at 37°C, and then fixed and processed for transmission electron microscopy as described in MATERIALS AND METHODS (see also Weidman *et al.*, 1993a). The cytosol added to each reaction mixture is as follows: (A) Control cytosol (1.0 mg/ml); (B) unfractionated CHO cytosol (1.3 mg/ml); (C) ARF(–) cytosol

Table 1. Coated vesicles and buds from Golgi membranes incubated with cytosols

Cytosol	Coated buds & vesicles (μm^2) (n)	% Complexes with long tubules
A. Control cytosol	9.2 ± 1.7 (23)	4
B. CHO cytosol	8.4 ± 1.0 (25)	0
C. ARF (-) cytosol	0.8 ± 0.2 (25)	0
D. ARF (-) + bARF1:		
1 $\mu\text{g}/\text{ml}$	3.2 ± 0.6 (25)	0
2 $\mu\text{g}/\text{ml}$	9.2 ± 1.5 (24)	0
3 $\mu\text{g}/\text{ml}$	11.0 ± 0.9 (22)	0
E. CHO cyt. (No ATP)	1.0 ± 0.2 (25)	12
F. No cytosol	0.6 ± 0.4 (19)	58

Values are $\pm$ SE.

Golgi membranes immobilized on polylysine/glutaraldehyde treated coverslips were subjected to a 10-min transport incubation at 37°C , processed for electron microscopy, and quantitative data scored from negatives. The cytosol added to each experiment was identical to that presented in Figure 6. The data in D for reactions with 1 and 2 $\mu\text{g}/\text{ml}$ bARF1 were obtained from a different experiment and contained complete ARF(-) cytosol (1.4 $\mu\text{g}/\text{ml}$).

the two reactions are identical. It is particularly striking that the length of the lag period was unaffected by removal of ARFs. Identical results were obtained when kinetics were measured in reactions containing a sub-saturating concentration of the cytosols. These results indicate that the observed lag in the transport reaction cannot be due to vesicle production on the donor Golgi membranes as previously thought (Balch *et al.*, 1984b). Furthermore, since it is extremely unlikely that the observation of identical kinetics in absence and presence of ARFs is purely coincidental, these results strongly suggest that depletion of ARFs from transport assays has no significant effect on the mechanism of the reaction.

Glycosylation Is Rate-determining for Transport but Is Not Responsible for the Lag Period

If the lag period is not a consequence of vesicle formation, what then is responsible for the lag? The recent studies of Wattenberg and colleagues (Hiebsch *et al.*, 1992) established that the glycosylation of VSV-G protein does not occur rapidly upon transfer to the acceptor Golgi as previously assumed (Balch *et al.*, 1984b). Rather, glycosylation occurs slowly and is most likely a result of the sluggish import of the radiolabeled precursor (UDP-GlcNAc) into the Golgi lumen. In light of

Figure 7. ARF(-) transport is NSF-dependent. Donor and acceptor Golgi membranes were treated with *N*-ethylmaleimide (NEM; 0.25 mM for 15 min on ice) to inactivate the transport factor NSF. Transport assays contained 2 μl each of these NEM-treated donor and acceptor Golgi and 3 μl equivalents of either ARF(-) or Control cytosol. Cytosols were pre-incubated at 37°C for 20 min to inactivate soluble NSF (Block *et al.*, 1988). Assays were supplemented with either buffer (no NSF) or 15 ng of pure NSF. Transport is restored when NSF is added to reactions containing either ARF(-) or Control cytosol. The addition of NSF restored transport to $\sim 85\%$ of that observed in reactions containing cytosols and untreated Golgi membranes (not shown).

these findings, we reexamined the mechanism of the reaction in more detail, to determine the extent to which glycosylation contributes to the lag period and to the overall rate of the transport reaction.

A glycosylation assay was developed using Golgi-enriched membrane fractions that were prepared from wild-type CHO cells infected with VSV ("wild-type donor"; Kanstein, 1992). Briefly, wild-type cells, rather than the GlcNAc-transferase 1-deficient mutant cells, were used for this assay to ensure that VSV-G protein was present throughout the Golgi stack, including the compartment(s) containing the enzyme GlcNAc-transferase 1. Transfer of radiolabeled GlcNAc to VSV-G in this system should be independent of membrane fusion events. Figure 9A demonstrates that cytosol is not required to observe glycosylation with this assay since the requirement for soluble proteins can be satisfied almost entirely by addition of commercially available UMP-kinase. This requirement is explained simply by the fact that import of UDP-GlcNAc, performed by an antiporter, is slow (Hirshberg and Snider, 1987) and that UMP, which accumulates in the absence of UMP-kinase, inhibits sugar import by this antiporter (Perez and Hirshberg, 1985; Waldman and Rudnick, 1990). A requirement for UMP-kinase had been demonstrated for

(1.7 mg/ml); (D) ARF(-) cytosol (1.7 mg/ml) plus pure bARF1 (3 $\mu\text{g}/\text{ml}$); (E) unfractionated CHO cytosol (1.3 mg/ml) in which the ATP-regenerating system was omitted; (F) no cytosol. The inset in D is a view of the punctate coats (magnified 2 $\times$) found on the vesicles formed during the transport incubations. The inset in C shows the smooth surface of an uncoated bud (magnified 2 $\times$) for comparison. A low density of uncoated buds or blunt-ended tubules were observed in all samples (arrows in A, B, and E). The bars are 0.2 μm .

