COPII Vesicles Derived from Mammalian Endoplasmic Reticulum Microsomes Recruit COPI

Tony Rowe,* Meir Aridor,* J. Michael McCaffery,‡ Helen Plutner,* Claude Nuoffer, and William E. Balch*

*Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037; and‡Division of Cellular and Molecular Medicine, University of California, San Diego, California 92093

Abstract. ER to Golgi transport requires the function of two distinct vesicle coat complexes, termed COPI (coatomer) and COPII, whose assembly is regulated by the small GTPases ADP-ribosylation factor 1 (ARF1) and Sar1, respectively. To address their individual roles in transport, we have developed a new assay using mammalian microsomes that reconstitute the formation of ER-derived vesicular carriers. Vesicles released from the ER were found to contain the cargo molecule vesicular stomatitis virus glycoprotein (VSV-G) and p58, an endogenous protein that continuously recycles between the ER and pre-Golgi intermediates. Cargo was efficiently sorted from resident ER proteins during vesicle formation in vitro. Export of VSV-G and p58 were found to be exclusively mediated by COPII. Subsequent movement of ER-derived carriers to the Golgi stack was blocked by a *trans*-dominant ARF1 mutant restricted to the GDP-bound state, which is known to prevent COPI recruitment. To establish the initial site of coatomer assembly after export from the ER, we immunoisolated the vesicular intermediates and tested their ability to recruit COPI. Vesicles bound coatomer in a physiological fashion requiring an ARF1–guanine nucleotide exchange activity. These results suggest that coat exchange is an early event preceding the targeting of ER-derived vesicles to pre-Golgi intermediates.

In eukaryotic cells, bidirectional vesicular traffic between the ER and the Golgi complex is mediated by two distinct vesicle coat complexes termed COPI and COPII, which promote selective transport (for reviews see Aridor and Balch, 1996; Schekman and Orci, 1996). COPII coats drive the export of the soluble secretory protein pre-pro α-factor from the ER in yeast and the cytosolic requirement for this reaction in vitro is satisfied by three soluble factors: the small GTPase Sar1p, Sec13p complex, and Sec23p complex (Barlowe et al., 1994). In mammalian cells, COPII is required for ER export of the type 1 transmembrane protein vesicular stomatitis virus glycoprotein (VSV-G)[1] (Kuge et al., 1994). COPI coats consist of a complex of seven polypeptides (α, β, β′, γ, δ, ϵ, and ζ) termed coatomer whose recruitment depends on the small GTPase ADP-ribosylation factor 1 (ARF1). Coatomer and ARF1 represent the minimum set of cytosolic proteins required to form COPI-coated vesicles in vitro (Orci et al., 1993*a*). GTPase activation is essential for the assembly of both COPI and COPII coats. The recruitment of coat components can be prevented by *trans*-dominant mutants that restrict the Sar1 and ARF1 GTPases to the inactive, GDP-bound forms (Tanigawa et al., 1993; Dascher and Balch, 1994; Elazar et al., 1994; Kuge et al., 1994; Aridor et al., 1995).

In mammalian cells, COPI is largely localized to the ER to Golgi transitional region, which includes pre-Golgi intermediates composed of vesicular tubular clusters (VTCs) (Oprins et al., 1993; Pind et al., 1994; Aridor et al., 1995; Griffiths et al., 1995). VTCs (also referred to as the ER-Golgi intermediate compartment, the 15°C compartment, or the salvage compartment) have been defined by resident marker proteins such as human p53 and its rat homologue, p58, and the KDEL receptor (for reviews see Pelham, 1991; Saraste and Kuismanen, 1992; Lippincott-Schwartz, 1993). These proteins continuously recycle between the ER, VTCs, and *cis*-Golgi elements (Pelham, 1988; Saraste and Svensson, 1991; Tang et al., 1993). While the exact site of COPI binding to the vesicular and tubular elements in VTCs is unknown because of the compact nature of these structures, it is now evident that VTCs represent the first site of recycling after export from the ER, implying that a

Address all correspondence to W.E. Balch, Departments of Cell and Molecular Biology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, Ca 92037. Tel.: (619) 784-2310. Fax: (619) 784-9126. E-mail: webalch@scripps.edu

Claude Nuoffer's current address is Biozentrum der Universität Basel, Abt. Pharmakologie, Kingelbersstrasse 70, CH-4056 Basel, Switzerland.

1. *Abbreviations used in this paper*: ARF1, ADP-ribosylation factor 1; BFA, brefeldin A; BiP, immunoglobulin binding protein; endo H, endoglycosidase H; HSP and MSP, high- and medium-speed pellet; GEF, guanine nucleotide exchange factor; MSS, medium speed supernatant; myr, myristoylated; NEM, *N*-ethylmaleimide; NRK, normal rat kidney; PIC, protease inhibitor cocktail; TEM, transmission electron microscopy; VSV-G, vesicular stomatitus virus glycoprotein; VTC, vesicular tubular cluster.

segregation event occurs early in the secretory pathway (Aridor et al., 1995; Tang et al., 1995).

While the role for COPII in selective transport from the ER is well established, the function of COPI is controversial. A number of lines of evidence suggest that COPI is required for anterograde traffic between the ER and Golgi and between Golgi compartments in vivo and in vitro (for review see Kreis et al., 1995). In contrast, it is becoming increasingly clear that COPI plays an essential role in retrograde transport (Pelham, 1994). Subunits of coatomer directly bind proteins bearing the di-lysine (KKXX) retrieval motif (Cosson and Letourner, 1994). Furthermore, yeast genetic studies have shown that mutations in γ-COP (Sec21p), β′-COP (Sec27p), α-COP (Ret1p), δ-COP (Ret2p), and ζ-COP (Ret3p) prevent retrieval of KKXX-tagged markers to the ER (Letourneur et al., 1995; Cosson et al., 1996). In mammalian cells, *trans*-dominant inhibitory mutants of ARF1, but not Sar1, block retrieval of a di-lysine–containing protein (p58) from VTCs to the ER (Aridor et al., 1995). Thus, COPI may either directly participate in both anterograde and retrograde transport or may be only indirectly involved in anterograde transport as a consequence of its key role in the segregation of cargo and transport components during recycling (Pelham, 1994). We have recently demonstrated that the sequential recruitment of COPII and COPI coats is coupled (Aridor et al., 1995), implying a close relationship between the function of the two coat complexes.

In this study, we have developed a new, general assay to measure ER export from microsomes prepared from mammalian cells. We use a combination of biochemical and morphological approaches to show that after COPII-dependent budding from the ER, COPI can be directly recruited to ER-derived vesicles. Our results suggest that ARF1 activation is necessary to initiate coat exchange, which may precede the fusion of ER-derived vesicles to pre-Golgi intermediates.

Materials and Methods

Materials

A polyclonal antibody to VSV-G was generated in rabbits immunized with the COOH-terminal 16 amino acids of VSV-G (Indiana serotype) coupled to KLH (Plutner et al., 1991). Anti-ARF1 polyclonal antibody was prepared as described previously (Aridor et al., 1995). Antibodies recognizing p58 and ribophorin II were supplied by J. Saraste (University of Oslo, Bergen, Norway) and D. Meyer (University of California, Los Angeles, CA), respectively. Anti β-COP (EAGE) and calnexin antibodies were obtained from M. Jackson (The Scripps Research Institute, La Jolla, CA). Recombinant Sar1a and ARF1 proteins were generated in *Escherichia coli* as described previously (Weiss et al., 1989; Rowe and Balch, 1995). Rat liver cytosol containing a protease inhibitor cocktail (PIC) was prepared as described (Davidson and Balch, 1993). Coatomer was purified from bovine liver down to the Mono Q pool as described (Waters et al., 1992).

Preparation of Microsomes

Normal rat kidney (NRK) cells were grown on 150-mm tissue culture dishes and at confluency infected with ts045 VSV for 4 h at the restrictive temperature (39.5°C) as described (Davidson and Balch, 1993). The cells were cooled rapidly (<5 s) by washing with ice-cold PBS and then were scraped from the culture dishes into 0.375 M sorbitol, 20 mM Hepes, pH 7.2, and collected by centrifugation (500 *g* for 3 min). After resuspension in 3–4 vol of the above sorbitol buffer (+ PIC) the cells were passed three times through a ball homogenizer (Balch and Rothman, 1985). The homogenate was diluted with an equal volume of the same buffer and then centrifuged at 720 *g* for 5 min. The postnuclear supernatant was collected and then adjusted to 0.25 M sorbitol, 70 mM KOAc, 1 mM $Mg(OAc)_2$, 20 mM Hepes, pH 7.2 (transport buffer), and centrifuged at 12,000 *g* for 2 min. The microsome pellet was washed once with transport buffer (+ PIC) and subsequently resuspended by repeated trituration in the same buffer at a protein concentration of 3–4 mg/ml. The membranes were divided into 50- or 100-μl aliquots, frozen in liquid N_2, and stored at −80°C.

Preparation of ARF1 and COPI-depleted Cytosol and Membrane Fractions

Coatomer-depleted cytosol was prepared by incubating 360 μl of 25 mg/ml rat liver cytosol with 0.5 mM ATP and 100 μg of anti-β-COP polyclonal IgG (EAGE) for 2 h on ice, followed by incubation at 4°C for a further 30 min in the presence of protein A–Sepharose beads. The mixture was centrifuged at 16,000 *g* for 10 min and the supernatant collected and frozen in 50-μl aliquots at −80°C. Cytosolic coatomer was depleted by roughly 75% by this procedure. Further attempts to reduce coatomer resulted in nonspecific inactivation of cytosol in mock controls lacking antibody.

To prepare ARF1-depleted rat liver cytosol, the tissue was homogenized in 3 vol (wt/wt) 0.25 M KCl, 10 mM Tris-HCl, pH 7.4, 1 mM MgATP, 2 mM DTT, and PIC. ARF1-depleted fractions were generated by chromatography on an anion-exchange column (Mono Q) and a gel-filtration column (Superose 6) as described (Taylor et al., 1994). The elution of ARF1 was followed by Western blotting of the column fractions using ARF1 polyclonal antiserum. The ARF1-depleted Superose 6 and Mono Q pools were concentrated by ultrafiltration on membranes (PM10; Amicon, Beverly, MA) to volumes of 300 and 150 μl, respectively. The pools were then dialyzed for 2 h against 125 mM KOAc, 25 mM, Hepes pH 7.2, centrifuged at 16,000 *g* for 10 min, and the supernatants combined to produce the ARF1-depleted cytosol. Under these conditions, we were able to generate cytosol that was depleted of ~90% of detectable ARF1 on Western blotting. To generate ER-derived vesicles in the presence of the ARF1-depleted cytosol, it was necessary to supplement the assay with wild-type Sar1 or Sar1[H79G] because of simultaneous depletion of endogenous Sar1.