Figure 8. The kinetics of transport with ARF(–) and control cytosols are identical. Transport reactions, performed as in Figure 4, containing either 0.75 or 2.5 μ l equivalents of cytosols, were incubated at 30°C for the times indicated. Reactions were then transferred to ice to arrest the transport reaction. Data for the experiments performed with 2.5 μ l of cytosol are shown and are averages of three independent experiments. Transport is represented as the percentage of the maximal transport observed with each cytosol. Identical results were obtained for the reactions containing subsaturating levels of cytosols (0.75 μ l per assay; not shown).

the standard transport assay by Wattenberg and colleagues (Hiebsch and Wattenberg, 1992). Further proof that glycosylation of VSV-G in the “wild-type donor” does not require membrane fusion comes from the fact that inactivation of NSF, a protein required for transport *in vitro* (Block *et al.*, 1988) has no effect on this reaction (Figure 9A, rightmost column). Also, this glycosylation assay is not affected by addition of neutralizing rab1 antibodies or rab3AL peptide, both of which inhibit the fusion-dependent standard transport assay (M. Kanstein, P. Melançon and W. Balch, unpublished observations; Kanstein, 1992).

Figure 9B compares the kinetics of glycosylation in 1) standard transport assays containing donor and acceptor Golgi membranes and cytosol, with 2) glycosylation assays containing “wild-type donor” Golgi lacking NSF and containing UMP-kinase but no cytosol. The kinetics are nearly identical except for the clear absence of a lag period in the fusion-independent reactions containing wild-type donor. These results clearly establish that glycosylation cannot be responsible for the lag observed in the standard transport reaction (up to ~8 min; see Figures 8 and 9B), whereas it appears to be the rate-determining step during the remainder of the reaction. Indeed, the linear portions of the two kinetic curves are parallel, which indicates that after the initial lag, the rate of the reaction is determined entirely by the rate of glycosylation and is no longer sensitive to events directly related to transport, such as activation of donor membranes or fusion.

We conclude that the lag, observed only in the standard intra-Golgi transport assay, is a sensitive measure of steps that are specific to the transport reaction (e.g., donor activation and/or fusion of transport intermediates with the acceptor). Therefore, the observation that ARF-depleted reactions, which exhibit a 10-fold lower vesicle density (Table 1), exhibit the same lag period as control reactions (Figure 8) strongly suggests that a similar coated vesicle-independent transport mechanism operates irrespective of the presence or absence of ARFs. Furthermore, rapid transfer of VSV-G protein via a coated vesicle-independent mechanism is consistent with the slow kinetics observed over the entire course of the reaction, since detection of early fusion events would be delayed due to the slow rate of glycosylation.

Transport Reactions Containing ARF(–) Cytosol Remain Sensitive to the Donor-specific Inhibitor Ilimaquinone

The sea sponge metabolite ilimaquinone (IQ) causes vesiculation of Golgi membranes *in vivo*, and has been shown to block intra-Golgi transport *in vitro* in a donor-specific manner (Takizawa *et al.*, 1993). This inhibition of donor activity was interpreted as the inhibition of the formation of competent transport vesicles by IQ (Takizawa *et al.*, 1993). Since IQ is thought to affect vesicle formation, we reasoned that the nonvesicular transport measured in the presence of the ARF(–) cytosol would be insensitive to IQ. The data presented in Figure 10 establish that, contrary to this hypothesis, the sensitivity of the intra-Golgi transport assay to IQ is independent of ARF concentration. Transport reactions containing either Control or ARF(–) cytosol were carried out with increasing concentrations of IQ. Identical IC_{50} values of ~2.5 μ M were measured in both cases. Furthermore, addition of pure ARFs from bovine brain to reactions containing ARF(–) cytosol had no effect on IQ sensitivity. Clearly, the effect of IQ cannot be limited to vesicle formation but may rather result from a block in a more general process critical to membrane fusion. These results further suggest that the IQ-sensitive mechanism of transport is the same in both the Control and ARF(–) reactions.

DISCUSSION

We have used chromatographic methods to produce a pool of soluble proteins from extracts of CHO cells which is 98% depleted of ADP-ribosylation factors. The extent of depletion was quantitated as a loss of ~21 kDa GTPases in the ARF(–) cytosol. This cytosol lacking ARFs is fully functional in the *in vitro* intra-Golgi transport assay. In fact, by all criteria examined, the characteristics of transport with ARF(–) cytosol are essentially indistinguishable from those of cytosol containing