Coatomer- and ARF1-depleted donor membranes were prepared by washing microsomes with 0.25 M KCl as follows. Thawed membranes (50 μl) were diluted into 0.2 ml of 312.5 mM KCl, incubated for 10 min on ice and then centrifuged at 16,000 *g* for 2 min. The membrane pellets were resuspended in 0.23 ml of 0.25 M sorbitol, 20 mM Hepes, pH 7.2 and then supplemented with KOAc and $Mg(OAc)_2$ to 70 and 1 mM, respectively (final volume of 0.25 ml), and repelleted. The membranes were resuspended as above in a final volume of 50 μl and used immediately for transport reactions. A coatomer-depleted, Golgi-enriched membrane (acceptor) fraction was prepared from CHO cells by flotation on a sucrose density gradient as described previously (Balch et al., 1994). Membranes at the 29–35% (wt/wt) sucrose interface were collected and diluted with 4 vol of 87.5 mM KOAc, 1.25 mM $Mg(OAc)_2$, 25 mM Hepes, pH 7.2, and centrifuged at 16,000 *g* for 10 min. The pellets were washed with 0.5 ml transport buffer and recentrifuged as above. The Golgi membranes were resuspended in transport buffer and stored in 50-μl aliquots at −80°C. In each case, depletion of coatomer and ARF1 was ~90% based on Western blotting.

In Vitro Vesicle Formation Assay

Thawed microsomes (10 μl) were incubated at 32°C in a 40-μl cocktail containing 200–250 μg of rat liver cytosol and 0.25 M sorbitol, 70 mM KOAc, 36 mM Hepes, pH 7.2, 5 mM EGTA, 1.8 mM $Ca(OAc)_2$, 2.5 mM $Mg(OAc)_2$, and an ATP-regenerating system (Davidson and Balch, 1993). Reactions were terminated by transfer to ice and the membranes collected by centrifugation at 20,000 *g* for 10 min. Membranes were resuspended by trituration (20×) in 50 μl of 0.25 M sorbitol, 20 mM Hepes, pH 7.2, and then supplemented with KOAc and $Mg(OAc)_2$ to final concentrations of 150 and 2.5 mM, respectively (final volume of 60.6 μl). Immediately after addition of the salts, differential centrifugation was performed at medium speed (16,000 *g*) for 2 min. The top 42-μl supernatant fraction (designated medium-speed supernatant [MSS]) was harvested and centrifuged at high speed (100,000 *g*) for 20 min. Membrane pellets from the medium-speed (MSP) and high-speed (HSP) spins were solubilized in Laemmli sample buffer at 95°C for 5 min, and proteins were separated by 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immu-

noblotted using the appropriate primary antibody followed by horseradish peroxidase–conjugated secondary antibody (Pierce, Rockford, IL). Blots were developed using the ECL kit (Amersham Corp., Arlington Heights, IL) and quantitated using a molecular imaging system (model GS-363; BioRad Labs, Hercules, CA).

ER to Golgi Transport Assay

Transport assays were performed as described above with the exception that 5 μl of microsomes and 150–200 μg of rat liver cytosol were used and the 40-μl reaction cocktail was supplemented with 0.25 mM UDP-*N*-acetylglucosamine. After incubation, membranes were sedimented at 20,000 *g* for 10 min and endoglycosidase H (endo H) digested (Davidson and Balch, 1993). Endo H–sensitive and –resistant forms of VSV-G were separated by SDS-PAGE and quantitated by immunoblotting as described above.

Two-Stage Fusion Assay

During stage 1, scaled-up (100 μl) vesicle formation reactions were performed as described above with the exception that after resuspension in 0.25 M sorbitol, 20 mM Hepes, pH 7.2, the membranes were supplemented with KOAc to a final concentration of 0.25 M before differential centrifugation. HSPs were resuspended in 0.25 M sorbitol, 20 mM Hepes, pH 7.2, and then supplemented with 70 mM KOAc (final concentration). During stage 2, the resuspended vesicle fractions (HSPs) were added to transport assay cocktails (in which ts045 VSV–infected microsomes were omitted; see above) containing 4 μl of noninfected microsomes or enriched Golgi membranes added as source of acceptor membranes. After incubation at 32°C, membranes were transferred to ice and then harvested by centrifugation at 20,000 *g* for 10 min. Delivery of VSV-G to Golgi membranes was determined after endo H digestion of the membranes as described above.

Immunoisolation of Vesicles

Dynabeads M-500 (DYNAL Inc., Great Neck, NY) magnetic beads were covalently coupled with goat anti–mouse IgG (Fc specific) (Sigma Chemical Co., St. Louis MO) secondary antibody at a density of ~10 μg antibody per 10^7 beads according to the manufacturer's instructions. The beads were coated with an mAb (p5D4) recognizing the cytoplasmic tail of VSV-G at a twofold molar excess over secondary antibody and then washed 4× with PBS + 1 mg/ml BSA. The p5D4-coated beads (1-mg beads) were added to MSS fractions (0.15–0.35 ml) and incubated in the presence of 0.25 M sorbitol, 0.15 M KOAc, 5% FBS, 2 mM EDTA, 20 mM Hepes, pH 7.2 (final volume of 0.4 ml), for 2–4 h at 4°C with continuous rotation. The immunoisolated vesicles were washed 5× with 0.25 M sorbitol, 0.15 M KOAc, 1 mg/ml BSA, 20 mM Hepes, pH 7.2, using a magnet and transferred to fresh tubes during the first and last washes. The beads were finally resuspended in 50 μl of 2× Laemmli sample buffer for analysis by SDS-PAGE and Western blotting or prepared for electron microscopy as described previously (Lucian and Palade, 1994).

COPI-binding Experiments

Immunoisolated vesicles were generated as described above with the exception that in the vesicle binding and washing steps, the KOAc concentration was increased from 0.15 to 0.25 M. The immunoisolated vesicles were incubated for 10 min at 32°C in the presence of a 0.1-ml cocktail containing 0.25 M sorbitol, 0.15 M KOAc, 2.5 mM $Mg(OAc)_2$, 20 mM Hepes, pH 7.2, and supplemented with either rat liver cytosol and 25 μM GTPγS, or purified ARF1, coatomer, and guanine nucleotides (as indicated in the figure legends). After incubation, the beads were washed an additional five times with 0.25 M sorbitol, 0.25 M KOAc, 1 mg/ml BSA, 20 mM Hepes, pH 7.2. (Fresh tubes were used during the first and last washes.) The beads were finally resuspended in 30 μl of Laemmli sample buffer and bound ARF1 and coatomer were determined by quantitative immunoblotting.

Results

Mammalian Microsomes Reconstitute ER to Golgi Transport

To examine the potential role(s) of COPI and COPII coat complexes in the formation of ER-derived vesicular carriers in mammalian cells, we have developed a new vesicle formation assay using microsomes prepared from NRK cells infected with VSV (Lafay, 1974). VSV strain tsO45 expresses a transmembrane surface glycoprotein, VSV-G, which has a thermoreversible folding defect preventing its exit from the ER at the restrictive temperature (39.5°C) (Lafay, 1974; Plutner et al., 1992). Transfer of cells to the permissive temperature (32°C) results in the synchronous folding, oligomerization, and transport of VSV-G to the Golgi complex in vivo (Doms et al., 1987) and in semiintact cells (Plutner et al., 1992; Balch et al., 1994). To follow export of VSV-G from the ER, a washed postnuclear microsome fraction is incubated at 32°C in the presence of cytosol and ATP. The fraction of VSV-G exported is measured using differential centrifugation to separate the more rapidly sedimenting ER and Golgi compartments that are recovered in a medium speed pellet (MSP) (16,000 *g*) from the slowly sedimenting ER-derived vesicular intermediates, which are released into the medium speed supernatant (MSS). Vesicular intermediates present in the MSS are recovered in a high speed pellet (HSP) (100,000 *g*) and the amount of cargo (VSV-G) quantitated by SDS-PAGE and immunoblotting. Transport to Golgi compartments is measured by the extent of processing of VSV-G from endo H–sensitive ER forms to the endo H–resistant form associated with delivery to *cis*- and *medial*-Golgi membranes (Schwaninger et al., 1991; Davidson and Balch, 1993).

Before incubation, VSV-G was confined to the MSP, whereas at the first time point (5 min) it could already be detected in the HSP (Fig. 1 *A*). Recovery in the HSP was maximal (17% of total VSV-G) after 20 min of incubation and then progressively decreased to less than 5% after 75 min. At the 20-min time point, endo H–resistant VSV-G was first detected in the MSP (~3% of total VSV-G). Further incubation for 75 min led to the recovery of ~15% endo H–resistant VSV-G in the MSP. Typically, 15–25% of the total VSV-G was converted to the endo H–resistant form after 75 min of incubation using different preparations of membranes and cytosol. Importantly, we were unable to detect endo H–resistant VSV-G in the HSP throughout the time course. Since resistance to endo H is a consequence of oligosaccharide processing by resident Golgi mannosidases and glycosyl transferases, it is evident that the HSP is largely free of Golgi membranes and Golgi-derived vesicles carrying VSV-G, even at later time points. The disappearance of VSV-G from the HSP reflects the fusion of ER-derived vesicular intermediates with rapidly sedimenting Golgi membranes. The appearance of VSV-G in the HSP was cytosol-dependent and inhibited by the ATP/GTP scavenger apyrase (data not shown).

To exclude the trivial possibility that the appearance of endo H–sensitive VSV-G in the HSP was due to nonspecific fragmentation of ER membranes, we compared the distribution of VSV-G with: (*a*) p58, (*b*) the resident ER protein ribophorin II, and (*c*) the molecular chaperones immunoglobulin binding protein (BiP) and calnexin over a 60-min time course at 32°C (Fig. 1 *B*). Like VSV-G, p58, ribophorin II, and the chaperones were confined to the MSP before incubation (Fig. 1 *B*, *0 min*). VSV-G recov-

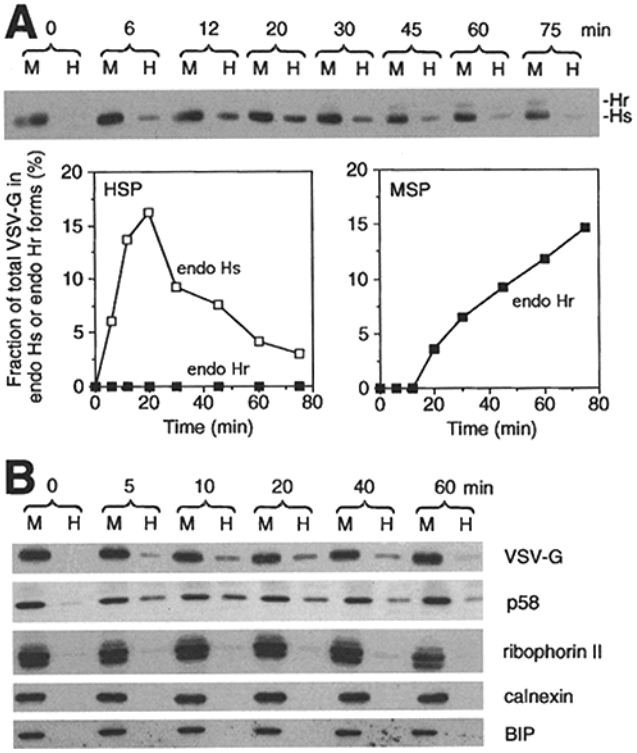


Figure 1. VSV-G is released into an HSP vesicle fraction upon incubation in vitro. Microsomes were prepared from NRK cells infected with ts045 VSV at the restrictive temperature (39.5°C) and then incubated at the permissive temperature (32°C) in the presence of unfractionated cytosol and an ATP-regenerating system as described under Materials and Methods. At the indicated times, reactions were terminated by transfer to ice and MSP (*M*) (16,000 *g*) and HSP (*H*) (100,000 *g*) fractions were prepared by differential centrifugation (see Materials and Methods). (*A*) The MSP and HSP fractions (33 and 70% of the total SDS-solubilized membranes, respectively) were digested with endo H (Davidson and Balch, 1993) and the proportions of VSV-G present in the endo H sensitive (*Hs, lower band*) and resistant (*Hr, upper band*) forms determined by quantitative immunoblotting. The immunoblot is shown in the upper panel and the results quantitated below. (*B*) The MSP and HSP fractions were solubilized in gel sample buffer (without endo H digestion) and equal amounts of each fraction analyzed by quantitative immunoblotting using primary antibodies to VSV-G, p58, ribophorin II, calnexin, and BiP as indicated.

ered in the HSP increased to ~19% after 20 min and then decreased over longer time periods. Incubation at 32°C also led to release of p58 into the HSP that peaked at ~20 min, representing 30–40% of the total pool present in the MSP before incubation. Thus, it is apparent that p58 is highly enriched in slowly sedimenting, vesicular intermediates. This released pool was redirected to the MSP at later time points, probably as a consequence of targeting to rapidly sedimenting Golgi membranes or, in the case of p58, recycling back to ER membranes. In contrast, the trace amount of ribophorin II detected in the HSP before incubation did not change significantly during incubation at 32°C (Fig. 1 *B*), indicating that the appearance of VSV-G and p58 in the HSP was not due to disruption of rough ER membranes. BiP and calnexin are chaperones that have been shown to associate transiently with a number of incompletely folded glycoproteins, including VSV-G (Ou et al., 1993; Hammond and Helenius, 1994*a*). We were unable to detect either of these chaperones in the HSP either before or after incubation at 32°C (Fig. 1 *B*), indicating that they are very efficiently sorted from VSV-G before export from the ER. Based on Western blotting, the ratio of VSV-G to calnexin in the MSP before incubation versus that found in vesicles in the HSP at the 10-min time point revealed at least a 100-fold change. Thus, vesicle budding from ER microsomes has all the hallmarks of the sorting and concentration events observed previously in semiintact cells (Balch et al., 1994).

Mammalian ER-derived Vesicular Intermediates Are Fusion Competent

The efficiency of targeting and fusion of intermediates formed during the ER budding reaction was assessed using a two-stage transport reaction (Fig. 2). Vesicles released into the HSP after incubation for 10 min at 32°C (stage 1) were incubated in a second stage in the presence of cytosol, ATP, and microsomes prepared from noninfected NRK cells as a source of Golgi membranes. As shown in Fig. 2 *A*, vesicle fusion initiated without a lag and was typically 40–45% efficient after 45–90 min of incubation at 32°C as judged by the rapid appearance of endo H–resistant VSV-G. The lack of a lag period in the second stage incubation suggests that vesicular intermediates formed in the first stage are competent to fuse with compartments of the Golgi stack without further rounds of vesicle budding. The fusion reaction was dependent on the addition of acceptor membranes and rat liver cytosol (Fig. 2 *B*). Delivery of VSV-G to Golgi membranes was completely inhibited by apyrase, and partially sensitive (~50%) to GTPγS or *N*-ethylmaleimide (NEM) (Fig. 2 *B*). Pretreatment of NEM with a twofold molar excess of glutathione before incubation at 32°C neutralized inhibition (data not shown). In addition, we have previously demonstrated that ER to Golgi transport in semiintact cells requires the small GTPase Rab1 and is potently inhibited by a *trans*-dominant mutant (Rab1[N124I]) that restricts Rab1 to the nucleotide-free form (Pind et al., 1994). As expected, we found that incubation of microsomes in the presence of the recombinant Rab1[N124I] mutant inhibited (~70%) stage 2 fusion of vesicles to the Golgi (Fig. 2 *B*). These results reflect the requirement for downstream components in targeting/fusion that are likely to include Rab GTPases and components involved in the assembly of soluble NSF attachment receptor (SNARE) complexes (Rothman, 1994).

Trans-dominant Mutants of ARF1 and Sar1 Block ER to Golgi Transport

To assess the potential requirement(s) for COPII and COPI coats in transport, we analyzed the effects of mutants of the small GTPases Sar1a and ARF1 on ER to Golgi traffic. These mutants have been extensively characterized. The GDP-restricted forms of both Sar1 (Sar1[T39N]) and myristoylated $ARF1_{myr}$ ($ARF1_{myr}$[T31N]) prevent coat assembly by interfering in a *trans*-dominant fashion with the function of Sar1- or ARF1-specific guanine nucleotide exchange factors (GEFs) in the recruitment and acti-

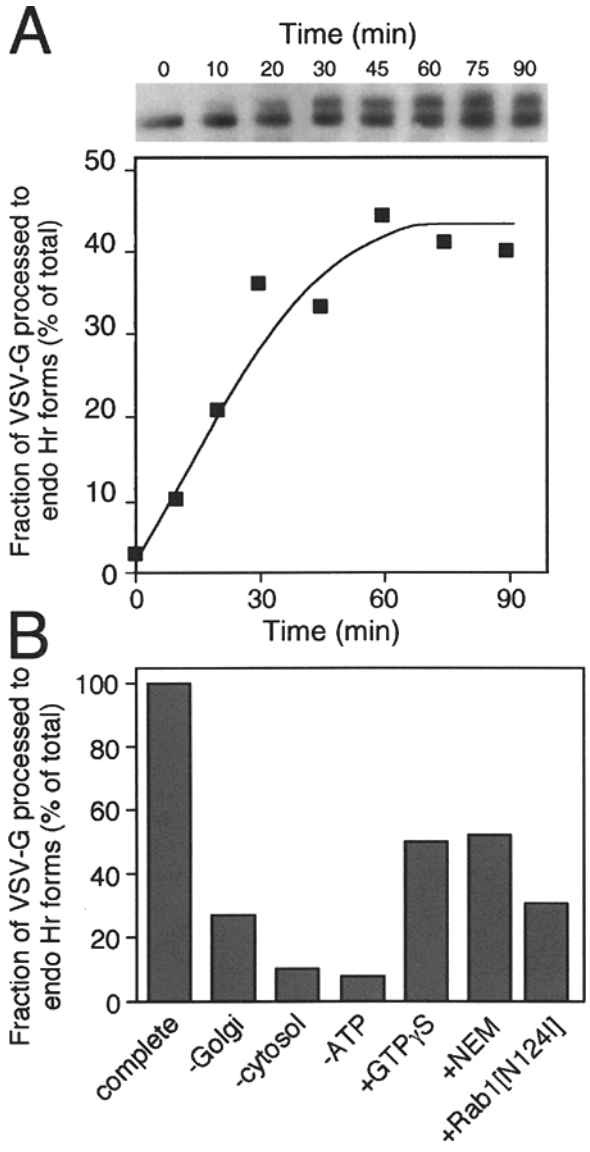


Figure 2. Mammalian ER-derived vesicles are functional intermediates of ER to Golgi transport. Two-stage fusion reactions were performed to assess the fusion competence of the carrier vesicles released into the MSS. In stage 1, a MSS fraction was prepared after incubation of microsomes in the presence of cytosol and ATP for 10 min at 32°C. The vesicles were sedimented at 100,000 *g*, and the HSP was subsequently incubated during stage 2 for 0–90 min at 32°C in the presence of cytosol, ATP, and microsomes prepared from noninfected NRK cells as a source of acceptor (Golgi) membranes. (*A*) Time course of the stage 2 reaction. Vesicle fusion was assessed by following the conversion of VSV-G from endo H–sensitive (*lower band*) to resistant (*upper band*) forms by immunoblotting of the endo H–digested membranes. The quantitation of the immunoblot is shown in the lower panel. (*B*) Biochemical properties of the stage 2 reaction. Vesicle fusion was assessed during a 60-min incubation either in the absence of acceptor membranes, cytosol, or ATP (plus 1 U of apyrase), or in complete reaction cocktails supplemented with GTPγS (25 μM) or 0.75 mM NEM, or 0.6 μg of the Rab1a[N124I] mutant. NEM-treated samples were pretreated for 10 min at 0°C with the reagent and then neutralized before incubation at 32°C with 1.5 mM glutathione. Fusion efficiency (%) is expressed relative to controls containing complete reaction cocktails.

vation of wild-type Sar1 and ARF1, respectively (Dascher and Balch, 1994; Elazar et al., 1994; Kuge et al., 1994; Aridor et al., 1995). The phenotype of $ARF1_{myr}$[T31N] is, in part, similar to that of brefeldin A (BFA) which is also believed to interfere with ARF1-GEF function (Donaldson et al., 1992*b*; Helms and Rothman, 1992). In contrast, the GTP-restricted forms (Sar1[H79G] and $ARF1_{myr}$[Q71L]) lead to efficient coat recruitment, but prevent coat disassembly by inhibiting GTP hydrolysis by their respective GTPase-activating proteins (Tanigawa et al., 1993; Dascher and Balch, 1994; Oka and Nakano, 1994; Aridor et al., 1995).