Figure 9. Glycosylation is the rate limiting step in the transport reaction. (A) Golgi membranes were prepared from VSV-infected, wild-type CHO cells, and a portion of the membranes was pre-treated with 0.25 mM NEM on ice to inactivate the transport factor NSF (NEM-don. [+]). Assays were performed as in Figure 4 except that incubation was at 37°C for 1 h, and 4 μ l of NEM-treated or untreated wt donor Golgi membranes were used and no acceptor Golgi was present. Reactions contained either 7.5 μ g CHO cytosol or 0.03 I.U. pure UMP-kinase (UK) as indicated. The identical incorporation of 3 H-GlcNAc observed in reactions containing inactivated NSF $\pm$ UK indicate that these reactions are measuring glycosylation of VSV-G already residing in the appropriate compartment, rather than transport to that compartment. (B) The kinetics of incorporation of 3 H-GlcNAc into VSV-G protein were measured in assays containing either 2 μ l each of donor and acceptor Golgi membranes and 7.5 μ g of cytosol ("standard" assay) or 4 μ l of wt donor Golgi pre-treated with 0.25 mM NEM and 0.03 I.U. pure UMP-kinase ("wt donor" + UK). Reactions containing wt donor + UK received no cytosol. Assays were incubated at 37°C for the times indicated and then transferred to ice to stop the reaction. The standard reaction exhibits a "lag" period (0–8 min at 37°C), which is not apparent in the reaction containing wt donor Golgi + UK.

ARFs. Nevertheless, the extent of ARF removal was sufficient to completely prevent the formation of coated buds and vesicles on Golgi cisternae during an in vitro incubation. These findings have important implications

on the role of COP-coated vesicles as transport intermediates in the in vitro intra-Golgi transport reactions.

ARFs Are Required for the Formation of Coated Vesicles from Golgi Membranes

Previous studies implicating ARFs in vesicle assembly relied on pharmacological agents such as peptides (Kahn *et al.*, 1992), BFA (Donaldson *et al.*, 1991) or GTP analogues (Serafini *et al.*, 1991, Orci *et al.*, 1993a,b) whose mechanisms of action have not been fully established. Other studies have demonstrated that high concentrations of recombinant ARFs stimulate association of Golgi vesicles coat proteins (COPs or coatomers) with Golgi membranes but did not establish a correlation with vesicle formation (Donaldson *et al.*, 1992a). In this study, we established that removal of cytosolic ARFs eliminates formation of coated vesicles on Golgi cisternae during otherwise normal transport incubations and that addition of pure ARF1 to physiological levels is both necessary and sufficient to reconstitute vesicle budding, in agreement with previous studies (Orci *et al.*, 1993b). Moreover, we have shown that the concentration of purified ARFs required to stimulate budding is similar to that found in unfractionated cytosol. The data presented here were acquired without the use of pharmacological reagents such as GTP γ S and therefore rep-

Figure 10. ARF(-) transport is sensitive to the drug ilimaquinone. Transport assays, containing 2 μ l each of donor and acceptor Golgi (not treated with NEM) were performed as in Figure 4. Assays contained 4 μ l equivalents of either ARF(-) or Control cytosol. Ilimaquinone (IQ; 60 μ M in a 25 mM HEPES, pH 7.4, 1% DMSO buffer) was titrated to a maximum of 12 μ M in 50 μ l assays. Both ARF(-) and Control reactions exhibited an IC_{50} of ~ 2.5 μ M for IQ. The IQ buffer did not affect transport at the levels assayed (not shown).

resent a definitive demonstration of the involvement of ARFs in the budding process.

ARFs Are Not Essential for Intra-Golgi Transport

Despite the fact that ARFs play an essential role in the formation of coated vesicles on Golgi cisternae, we find that cytosolic ARFs are not required to drive transport between Golgi membranes in the cell-free system. This is particularly evident in the observation that the concentration of cytosol needed to optimally drive transport is independent of the ARF concentration. The slightly lower maximal extent of transport observed with ARF(−) cytosol results from the loss during chromatography of a small protein factor, which is distinct from ARFs.

Although the removal of soluble ARFs was essentially complete in the ARF(−) cytosol, our conclusions are limited by the fact that a very small amount of ~21 kDa GTPases are detected on Golgi membranes. If these proteins are ARFs, then either GTP hydrolysis is not required for their function, or these ARFs do not bind GTP γ S and are present as ARF-GTP, since GTP γ S has no effect on transport reactions containing ARF(−) cytosol. Neither possibility is consistent with known properties of ARFs. First, GTP γ S binding to ARFs blocks transport (Taylor *et al.*, 1992). Second, ARF-GDP does not bind to membranes (Kahn, 1991; Walker *et al.*, 1992) and GTP hydrolysis occurs rapidly on ARFs in the presence of Golgi membranes (Donaldson *et al.*, 1992b; Helms *et al.*, 1993). Thus a more likely explanation is that these Golgi-associated 21 kDa GTPases represent members of other families such as rabs or sar1p. Immunoblotting with antibodies to various membrane-associated 21 kDa GTPases may confirm this hypothesis.

Our conclusion that ARFs are not required for transport is apparently inconsistent with the observation that synthetic peptides corresponding to the N-terminus of ARF1 concertedly block intra-Golgi transport, coat protein binding to Golgi membranes and Golgi vesicle formation (Kahn *et al.*, 1992). It was assumed that this inhibition resulted from competition between ARFs and the peptide for an essential effector, and therefore concluded that ARF is required for transport. Here we have shown that the concentration of peptide required to block transport is independent of the level of cytosolic ARFs. Inhibition of transport by these peptides is therefore unlikely to result from competition between ARFs and peptide. We and others have obtained evidence that this peptide blocks protein glycosylation, possibly by disrupting membrane integrity (Kahn *et al.*, 1992; Weidman *et al.*, 1993b). Therefore, we suggest that the peptide acts independently of ARFs and that its inhibitory activity does not accurately address a role for ARF in transport.

If ARFs are not required for transport, then why do they inhibit transport in the presence of GTP γ S (Taylor

et al., 1992)? It has recently been demonstrated that incubations containing GTP γ S induce rapid and near complete vesiculation of immobilized Golgi cisternae (Weidman *et al.*, 1993a). This massive vesiculation occurs in the virtual absence of opportunity for vesicle transfer, suggesting that vesicles accumulate primarily at their sites of formation and not at their sites of fusion, as previously thought (Orci *et al.*, 1989). The selective inhibition of acceptor membrane function by ARF and GTP γ S might thus be due to obliteration of membrane fusion sites by this aberrant vesiculation. The complete refractility of donor membrane function to GTP γ S (Melançon *et al.*, 1987) is difficult to reconcile with either vesicular or nonvesicular intra-Golgi transport. An alternative explanation for this inconsistency is presented in the discussion that follows.

Intra-Golgi Transport In Vitro May Not Involve Coated Vesicles even when ARFs Are Present

Coated vesicles form on the rims of Golgi membranes when incubated with ATP and cytosol containing ARFs. Previously, a correlation between conditions which favor the appearance of these vesicles and in vitro transport, as well as the association of VSV-G containing vesicles with acceptor membranes, led to the proposal that these vesicles mediate transport (Rothman and Orci, 1992). We have removed ARFs from transport reactions and reduced the density of coated buds and vesicles to background levels. This dramatic decrease in vesicle density is not accompanied by any detectable change in the characteristics of the transport reactions, in particular the length of the lag period, as measured in kinetic experiments. The addition of purified ARFs to physiological levels restores coated vesicle density to values observed in the Control but, again, this has no effect on the extent or the kinetics of the transport reaction. This lack of correlation between coated vesicle density and the rate and extent of transport is inconsistent with a transport mechanism involving coated vesicles. Although it is possible that the mechanism of transport could switch from vesicular to direct cisternal fusion upon ARF removal, it seems unlikely that the similarity of the kinetics measured under these various conditions is purely coincidental. A much simpler explanation is that the principal mechanism of transport in the Golgi in vitro does not involve coated vesicles, whether or not ARFs are present.

What, then, is the mechanism of protein transfer? Transport could occur via direct fusion events between Golgi or Golgi fragments, as proposed by Mellman and Simons (1992). Morphological and biochemical studies with Golgi membranes in the presence of BFA suggest that the formation of tubular structures within and between Golgi membranes could mediate intra-Golgi protein transfer (Orci *et al.*, 1991). If such tubular networks form during transport reactions with ARF-free

cytosol, they would not have been detected in our morphological analysis since the membranes were immobilized prior to the transport incubation. Nevertheless, if tubular networks form between cisternae in the absence of ARF, our biochemical data indicate that they do not contribute to the transport observed. The biochemical characteristics of transport in the absence of ARFs are identical to those of the normal assay, and the formation of tubular networks is not observed in normal transport incubations (Orci *et al.*, 1991). We think it extremely unlikely that this identical behavior is purely coincidental and offer an alternate explanation. Namely, the donor membranes for *in vitro* transport may not be Golgi cisternae, but rather mature transport intermediates such as tubulovesicular elements of the intermediate compartment (IC). Transport could thus occur as a result of the direct fusion of IC elements with the CGN of the Golgi stack. In support of this possibility, markers for the IC do co-purify with stacked Golgi membranes (D. Mundy, unpublished observations) and are thus present in the donor membrane fraction. Furthermore, GlcNAc transferase 1 activity in CHO cells is present in a compartment also containing Golgi mannosidase 1, presumably *cis*-Golgi cisternae (Schwaninger *et al.*, 1991).

A model of transport involving fusion of morphologically and biochemically distinct membranes, in contrast to tubular or general cisternal fusion, is consistent with the differential properties of donor and acceptor membranes (Balch *et al.*, 1984b; Balch and Rothman, 1985; Hiebsch *et al.*, 1991). For example, it accounts for the irreversible inhibition of donor, but not acceptor function by IQ (Takizawa *et al.*, 1993). In addition, this model explains the insensitivity of donor membrane function to ARF-GTP γ S (Melançon *et al.*, 1987) if IC membranes lack the nucleotide exchange activity responsible for ARF activation. Finally, the unexpected finding that BFA-induced cisternal membrane fusion does not alter the extent of transport (Orci *et al.*, 1991) is reconciled if donor cisternae do not substantially contribute to the VSV-G transported in the assay.