Addition of both Sar1 GDP- and GTP-restricted mutants completely blocked ER to Golgi transport as measured by the appearance of endo H–resistant VSV-G (Fig. 3, *b* and *c*). Addition of an equivalent concentration of the wild-type protein did not significantly affect the reaction (Fig. 3 *a*). While the wild-type $ARF1_{myr}$ has no inhibitory effect on ER to Golgi transport in vivo or in vitro (Dascher and Balch, 1994), transport to Golgi membranes was completely inhibited by both GDP- and GTP-restricted

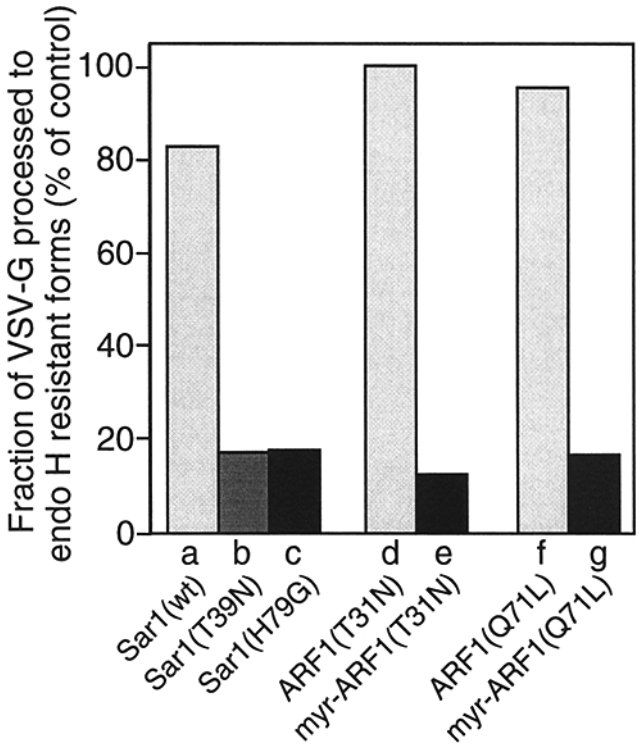


Figure 3. Mutants of Sar1a and ARF1 restricted to the GDP- or GTP-bound forms are *trans*-dominant inhibitors of the microsome-based ER to Golgi transport reaction. Recombinant proteins were added to ER to Golgi transport reactions at final concentrations of 1 μM for wild-type Sar1a, Sar1[T39N] (GDP-restricted mutant), or Sar1[H79G] (GTP-restricted mutant) (*a–c*), and 2 μM for the myr (active) (*e* and *g*) or nonmyr (inactive) (*d* and *f*) forms of ARF1[T31N] (GDP-restricted mutant) (*d* and *e*) and ARF1[Q71L] (GTP-restricted mutant) (*f* and *g*). Transport reactions were incubated for 90 min at 32°C and the fraction of VSV-G processed to endo H–resistant forms determined as described under Materials and Methods. Representative data from four independent experiments are shown in which transport efficiencies were 16–25%.

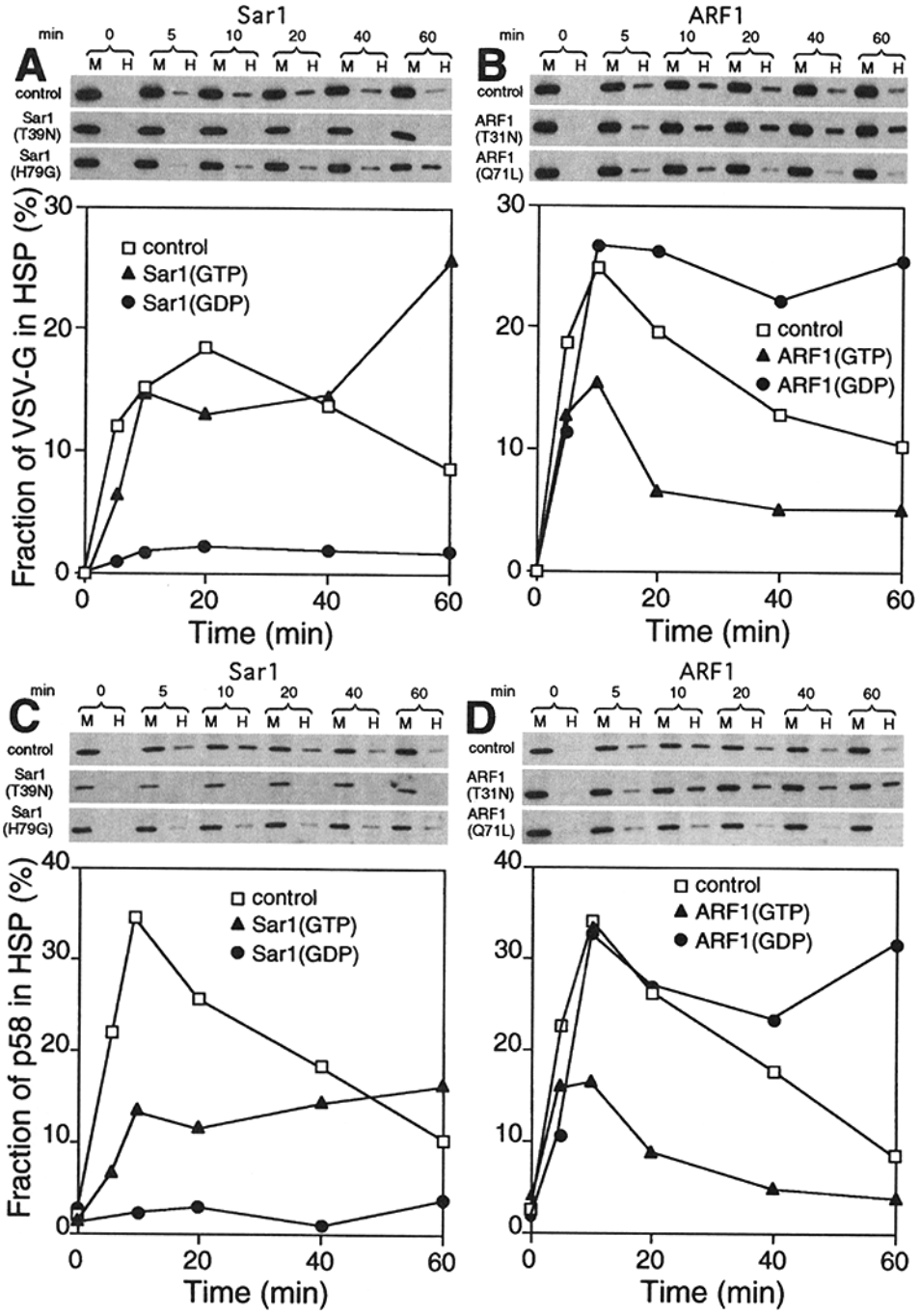


Figure 4. Effects of the GDP- and GTP-restricted mutants of Sar1a and ARF1 on vesicle formation. Vesicle formation reactions were performed in the absence (control) or presence of the Sar1 or ARF1 mutants as indicated. Recombinant mutant proteins were added at the final concentrations described in Fig. 3 legend. Incubations were performed for the indicated times and the release of ER-derived vesicles containing VSV-G (*A* and *B*) or p58 (*C* and *D*) from the MSP (*M*) into the HSP (*H*) was determined as described under Materials and Methods. These experiments measure the release of endo H–sensitive VSV-G into the HSP (vesicle fraction) as shown in Fig. 1, and therefore endo H digests were not performed. The results shown are representative of three independent experiments.

forms of ARF1 (Fig. 3, *e* and *g*). Inhibition was dependent on myristoylation (Fig. 3, *d* and *f*), a posttranslational modification essential for ARF1 function (Balch et al., 1992; Kahn et al., 1992). The complete inhibition of VSV-G processing to endo H–resistant forms observed in the presence of $ARF1_{myr}$[T31N] in vitro is consistent with previous in vivo studies (Dascher and Balch, 1994) and indicates that this mutant was not promoting the microtubule-dependent fusion of Golgi to ER membranes as observed with BFA in vivo (Lippincott-Schwartz et al., 1989).

COPI Is Not Required for Budding

Having established that the Sar1 and ARF1 mutants inhibit ER to Golgi transport using the microsome-based assay, we analyzed their potential effects on ER budding (Fig. 4). Addition of the Sar1 GDP–restricted mutant (Sar1[T39N]) strongly inhibited vesicle release into the HSP (Fig. 4 *A*, *closed circles*), demonstrating that budding is blocked in mammalian cells in the absence of COPII coat assembly. This result is consistent with previous indirect immunofluorescence experiments in semiintact mammalian cells, which suggested that VSV-G was retained in an ER distribution in the presence of the mutant protein (Kuge et al., 1994). In contrast, the Sar1 GTP–restricted mutant (Sar1[H79G]), while slightly reducing the efficiency of vesicle release at early time points, led to a pronounced accumulation of ER-derived vesicular intermedi-

ates in the HSP after 60 min of incubation (Fig. 4 *A*, *closed triangles*). The extensive accumulation of vesicles in the presence of the Sar1 GTP–restricted mutant was identical to the effects of the Rab1a[N124I] mutant, which specifically prevents a step in vesicle docking and/or fusion (Pind et al., 1994) (data not shown).

The effects of the ARF1 mutants on the ER budding reaction were very different. Strikingly, neither mutant prevented the formation of VSV-G containing vesicular intermediates (Fig. 4 *B*, *closed circles* and *triangles*). These results demonstrate that VSV-G is exported from the ER in a COPI-independent fashion and are consistent with the observation in semiintact cells that Sar1 function is epistatic to ARF1 (Aridor et al., 1995). Moreover, the ARF1 GDP–restricted mutant ($ARF1_{myr}$[T31N]) caused VSV-G to accumulate in the HSP (Fig. 4 *B*, *closed circles*), indicating that the ER to Golgi transport block exerted by the ARF1 GDP–restricted mutant (Fig. 3 *e*) was due to inhibition of vesicle consumption. This result suggests that the recruitment and activation of wild-type ARF1 is critical for an event downstream of budding from the ER, which in in vivo (Dascher et al., 1994) or semiintact cells (Aridor et al., 1995) corresponds to the delivery of cargo to VTCs.

The ARF1 GTP–restricted mutant ($ARF1_{myr}$[Q71L]) did not interfere with the initial rate of vesicle budding but led to a reduced level of VSV-G release and a loss of VSV-G from the HSP (Fig. 4 *B*, *closed triangles*) at later time points. The reduced levels of VSV-G in the HSP at 10–20 min could result from a partial inhibitory effect on vesicle formation by the mutant, although this would clearly be inconsistent with the effect of the ARF1 GDP–bound mutant that did not block vesicle budding, but potently inhibited consumption (Fig. 4 *B*). A more likely explanation is that the stable recruitment of COPI promoted by the activated GTPase (Aridor et al., 1995) leads to the formation of a population of vesicle clusters or aggregates that sediment more rapidly than free vesicles during differential centrifugation. This is consistent with morphological studies in living cells (Dascher and Balch, 1994) and in semiintact cells (Aridor et al., 1995), where the ARF1 GTP–restricted mutant causes VSV-G to accumulate in compact pre-Golgi VTCs that show exaggerated staining for β-COP. Thus, it is apparent that after the COPII-mediated budding step, the orderly progression of VSV-G from the ER via vesicular carriers to the Golgi stack may require guanine nucleotide exchange and GTP hydrolysis by ARF1.

p58 Is Exported in a Sar1-dependent Fashion

To establish whether the sequential requirement for COPII and COPI coats was a general attribute of membrane flow through the early secretory pathway, we tested the effects of the Sar1 and ARF1 mutants on the export of p58 (Fig. 4, *C* and *D*). The appearance of this endogenous recycling protein in the HSP was potently inhibited by the Sar1 GDP–restricted mutant (Fig. 4 *C*, *closed circles*), while the Sar1 GTP–restricted mutant led to the accumulation of p58 in the HSP (Fig. 4 *C*, *closed triangles*). The result obtained with the Sar1 GDP–restricted mutant implies that the appearance of p58 in the HSP is due to budding from ER microsomes rather than from recycling from VTCs or Golgi membranes. The effects of the ARF1 mutants on p58 export were also identical to those observed for VSV-G. While the GDP-restricted form led to the accumulation of p58-containing carriers (Fig. 4 *D*, *closed circles*), the GTP-restricted mutant led to a loss of p58 from the HSP at later time points (Fig. 4 *D*, *closed triangles*). Thus, the combined action of COPII and COPI coats may be essential for mobilization of both cargo and recycling components from the ER through pre-Golgi VTCs.