The view that emerges is one where there is a production of intermediates of unknown nature, followed by their fusion with the acceptor and the slow modification of the transferred cargo. Activation of these intermediates for fusion most likely involves recruitment of the several factors identified to date (e.g., NSF, SNAPs, and SNAREs). Vesicles are certainly produced from the cisternae of donor Golgi stacks throughout the course of the reaction but may carry a relatively small fraction of the VSV-G transferred to acceptor compartments. This interpretation in no way invalidates the highly significant work which led to the identification of transport factors essential for fusion of donor-derived intermediates, and the formulation of the SNARE hypothesis (Söllner *et al.*, 1993a,b; reviewed in Rothman and Orci, 1992; Südhof *et al.*, 1993; Takizawa and

Malhotra, 1993; Warren, 1993), but may cause reevaluation of the kinetics of these reactions as previously suggested (Hiebsch and Wattenberg, 1992).

Function of ARFs in the Golgi and Other Endomembrane Systems

The lack of requirement for ARFs and coated vesicles observed *in vitro* in no way precludes an essential function for GCVs *in vivo*. Indeed, much genetic and biochemical evidence demonstrates that ARFs and coatomers are required for protein transport *in vivo* (Stearns *et al.*, 1990; Hosobuchi *et al.*, 1992; Pepperkok *et al.*, 1993). In addition to GCVs, the tubular structures visualized on Golgi membranes (Rambourg *et al.*, 1991; Cooper *et al.*, 1990) could also play an important role in this process. A regulatable switch between vesicular and tubular modes of transport could control the rate and specificity of traffic through a given organelle. Our observation that ARFs are required for the formation of coated buds and vesicles, along with the fact that hydrolysis of GTP by ARF triggers uncoating of membranes (Tanigawa *et al.*, 1993) suggests that regulating the density of ARFs on a specific membrane could serve this function.

Assembly of a protein coat on membranes appears to be required for initiation of bud and vesicle formation (Weidman *et al.*, 1993a). However, abortion of the assembly process after it has initiated, by a signal indicating presence of inappropriate cargo, for example, could leave a naked bud free to extend into a tubule and fuse back with the organelle of origin. Fusion of such tubules on the periphery would lead to the characteristic fenestrated structure of the rims of Golgi membranes. ARFs appear ideally suited to regulate this switch between tubular fusion with like-membranes and production of vesicles destined for the next compartment. Additional functions for members of this multigene family have been identified (Brown *et al.*, 1993; Kahn *et al.*, 1993), and further biochemical and cell biological characterization of individual ARFs will more clearly define their putative multi-functional roles in intracellular processes.

Note added in proof. While this article was in press, a manuscript was brought to our attention that also reports that coat assembly is not required for cell-free transport (Elazar *et al.*, 1994). These authors reach different conclusions about the mechanistic nature of the reaction, however. Elazar *et al.* propose that the absence of coat proteins uncouples fusion from budding and results in a drastic switch in mechanism from vesicle-mediated transport to cisternal fusion. These authors show that addition of competing membranes lacking VSV-G and GlcNAc transferase 1 reduces the extent of VSV-G glycosylation, presumably by sequestering vesicular intermediates. This competition is not observed under conditions that preclude coat assembly, which Elazar *et al.* take as proof of cisternal fusion. First, we caution that this method cannot distinguish between entrapment of *de novo* assembled COP vesicles or of the preexisting pleiomorphic IC vesicles we propose as the major carrier of VSV-G in this assay. Furthermore, the observation that conditions that eliminate competition by trap membranes do not increase either the rate or the extent of the reaction

seems inconsistent with the proposed fusion of donor and acceptor cisternae. It may be important to note that morphological analysis by these authors shows no evidence for the predicted uncoupled cisternal fusion. On the other hand, a recent morphological study (Balch *et al.*, 1994) identifies vesiculotubular clusters (VTCs) as transport intermediates for the transfer of proteins from the ER to the Golgi complex and thus supports our hypothesis. ARFs and COPs are most likely required for formation but not fusion of these structures, which is consistent with the observations reported here.

ACKNOWLEDGMENTS

We are very grateful to John Heuser for use of electron microscopic facilities and to Robyn Roth for preparation of replicas. We thank Debbie McDonald and Bill Winter for the preparation of membrane fractions used in transport and budding assays, respectively, and M. Kissinger for preparation of culture media and cell culture. We acknowledge Doug Clary for the generous gift of purified NSF and Rick Kahn for providing ARF peptide 13. Gerry Waters and Binks Wattenberg provided helpful comments on the manuscript. This research was supported by National Institutes of General Medical Sciences Grants 5R01GM-43378 (P.M.) and 5R01GM-29647 (Dr. J. Heuser), by the American Cancer Society grant BE-87 (P.W.), and by an NIH predoctoral fellowship (T.C.T.).

REFERENCES

Balch, W.E., Dunphy, W.G., Braell, W.A., and Rothman, J.E. (1984a). Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell* 39, 405–416.

Balch, W.E., Glick, B.S., and Rothman, J.E. (1984b). Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. *Cell* 39, 525–536.

Balch, W.E., McCaffery, J.M., Plutner, H., and Farquhar, M.G. (1994). Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum. *Cell* 76 (*in press*).

Balch, W.E., and Rothman, J.E. (1985). Characterization of protein transport between successive compartments of the Golgi apparatus: asymmetric properties of donor and acceptor activities in a cell-free system. *Arch. Biochem. Biophys.* 240, 413–425.