Immunoisolation of ER-derived Vesicular Intermediates

To characterize the vesicular intermediates that appear in the HSP upon incubation of microsomes in vitro, we immunoisolated the VSV-G–containing carriers by incubating MSS fractions with magnetic beads coated with an mAb (p5D4) specific for the cytoplasmic tail of VSV-G (Kreis, 1986). Vesicles recovered on the beads were washed extensively, solubilized in gel sample buffer, and analyzed for their content of VSV-G and p58 by immunoblotting (Fig. 5). We found that p58 copurified with VSV-G on p5D4-coated beads (Fig. 5, lane *b*), indicating that it is a component of VSV-G–containing vesicles. Roughly 30% of the total VSV-G or p58 present in the starting fraction (MSS) was recovered on the beads. Neither marker was recovered in the absence of p5D4 (Fig. 5, lane *a*), attesting to the specificity of the coimmunoisolation. Upon solubilization of the immunoisolated vesicles in the presence of Triton X-100 (Fig. 5, lane *c*), the stoichiometry of VSV-G binding to beads was markedly reduced, demonstrating that ER-derived vesicular carriers contain multiple copies of VSV-G. In contrast, in the presence of detergent, p58 could no longer be detected (Fig. 5, lane *c*), suggesting that the protein is not stably associated with VSV-G in the ves-

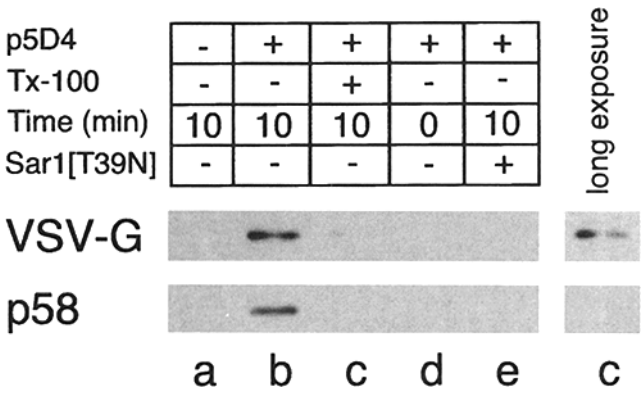


Figure 5. Immunoisolation of ER-derived vesicles. Microsomes were incubated in the presence of cytosol and ATP on ice (lane *d*) or for 10 min at 32°C (lanes *a*, *b*, *c*, and *e*). In lane *e*, the reaction was supplemented with 1 μM Sar1[T39N]. The resulting MSS fractions were incubated with magnetic beads either not coated (lane *a*) or coated (lanes *b–e*) with p5D4, a mAb specific for the cytoplasmic tail domain of VSV-G, as described under Materials and Methods. The immunoisolated vesicles were washed, solubilized in Laemmli sample buffer, and analyzed for their content of VSV-G and p58 by Western blotting. In lane *c*, the immunoisolated vesicles were solubilized with 1% Triton X-100 (*Tx-100*) before washing.

icles. When vesicle budding was prevented by incubation of microsomes on ice (Fig. 5, lane *d*), or at 32°C in the presence of Sar1[T39N] (Fig. 5, lane *e*), neither VSV-G nor p58 were recovered from the MSS, indicating that immunoisolation is dependent on vesicle formation.

Vesicles accumulated in the absence or presence of the Sar1[H79G] mutant were bound to magnetic beads and examined using transmission electron microscopy (TEM) (Fig. 6, Table I). ER-derived buds and vesicles observed in vivo are typically 60–70 nm in diameter. As shown in Fig. 6 *A*, the immunoisolated membranes clearly contained a population of vesicles of 50–70 nm in diameter (*arrowheads*), although some larger structures were occasionally observed (*asterisks*). After export from the ER in vivo, VSV-G becomes associated with VTCs that contain numerous vesicles and small tubular elements (Saraste and Svensson, 1991; Balch et al., 1994). The tubular components of VTCs may form as a consequence of homotypic fusion of ER-derived vesicles (Balch et al., 1994), although it cannot be excluded that they represent a discrete compartment, thus requiring a heterotypic fusion event. Given the possibility that ER-derived COPII carriers may undergo homotypic fusion or fuse with elements of VTCs that may be present in the microsome preparation, it was important to characterize the size distribution of the immunoisolated VSV-G–containing vesicular intermediates. Fusion of 2, 3, or 4 vesicles of 60-nm diameter would be expected to give rise to structures with diameters of ~85, 100, and 110 nm, respectively. Analysis of immunoisolated vesicles generated in the absence of Sar1[H79G] revealed that 67% were <80 nm in diameter, 11% were between 80 and 100 nm, and 22% were >100 nm in diameter (Table I). Those of >100 nm diameter typically did not contain VSV-G (see below). Excluding these large, contaminating membranes, we conclude that 14% of the total vesicles bound to beads may have undergone fusion to form elements that fall in the 80–100 nm size class (Table I). Vesicles immunoisolated from incubations containing the Sar1 GTP–restricted mutant showed a marked reduction in the percentage of structures in the 80–100 nm class (4%) (Table I), suggesting that the predominant population of structures immunoabsorbed to beads under these conditions are ER-derived carrier vesicles.

To assess the purity of the immunoisolated fractions, beads were immunolabeled with a polyclonal antibody specific for the cytoplasmic tail of VSV-G. A large proportion (>90%) of the structures of <100 nm in diameter showed immunogold labeling for VSV-G (Fig. 6 *B*). The larger membranes (>100 nm in diameter) that were occasionally observed (Fig. 6 *A*, *asterisks*) consistently lacked detectable VSV-G, indicating that they are nonspecific contaminants of the immunoisolation procedure. Therefore, it is evident that for the first time we can successfully isolate a population of mammalian ER-derived vesicles involved in selective transport from the ER.

Consumption of ER-derived Intermediates Is Prevented by the ARF1 GDP–restricted Mutant

To examine the specific role of COPI coats in ER to Golgi transport, we performed two stage reactions. During stage 1, vesicles were generated in the absence or in the presence of Sar1[H79G], $ARF1_{myr}$[T31N], or wild-type $ARF1_{myr}$. The fusion competence of the respective washed HSP fractions was monitored during stage 2 by following the appearance of endo H resistant VSV-G upon incubation in the presence of cytosol, ATP, and Golgi membranes prepared from noninfected cells (Fig. 7 *A*). Vesicular intermediates accumulated in the presence of the Sar1 GTP–restricted mutant delivered VSV-G to Golgi membranes with an efficiency comparable to those generated under wild-type conditions (Fig. 7 *A*, compare *a* with *b*). This result is likely to be due to the loss of the unstable Sar1 coat during washing of the HSP fraction after the stage 1 incubation. A similar result has been observed in yeast where the COPII coat is lost from ER-derived vesicles after washing in sorbitol buffers (Oka and Nakano, 1994).

In contrast to vesicles generated under wild-type conditions or in the presence of Sar1[H79G], those accumulated in the presence of the ARF1 GDP–restricted mutant delivered VSV-G to Golgi membranes in the second stage with very low efficiency (~20% of that of control incubations) (Fig 7 *A*, *c*). Vesicles generated in the presence of wild-type $ARF1_{myr}$ delivered VSV-G to Golgi membranes with an efficiency of ~80% of those generated in the absence of recombinant protein (Fig. 7 *A*, compare *a* to *d*), demonstrating that the effect of the mutant ARF1 on transport was a consequence of the T31N mutation. The effect of the ARF1 GDP–restricted mutant is consistent with the results observed in sedimentation assays (Fig. 4 *B* and *D*), where it promoted vesicle accumulation in the HSP. Moreover, these results demonstrate that inhibition of anterograde traffic by $ARF1_{myr}$[T31N] observed in the single stage transport reaction (Fig. 3) is an effect on ER-derived intermediates rather than an effect on the acceptor Golgi fraction.

To determine whether ER-derived intermediates formed in the absence of recombinant protein or in the presence of Sar1[H79G] were past the step sensitive to the recruitment of COPII coats, the second stage reaction was supplemented with Sar1[T39N] at a concentration that com-

Figure 6. Morphology of immunoisolated ER-derived transport vesicles. (*A*) Vesicle formation reactions were performed in the presence of the GTP-restricted Sar1 mutant (Sar1[H79G]) as described under Materials and Methods. The accumulated vesicles were immobilized on magnetic beads and processed for electron microscopy as described (see Materials and Methods). Membranes bound to beads consisted largely of a homogeneous population of 50–70 nm vesicles (*arrowheads*). Occasionally larger structures (>100 nm) were observed (*asterisks*). (*B*) Immunogold labeling for VSV-G. The beads were incubated in the presence of polyclonal antibodies against VSV-G followed by 5-nm gold-conjugated anti–rabbit IgG secondary antibodies before sectioning in epon resin as described (Lucian and Palade, 1994). The gallery illustrates that >90% of the vesicles labeled positively for VSV-G. The dark staining structures that are continuous with the surface of the beads represent displaced iron particles that are also present on beads that are not incubated with vesicle fractions (data not shown). Bars, 0.1 μm.