Block, M.R., Glick, B.S., Wilcox, C.A., Wieland, F.T., and Rothman, J.E. (1988). Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. *Proc. Natl. Acad. Sci.* 85, 7852–7856.

Braell, W.A., Balch, W.E., Dobbertin, D.C., and Rothman, J.E. (1984). The glycoprotein that is transported between successive compartments of the Golgi in a cell-free system resides in stacks of cisternae. *Cell* 39, 511–524.

Brown, H.A., Gutowski, S., Moomaw, C.R., Slaughter, C., and Sternweis, P.C. (1993). ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. *Cell* 75, 1137–1144.

Clary, D.O., and Rothman, J.E. (1990). Purification of three related peripheral membrane proteins needed for vesicular transport. *J. Biol. Chem.* 265, 10109–10117.

Colombo, M.I., Gonzallo, S., Weidman, P., and Stahl, P. (1991). Characterization of trypsin-sensitive factor(s) required for endosome-endosome fusion. *J. Biochem.* 266, 23438–23445.

Cooper, M.S., Cornell-Bell, A.H., Chernjavsky, A., Dani, J.W., and Smith, S.J. (1990). Tubulovesicular processes emerge from trans-Golgi cisternae, extend along microtubules, and interlink adjacent trans-Golgi elements into a reticulum. *Cell* 61, 135–145.

de Curtis, I., and Simons, K. (1989). Isolation of exocytic carrier vesicles from BHK cells. *Cell* 58, 719–727.

Donaldson, J.G., Lippincott-Schwartz, J., and Klausner, R.D. (1991). Guanine nucleotides modulate the effects of Brefeldin A in semi-permeable cells: regulation of the association of a 110-kD peripheral membrane protein with the Golgi apparatus. *J. Cell Biol.* 112, 579–588.

Donaldson, J.G., Cassel, D., Kahn, R.A., and Klausner, R.D. (1992a). ADP-ribosylation factor, a small GTP-binding protein, is required for binding of the coatamer protein β -COP to Golgi membranes. *Proc. Natl. Acad. Sci.* 89, 6408–6412.

Donaldson, J.G., Finazzi, D., and Klausner, R.D. (1992b). Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein. *Nature* 360, 350–352.

Duden, R., Allan, V., and Kreis, T. (1991). Involvement of β -COP in membrane traffic through the Golgi complex. *Trends Cell Biol.* 1, 14–19.

Elazar, Z., Orci, L., Ostermann, J., Amherdt, M., Tanigawa, G., and Rothman, J.E. (1994). ADP-ribosylation factor and coatamer couple fusion to vesicle budding. *J. Cell Biol.* (*in press*).

Farquhar, M.G., and Palade, G.E. (1981). The Golgi apparatus (complex)-(1954–1981)-from artifact to center stage. *J. Cell Biol.* 91, 77s–103s.

Groesch, M.E., Ruohola, H., Bacon, R., Rossi, G., and Ferro-Novick, S. (1990). Isolation of a functional vesicular intermediate that mediates ER to Golgi transport in yeast. *J. Cell Biol.* 111, 45–53.

Gruenberg, J., and Howell, K.E. (1989). Membrane traffic in endocytosis: insights from cell-free assays. *Annu. Rev. Cell Biol.* 5, 453–481.

Helms, J.B., and Rothman, J.E. (1992). Inhibition by brefeldin A of a Golgi membrane enzyme that catalyzes exchange of guanine nucleotide bound to ARF. *Nature* 360, 352–354.

Helms, J.B., Palmer, D.J., and Rothman, J.E. (1993). Two distinct populations of ARF bound to Golgi membranes. *J. Cell Biol.* 121, 751–760.

Heuser, J., and Evans, L. (1980). Three-dimensional visualization of coated vesicle formation in fibroblasts. *J. Cell Biol.* 84, 560–583.

Heuser, J.E., Jan, T.S., Dennis, M.J., and Evans, L. (1979). Synaptic vesicle exocytosis captured by quick freezing and correlated with the quantal transmitter release. *J. Cell Biol.* 81, 275–300.

Hiebsch, R.H., Raub, T.J., and Wattenberg, B.W. (1991). Primaquine blocks transport by inhibiting the formation of functional vesicles. *J. Biol. Chem.* 266, 20323–20328.

Hiebsch, R.R., and Wattenberg, B.W. (1992). Vesicle fusion in protein transport through the Golgi does not involve long-lived prefusion intermediates. A reassessment of the kinetics of transport as measured by glycosylation. *Biochemistry* 31, 6111–6118.

Hirschberg, C.B., and Snider, M.D. (1987). Topography of glycosylation in the rough endoplasmic reticulum and Golgi apparatus. *Ann. Rev. Biochem.* 56, 63–87.

Hosobuchi, M., Kreis, T., and Schekman, R. (1992). SEC21 is a gene required for ER to Golgi protein transport that encodes a subunit of a yeast coatamer. *Nature* 360, 603–605.

Jamieson, J., and Palade, G. (1967). Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. *J. Cell Biol.* 34, 577–596.

Kahn, R.A. (1991). Fluoride is not an activator of the smaller (20–25 kDa) GTP-binding proteins. *J. Biol. Chem.* 266, 15595–15597.

Kahn, R.A., Randazzo, P.A., Weiss, O., Rulka, C., Clark, J., Amherdt, M., Roller, P.P., Orci, L., and Rothman, J.E. (1992). The amino-terminus of ADP-ribosylation factor (ARF) is a critical determinant of ARF activities and is a potent and specific inhibitor of protein transport. *J. Biol. Chem.* 267, 13039–13046.

- Kahn, R.A., Yucel, J.K., and Malhotra, V. (1993). ARF signaling: a potential role for phospholipase D in membrane traffic. *Cell* 75, 1045–1048.
- Kanstein, M. (1992). Biochemical function of rab1b in an intra-Golgi transport assay. Masters Thesis. Boulder, CO: University of Colorado.
- Klausner, R.D., Donaldson, J.G., and Lippincott-Schwartz, J. (1992). Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* 116, 1071–1080.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lang, L., and Kornfeld, S. (1984). A simplified procedure for synthesizing large quantities of highly purified uridine [β - 32 P]diphospho-N-acetylglucosamine. *Anal. Biochem.* 140, 264–269.
- Lapetina, E.G., and Reep, B.R. (1987). Specific binding of [α - 32 P]GTP to cytosolic and membrane-bound proteins of human platelets correlates with the activation of phospholipase C. *Proc. Natl. Acad. Sci.* 84, 2261–2265.
- Lippincott-Schwartz, J. (1993). Bidirectional membrane traffic between the endoplasmic reticulum and Golgi apparatus. *Trends Cell Biol.* 3, 81–88.
- Melançon, P., Glick, B.S., Malhotra, V., Weidman, P.G., Serafini, T., Gleason, M.L., Orci, L., and Rothman, J.E. (1987). Involvement of GTP-binding "G" proteins in transport through the Golgi stack. *Cell* 51, 1053–1062.
- Mellman, I., and Simons, K. (1992). The Golgi complex: in vitro veritas? *Cell* 68, 829–840.
- Orci, L., Glick, B.S., and Rothman, J.E. (1986). A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell* 46, 171–184.
- Orci, L., Malhotra, V., Amherdt, M., Serafini, T., and Rothman, J.E. (1989). Dissection of a single round of vesicular transport: sequential coated and uncoated intermediates mediate intercompartmental movements in the Golgi stack. *Cell* 56, 357–368.
- Orci, L., Tagaya, M., Amherdt, M., Perrelet, A., Donaldson, J.G., Lippincott-Schwartz, J., Klausner, R.D., and Rothman, J.E. (1991). Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell* 64, 1183–1195.
- Orci, L., Palmer, D.J., Ravazzola, A.P., Amherdt, M., and Rothman, J.E. (1993a). Budding from Golgi membranes requires the coatamer complex of non-clathrin coat proteins. *Nature* 362, 648–652.
- Orci, L., Palmer, D.J., Amherdt, M., and Rothman, J.E. (1993b). Coated vesicle assembly in the Golgi requires only coatamer and ARF proteins from the cytosol. *Nature* 364, 732–734.
- Osterman, J., Orci, L., Tani, K., Amherdt, M., Ravazzola, M., Elazar, Z., and Rothman, J.E. (1993). Stepwise assembly of functionally active transport vesicles. *Cell* 75, 1015–1025.
- Palade, G. (1975). Intracellular aspects of the process of protein synthesis. *Science* 189, 347–358.
- Pepperkok, R., Scheel, J., Horstman, H., Hauri, H.P., Griffiths, G., and Kreis, T.E. (1993). β -COP is essential for biosynthetic membrane transport from the endoplasmic reticulum to the Golgi complex in vivo. *Cell* 74, 71–82.
- Perez, M., and Hirschberg, C.B. (1985). Translocation of UDP-N-acetylglucosamine into vesicles derived from rat liver rough endoplasmic reticulum and Golgi apparatus. *J. Biol. Chem.* 260, 4671–4678.
- Randazzo, P.A., Yang, Y.C., Rulka, C., and Kahn, R.A. (1993). Activation of ADP-ribosylation factor by Golgi membranes. Evidence for a Brefeldin A- and protease-sensitive activating factor on Golgi membranes. *J. Biol. Chem.* 268, 9555–9563.
- Rexach, M.F., and Schekman, R.W. (1991). Distinct biochemical requirements for the budding, targeting, and fusion of ER-derived transport vesicles. *J. Cell Biol.* 114, 219–229.
- Rothman, J.E., Urbani, L.J., and Brands, R. (1984a). Transport of protein between cytoplasmic membranes of fused cells: correspondence to processes reconstituted in a cell-free system. *J. Cell Biol.* 99, 248–259.
- Rothman, J.E., Miller, R.L., and Urbani, L.J. (1984b). Intercompartmental transport in the Golgi complex is a dissociative process: facile transfer of membrane protein between two Golgi populations. *J. Cell Biol.* 99, 260–271.
- Rothman, J.E., and Orci, L. (1990). Movement of proteins through the Golgi stack: a molecular dissection of vesicular transport. *FASEB J.* 4, 1460–1468.
- Rothman, J.E., and Orci, L. (1992). Molecular dissection of the secretory pathway. *Nature*, 355, 409–415.
- Saraste, J., and Kuismanen, E. (1992). Pathways of protein sorting and membrane traffic between the rough endoplasmic reticulum and the Golgi complex. *Sem. Cell Biol.* 3, 343–355.
- Schmitt, H.D., Wagner, P., Pfaff, E., and Gallwitz, D. (1986). The ras-related YPT1 gene product in yeast: a GTP-binding protein that might be involved in microtubule organization. *Cell* 47, 401–412.
- Schwaninger, R., Beckers, C.J.M., and Balch, W.E. (1991). Sequential transport of protein between the endoplasmic reticulum and successive Golgi compartments in semi-intact cells. *J. Biol. Chem.* 266, 13055–13063.
- Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R.A., and Rothman, J.E. (1991). ADP-ribosylation factor is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. *Cell* 67, 239–253.
- Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993a). SNAP receptors implicated in vesicle targeting and fusion. *Nature* 362, 318–324.
- Söllner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H., and Rothman, J.E. (1993b). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation and fusion. *Cell* 75, 409–418.
- Südhof, T.C., De Camilli, P., Niemann, H., and Jahn, R. (1993). Membrane fusion machinery: insights from synaptic proteins. *Cell* 75, 1–4.
- Stanley, P., Caillibot, V., and Siminovitch, L. (1975). Selection and characterization of eight phenotypically distinct lines of lectin-resistant Chinese hamster ovary cells. *Cell* 6, 121–128.
- Stearns, T., Kahn, R.A., Botstein, D., and Hoyt, M.A. (1990). ADP ribosylation factor is an essential protein in *Saccharomyces cerevisiae* and is encoded by two genes. *Mol. Cell Biol.* 10, 6690–6699.
- Sztul, E., Kaplin, A., Saucan, L., and Palade, G. (1991). Protein traffic between distinct plasma membrane domains: isolation and characterization of vesicular carriers involved in transcytosis. *Cell* 64, 81–89.
- Takizawa, P.A., Yucel, J.K., Veit, B., Faulkner, D.J., Deerinck, T., Soto, G., Ellisman, M., and Malhotra, V. (1993). Complete vesiculation of Golgi membranes and inhibition of protein transport by a novel sea sponge metabolite, limaquinone. *Cell* 73, 1079–1090.
- Takizawa, P.A., and Malhotra, V. (1993). Coatamers and SNAREs in promoting membrane traffic. *Cell* 75, 593–596.
- Tanigawa, G., Orci, L., Amherdt, M., Ravazzola, M., Helms, J.B., and Rothman, J.E. (1993). Hydrolysis of bound GTP by ARF protein triggers uncoating of Golgi-derived COP-coated vesicles. *J. Cell Biol.* 123, 1365–1371.