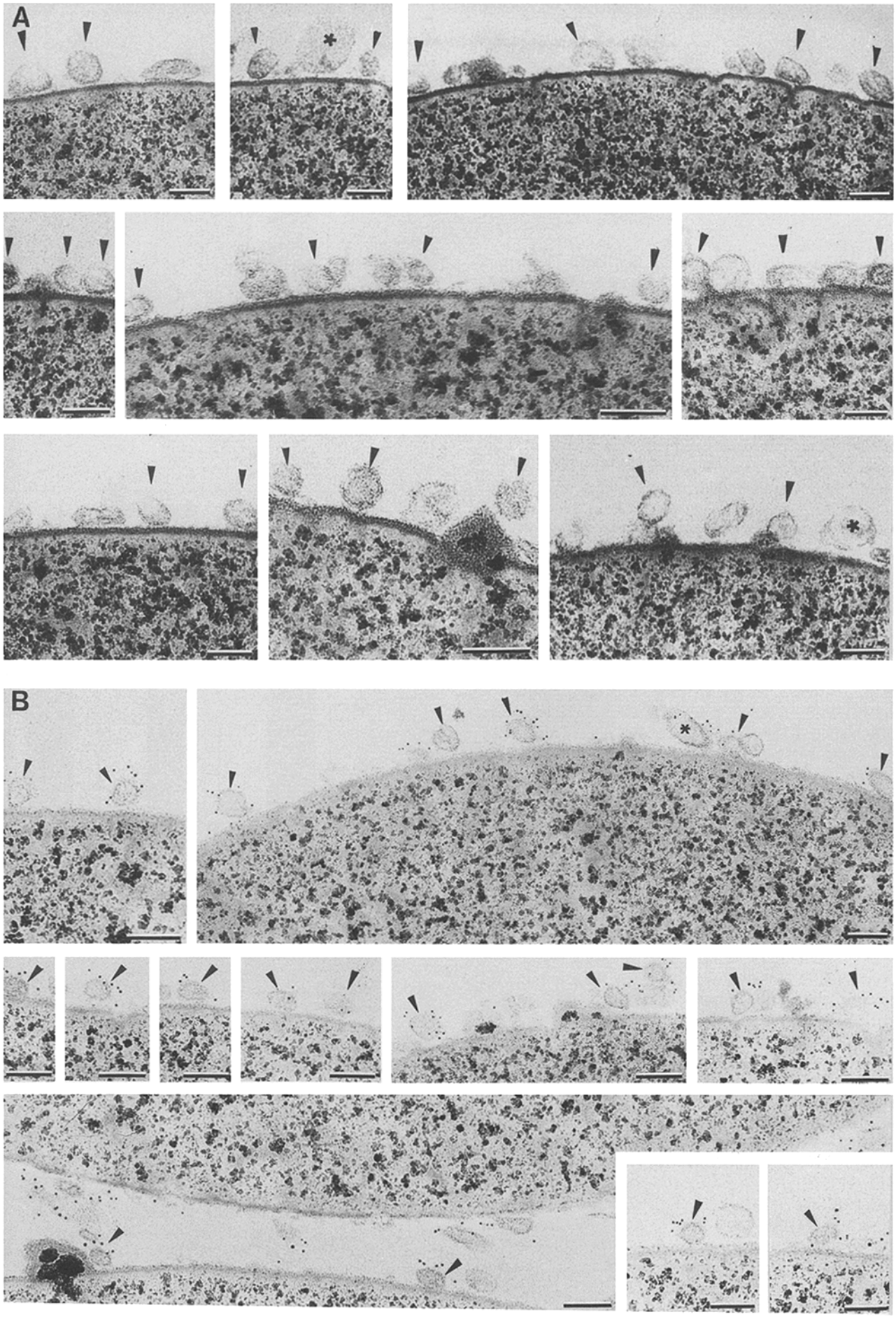

A
B

Table I. Size Distribution of Immunoisolated Vesicles

Vesicle fraction	Percentages of vesicles of diameter		
	<80	80–100 nm	>100 nm
Wild type	67	11	22
Sar1[H79G]	78	3	19

ER-derived vesicles were synthesized in the absence (wild type) or presence of Sar1[H79G] and then recovered on magnetic beads and processed for electron microscopy. The diameters of ~250 randomly selected vesicles on ~20 different fields were determined.

pletely inhibited the overall ER to Golgi transport reaction (Fig. 3). Fusion of wild-type and Sar1[H79G] accumulated vesicles was 90 and 75% efficient (Fig. 7 *B*, *a* and *b*), respectively, compared to control incubations. Similar data were observed when an equivalent concentration of wild-type Sar1 or the Sar1-GTP–restricted mutant were included in stage 2 (data not shown). These results indicate that after vesicle formation and loss of COPII coats, transit to the Golgi stack is independent of COPII.

The inhibitory effect of adding the $ARF1_{myr}$[T31N] mutant during stage 2 was more prominent than that of the Sar1 mutants. It reduced the fusion efficiency of wild-type or Sar1[H79G] accumulated vesicles to ~55 and ~33%, respectively, of the control value (Fig. 7 *B*, *c* and *d*). A similar level of inhibition was observed in the presence of $ARF1_{myr}$[Q71L] (not shown), whereas wild-type $ARF1_{myr}$ only weakly inhibited processing to the endo H resistant form (Fig. 7 *B*, *e*). The increased sensitivity of Sar1[H79G] accumulated vesicles to the presence the ARF1 GDP–restricted mutant in the second stage is consistent with the interpretation that COPII coat disassembly precedes COPI function. Furthermore, the striking inhibition (~80%) of transport to the Golgi observed when vesicles were formed in stage 1 in the presence of $ARF1_{myr}$[T31N] (Fig. 7 *A*, *c*) compared to that observed upon addition of the mutant during stage 2 after a normal stage 1 incubation (~55%) (Fig. 7 *B*, *c*) suggests that COPI assembly initiates rapidly after vesicle budding from the ER. Although the irreversible nature of inhibition by the ARF1 mutant was surprising, inhibition by the Sar1 GDP–bound mutant was similarly irreversible (not shown), suggesting that the inactive mutants may be relatively resistant to washing procedures because of a strong association with their respective exchange factors.

To address whether coatomer or ARF1 were directly required for ER to Golgi transport in vitro, we attempted to remove the endogenous pools of these proteins from the cytosol and membrane fractions used in the two-stage assay (see Materials and Methods). While we were able to deplete ~80 and 90% of the endogenous coatomer and ARF1 pools, respectively, transport was unaffected (not shown). The transport observed may have resulted from the residual pools that could not be removed from the assay. An alternative possibility is that ER-derived vesicles may undergo uncoupled fusion with Golgi elements at reduced COPI or ARF1 concentrations (Elazar et al., 1994; Taylor et al., 1994) (see Discussion). Since we were unable to demonstrate a direct coatomer or ARF1 requirement in the assay, we cannot exclude the possibility that the inhibition of transport exerted by $ARF1[T31N]_{myr}$ is indirect.

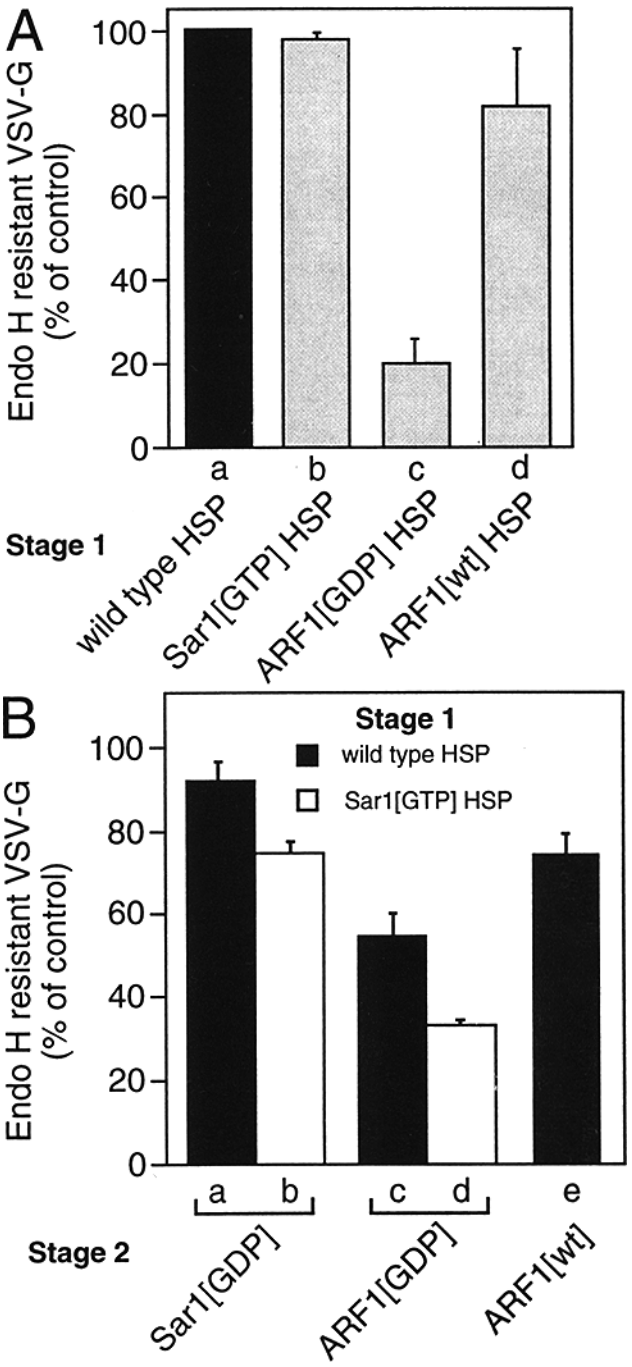


Figure 7. Effects of the Sar1 and ARF1 mutants on the fusion of ER-derived vesicles assessed using two-stage assays. (*A*) Vesicles were generated during stage 1 by incubation of microsomes at 32°C for 10 min in the absence of recombinant proteins (*a*), 45 min in the presence of Sar1[H79G] (*b*), $ARF1_{myr}$[T31N] (*c*), or 10 min in the presence of $ARF1_{myr}$[wild-type (wt)] (*d*). HSP fractions were prepared and fusion with Golgi membranes was followed during a 60-min stage 2 reaction with fresh reaction cocktails lacking recombinant proteins. (*B*) Vesicles generated under wild-type conditions (*a*, *c*, and *e*) or accumulated in the presence of Sar1[H79G] (*b* and *d*) during stage 1 were washed and incubated during stage 2 in the presence of Sar1[T39N] (*a* and *b*), $ARF1_{myr}$[T31N] (*c* and *d*), or $ARF1_{myr}$[wild-type (wt)] (*e*). Fusion was measured by following the appearance of endo H–resistant VSV-G as described in Fig. 2 legend. Data are expressed relative to control reactions that measured the fusion of wild-type vesicles in the absence of recombinant proteins in stage 2. The mean ± SEM from three independent experiments is shown.

The fact that wild-type $ARF1_{myr}$ did not significantly block fusion when added to either stage 1 or 2 suggests that the inhibitory effect of the GDP-restricted mutant on transport results from the T31N mutation.

Coatomer Is Recruited to Immunoisolated Vesicles

A direct prediction of the above results is that COPI may be recruited to ER-derived vesicular carriers after COPII coat disassembly. We first examined by Western blotting whether immunoisolated vesicular intermediates generated under normal incubation conditions contained β-COP after washing with a high-salt (0.25 M KOAc) buffer. Resistance to high-salt extraction is a hallmark of "activated" COPI recruited to Golgi budding structures and vesicles after GTPγS-induced coat assembly (Malhotra et al., 1989; Ostermann et al., 1993). While β-COP was detected on the vesicles after a low-salt (0.15 M KOAc) wash, it was nearly completely removed after a 0.25 M KOAc wash (data not shown).

To determine whether the high-salt–washed, immunoisolated ER-derived intermediates recovered from the MSS were able to bind activated coatomer, they were first incubated at 32°C for 10 min in the presence of increasing cytosol concentrations and GTPγS. Subsequently, the membranes were washed with 0.25 M KOAc to remove loosely associated COPI, and bound coatomer was measured by quantitative immunoblotting for β-COP. Under these conditions, coatomer was efficiently recruited to the ER-derived intermediates and binding appeared saturable at high concentrations of cytosol (Fig. 8 *A*). Increasing the concentration of cytosol did not increase binding of COPI to vesicles (not shown). Similar levels of coatomer recruitment were observed in the presence of the $ARF1_{myr}$ [Q71L] mutant. In this case, binding was independent of added GTPγS (data not shown), consistent with its expected GTP-bound, activated phenotype. Coatomer did not bind to p5D4-coated beads when the immunoisolated membranes were absent (data not shown).

To provide further evidence for COPI recruitment, we tested whether immunoisolated vesicles were able to bind COPI components from a mixture of purified coatomer and wild-type $ARF1_{myr}$, the minimum set of cytosolic proteins required to form Golgi-derived buds and vesicles in vitro (Orci et al., 1993*a*; Ostermann et al., 1993). In the presence of excess ARF1, ER-derived intermediates exhibited saturable GTPγS-dependent coatomer binding (Fig. 8 *B*), and binding did not increase at higher concentrations of COPI components (not shown). The biochemical properties of the recruitment reaction were assessed by incubating the immunoisolated vesicles with a fixed amount of purified coatomer in the absence or presence of $ARF1_{myr}$ and guanine nucleotides, followed by washing with 0.25 M KOAc and quantitative blotting for β-COP (Fig. 8 *C*, *stippled bars*) or ARF1 (Fig. 8 *C*, *closed bars*). In the presence of 25 μM GTP, substantial coatomer binding was observed, representing ~40% of the GTPγS-stimulated signal (Fig. 8 *C*, compare *a* and *b*). Coatomer binding to immunoisolated vesicles in the presence of GTPγS was ARF1-dependent (Fig. 8 *C*, *d*) as observed previously for enriched Golgi membrane fractions (Donaldson et al., 1992*a*; Kahn et al., 1992). Binding was reduced by roughly

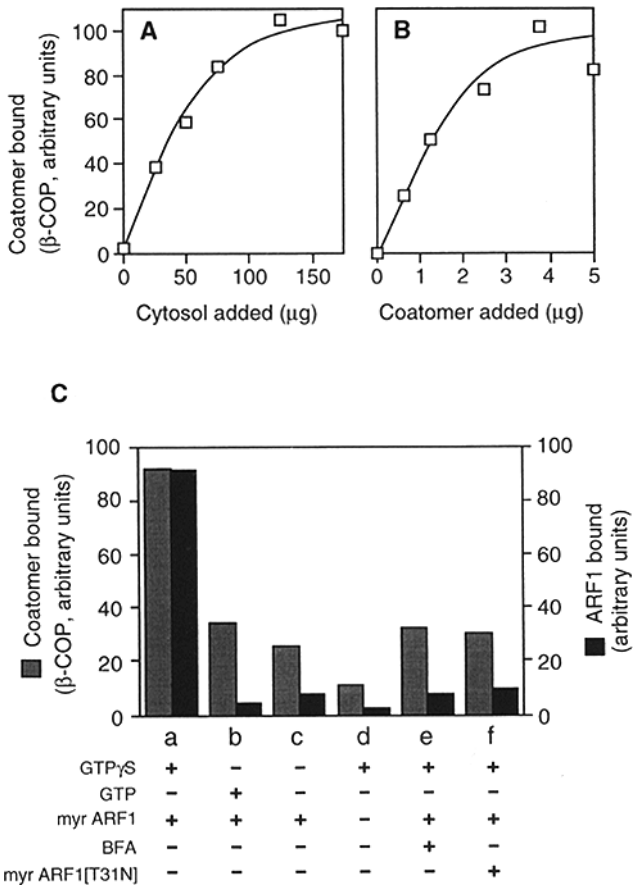
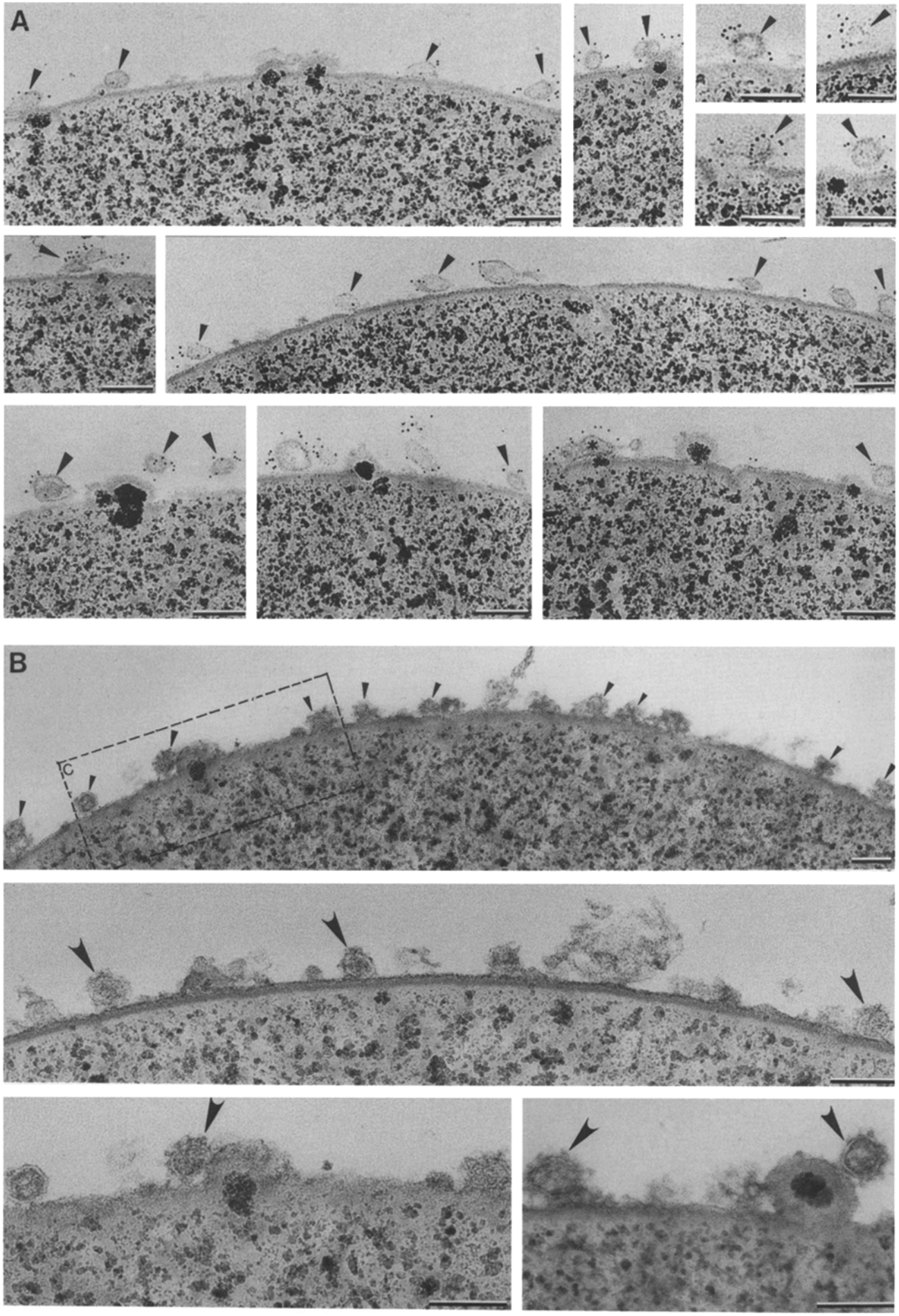


Figure 8. Coatomer is recruited to ER-derived vesicles. Immunoisolated vesicles prepared from MSS fractions after a 10-min incubation at 32°C in the presence of cytosol and ATP were washed with a 0.25 M KOAc–containing buffer to remove low affinity bound coatomer (see Results). The vesicles were subsequently incubated for 10 min at 32°C in the presence of GTPγS (25 μM) with either (*A*) increasing amounts of crude cytosol or (*B*) increasing amounts of purified coatomer and wild-type $ARF1_{myr}$ (at a constant molar ratio of 2.5:1 [ARF1/coatomer]). (*C*) Immunoisolated vesicles were incubated with a fixed amount of coatomer (37.5 μg/ml) and different combinations of the indicated guanine nucleotide (25 μM) (*a*, *b*, and *d–f*), 250 μM BFA (*e*), 90 μg/ml wild-type $ARF1_{myr}$ (*a–c*, *e*, and *f*), or 2 μM $ARF1_{myr}$[T31N] (*f*). Samples containing BFA or $ARF1_{myr}$[T31N] were preincubated for 5 min at 32°C before addition of purified COPI components and GTPγS. (*A–C*) After incubation, vesicles were washed with the 0.25 M KOAc–containing buffer, and bound coatomer and ARF1 were measured by quantitative immunoblotting as described under Materials and Methods. The results shown are representative of three independent experiments.

65% in the presence of BFA (Fig. 8 *C*, *e*), a drug known to inhibit ARF1-GEF activity (Donaldson et al., 1992b; Helms and Rothman, 1992) and promote rapid coatomer dissociation from membranes in vivo (Donaldson et al., 1990). Recruitment was also blocked by the $ARF1_{myr}$[T31N] mutant (Fig. 8 *C*, *f*), a result consistent with previous reports that this mutant blocks coatomer binding to Golgi compartments in vitro (Elazar et al., 1994; Aridor et al., 1995) and in vivo (Dascher and Balch, 1994). These observations imply that ER-derived vesicular intermediates possess a membrane-bound COPI receptor(s) and ARF1-GEF ac-


A
B
C

tivity that function in a physiological fashion to recruit coatomer after vesicle budding.

Although ARF1 binding was detected in the presence of GTPγS, binding was reduced to background levels when incubated in the presence of GTP (Fig. 8 *C*, *b*). ARF1 association with the membranes was not increased by raising the GTP concentration to 1 mM, making it unlikely that the low level of binding observed was a consequence of hydrolysis and loss of the GTP pool. The fact that ARF1 binding relative to coatomer was markedly reduced in the presence of GTP compared to GTPγS (Fig. 8 *C*, *a* and *b*) raises the possibility that ARF1 plays a catalytic rather than stoichiometric role in coatomer recruitment and that coatomer remains associated with the ER-derived intermediates after recruitment and cycling of ARF1 to the GDP-bound form. An alternative explanation is that incubation in the presence of GTPγS may lead to the membrane association of a "loosely bound" pool of ARF1 that may not contribute to coatomer recruitment, as shown previously in the case of Golgi membranes (Helms et al., 1993).