- Taylor, T.C., Kahn, R.A., and Melançon, P. (1992). Two distinct members of the ADP-ribosylation factor family of GTP-binding proteins regulate cell-free intra-Golgi transport. *Cell* 70, 69–79.
- Tsuchiya, M., Price, S.R., Tsai, S.-C., Moss, J., and Vaughan, M. (1991). Molecular identification of ADP-ribosylation factor mRNAs and their expression in mammalian cells. *J. Biol. Chem.* 266, 2772–2777.
- Waldman, B.C., and Rudnick, G. (1990). UDP-GlcNAc transport across the Golgi membrane: electroneutral exchange for dianionic UMP. *29*, 44–52.
- Walker, M.W., Bobak, D.A., Tsai, Su-C., Moss, J., and Vaughan, M. (1992). GTP but not GDP analogues promote association of ADP-ribosylation factors, 20-kDa protein activators of cholera toxin, with phospholipids and PC-12 cell membranes. *J. Biol. Chem.* 267, 3230–3235.
- Walworth, N.C., and Novick, P.J. (1987). Purification and characterization of constitutive secretory vesicles from yeast. *J. Cell Biol.* 105, 163–174.
- Warren, G. (1993). Bridging the gap. *Nature* 362, 297–298.
- Waters, M.G., Serafini, T., and Rothman, J.E. (1991). “Coatomer”: a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature* 349, 248–251.
- Waters, M.G., Clary, D.O., and Rothman, J.E. (1992). A novel 115-kD peripheral membrane protein is required for intercisternal transport in the Golgi stack. *J. Cell Biol.* 118, 1015–1026.
- Weidman, P., Melançon, P., Block, M.R., and Rothman, J.E. (1989). Binding of an N-ethylmaleimide-sensitive fusion protein to Golgi membranes requires both a soluble protein(s) and an integral membrane receptor. *J. Cell Biol.* 108, 1589–1596.
- Weidman, P., Roth, R., and Heuser, J. (1993a). Golgi membrane dynamics imaged by freeze-etch electron microscopy: views of different membrane coatings involved in tubulation versus vesiculation. *Cell* 75, 123–133.
- Weidman, P., Winter, B., Roth, R., and Heuser, J. (1993b). Peptide inhibitor of Golgi transport: sorting out fact from artefact. *Mol. Biol. Cell* 4S, 321a.
- Woodman, P.G., and Warren, G. (1991). Isolation of functional, coated, endocytic vesicles. *J. Cell Biol.* 112, 1133–1141.