Since the immunoisolated membranes consisted of a population of 50–70 nm ER-derived vesicles and some larger structures (~14% of the VSV-G–containing intermediates were 80–100 nm in diameter; Table I) that may correspond to fused vesicles, it was important to determine whether COPI was recruited to structures falling into one or both size classes. Therefore, the immunoisolated vesicles were examined by immunoelectron microscopy after incubation in the presence of purified COPI components and GTPγS. Under these conditions, >90% of the vesicular profiles were decorated with gold particles when labeled with an antibody specific for β-COP (Fig. 9 *A*). Thus, it is clear that COPI is recruited to both 50–70 nm vesicles and the 80–100 nm structures. Although for technical reasons, a direct colocalization was not possible, the fact that extremely high percentages (>90%) of the vesicles labeled positively for both VSV-G (Fig. 6 *B*) and β-COP (Fig. 9 *A*), when tested individually, indicates that COPI can bind directly to VSV-G–containing vesicles. While these results indicate that in the biochemical COPI recruitment assay (Fig. 8), the 80–100 nm structures can contribute to the total amount of COPI bound, the more abundant 50–70 nm vesicles are likely to be the major source of COPI binding. Since the majority of structures bound to beads before incubation with purified COPI components were <70 nm in diameter, it is unlikely that incubation of beads with coatomer and ARF1 leads to a second round of vesicle budding. We conclude that coatomer can be recruited to ER-derived carriers.

To demonstrate that vesicles had indeed recruited a characteristic COPI coat, the immunoisolated vesicles were stained with tannic acid before examination by TEM to enhance coat visualization (Orci et al., 1986). As shown in Fig. 9 *B* (*arrowheads*), we observed a population of 50–70 nm vesicles that possessed an 8–10 nm thick electron-dense coat surrounding the lipid bilayer. This coat closely resembles the coatomer coat observed on Golgi-derived vesicles generated in vitro in the presence of GTPγS (Malhotra et al., 1989; Orci et al., 1993*a*). Before incubation, COPI coats were clearly not observed on the vesicles (Fig. 10 *A*). Because of the concern that COPI recruitment in the presence of GTPγS may not be physiologically relevant, we also performed the binding reaction in the presence of GTP. Under this condition, a coat was present on the 50–70 nm vesicles (Fig. 10 *B*, *arrowheads*), although in general it appeared less prominent than to that observed in the presence of GTPγS (Fig. 9 *B*). This may reflect GTP hydrolysis and partial loss of coat during preparation for TEM. The 80–100 nm structures were also less prominently coated and ~50% of these structures (compared to the GTPγS condition) appeared uncoated. While the nonhydrolyzable GTP analog may lead to some exaggeration of COPI binding, the fact that a COPI coat was observed on 50–70 nm vesicles incubated in the presence of GTP suggests that recruitment is physiologically relevant.

Taken together, our results indicate that COPI can be readily assembled in an ARF1- and GTP-dependent manner onto vesicles released into the MSS by a COPII-dependent mechanism. While it has been proposed that the role of ARF1 and coatomer binding to Golgi membranes is to promote the membrane deformation required for vesicle budding (Orci et al., 1993*b*), our results showing COPI binding to preformed ER-derived vesicles suggest that the recruitment of COPI can be independent of such events.

Discussion

In this study we describe a new biochemical assay that efficiently reconstitutes ER to Golgi transport using microsomes prepared from mammalian cells. These results demonstrate that the extensive reticular network of the ER observed in living cells is not necessary for vesicle formation. One of the main advantages of this assay over previous semiintact cell assays is that it can be used to measure independently the formation and consumption of ER-derived transport vesicles. In particular, the requirements for ER budding in semiintact cells are more difficult to interpret because of the low resolution indirect immunofluorescence techniques used to follow the appearance of cargo in VTCs as a morphological measure for ER export. To focus exclusively on export from the ER in the new assay, microsomes derived from NRK cells infected at the restrictive temperature with the ts045 mutant of VSV are used as

Figure 9. Coatomer is present on ER-derived vesicles when examined using TEM. (*A*) Immunogold labeling for β-COP after incubation with purified $ARF1_{myr}$ and coatomer in the presence of GTPγS (25 μM) to stabilize recruitment as described in Fig. 8 legend. The beads were incubated in the presence of polyclonal antibodies against β-COP followed by 5-nm gold-conjugated anti–rabbit IgG secondary antibodies before sectioning in epon resin as described (Lucian and Palade, 1994). The gallery illustrates that >90% of the vesicles labeled positively for β-COP. (*B*) Purified COPI components were recruited onto immunoisolated vesicles as described in *A*. Samples were prepared for electron microscopy with tannic acid treatment as described (Lucian and Palade, 1994). The gallery shows an electron-dense COPI coat present on the vesicles (*arrowheads*). The region enclosed by a box in top image is shown at higher magnification in the bottom left image. Bars, 0.1 μm.

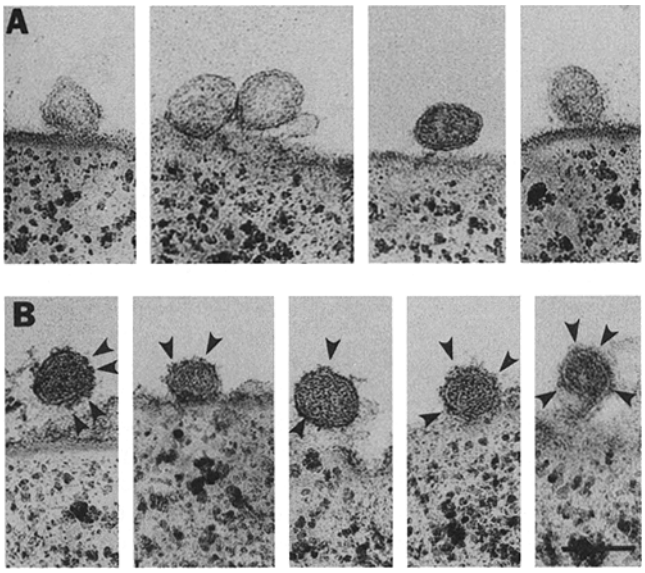


Figure 10. A COPI coat is recruited to ER-derived vesicles in the presence of GTP. Immunoisolated vesicles were incubated in the absence of COPI components (*A*), or in the presence of purified ARF1 and coatomer and GTP (*B*). The presence of coat components is indicated by arrowheads. Samples were examined by TEM after tannic acid staining as described in Fig. 9. Bar, 0.1 μm.

a source of membranes for the vesicle formation reaction. However, we anticipate that adaptation of the assay conditions developed in the present study will allow the analysis of the requirements for the export of a wide range of endogenous proteins from the ER using microsomes prepared from different mammalian cell lines.

We also report for the first time the immunoisolation of mammalian ER-derived transport vesicles. This was not possible using semiintact cells as they do not release transport intermediates (Plutner et al., 1992). We have shown that vesicles formed in vitro contained both VSV-G and p58 and that both proteins exit the ER via a COPII-dependent mechanism. Although the function of p58 is unknown, it has recently been shown to be mannose-binding lectin and suggested to potentially facilitate egress of glycosylated cargo from the ER (Arar et al., 1995). The potential to now identify additional vesicle components provides a powerful new tool for increasing our understanding of ER to Golgi transport in mammalian cells.

Export of Cargo from the ER Is Mediated by COPII

Using the microsome-based assay, we have shown that ER export is blocked by a Sar1 mutant restricted to the GDP-bound form that prevents COPII coat assembly. Although this mutant also appeared to block export in semiintact mammalian cells as measured by indirect immunofluorescence (Kuge et al., 1993; Aridor et al., 1995), the inability to detect individual vesicles in these cells necessitated the development of the quantitative budding assay described in this study to confirm this point. Our results with VSV-G and p58 are fully consistent with the previous demonstration in yeast that interaction of Sar1p with Sec12p (a Sar1-specific GEF) is essential for export of the secretory marker pre-pro α-factor from the ER (d'Enfert et al., 1991; Barlowe et al., 1993). In contrast to the effects of the GDP-restricted mutant, the Sar1 mutant restricted to the GTP-bound form allowed vesicle formation, but prevented consumption. Although previous studies in yeast have shown that uncoating and fusion of COPII vesicles is blocked by nonhydrolyzable GTP analogs, it could not be excluded that the block in fusion could result from inhibition by the Ypt1p GTPase. Our observations reinforce the essential need for GTP hydrolysis by Sar1p to allow vesicles to become competent for fusion with Golgi compartments (Barlowe et al., 1994; Oka and Nakano, 1994). In general, it is now clear that the COPII machinery is the basic coat complex driving the export of a diverse group of proteins from the ER in yeast and mammalian cells.

Export of VSV-G from ER microsomes involves sorting and concentration (Balch et al., 1994). We now refer to these events as selective transport (Aridor and Balch, 1996), a concept that replaces bulk flow as a working model for membrane vesicular traffic through the exocytic pathway. Under conditions where VSV-G– and p58-containing vesicles were efficiently formed, neither BiP nor calnexin were released from the ER. Such results demonstrate the remarkable capability of the export machinery to discriminate between resident ER proteins and exportable cargo. Although a previous study demonstrated that BiP–VSV-G complexes can be transported to pre-Golgi intermediates at 39.5°C (Hammond and Helenius, 1994*b*), this is clearly an anomaly associated with the excessive viral infection conditions used in this particular analysis. Under normal infection conditions we have never been able to detect in vivo or in vitro the release of tsO45 VSV-G from the ER to pre-Golgi intermediates at the restrictive temperature (Plutner et al., 1992; Balch et al., 1994). Thus, it is evident that the new assay reconstitutes the normal folding pathway leading to the efficient release of mature forms of VSV-G from both BiP and calnexin. Indeed, the high degree of retention of chaperones in the ER indicates that they are predominantly resident ER proteins with a very low "escape" rate when compared to egress of itinerant cargo.

ARF1 Activation Is Essential for COPI Recruitment to ER-derived Vesicles

While export of VSV-G and p58 from the ER required COPII, we found no evidence for COPI involvement in this event. Vesicular intermediates were efficiently formed and accumulated in the presence of the $ARF1_{myr}$[T31N] mutant, which would be expected to inhibit the assembly of COPI coats (Donaldson et al., 1992*b*; Helms and Rothman, 1992; Dascher and Balch, 1994; Elazar et al., 1994; Aridor et al., 1995). In yeast, the formation of COPII-coated vesicles containing pre-pro α-factor is also COPI independent (Barlowe et al., 1994). These results now provide a plausible explanation for a previous indirect immunofluorescence study of transport in semiintact cells in which we reported an apparent block of VSV-G export from the ER in the presence of an antibody raised against β-COP (Peter et al., 1993). It is now clear that inhibition of COPI recruitment does not affect export of VSV-G from the ER, rather it appears to prevent efficient accumulation of cargo in pre-Golgi VTCs in vitro (Aridor et al., 1995)

and in vivo (Dascher and Balch, 1994), thus preventing detection of export using morphological approaches.

The initial site of COPI recruitment in living cells has been difficult to assess. While COPI is highly abundant on pre-Golgi VTCs (Oprins et al., 1993; Balch et al., 1994), these are compact structures in which the distribution of COPI to tubules and/or vesicles based on immunoelectron microscopy cannot be resolved. Using an entirely new biochemical approach to generate immunopurified ER-derived vesicles in vitro, we have found that COPI can be directly recruited to immunoisolated ER-derived vesicles in an ARF1- and GTP-dependent manner. While COPI recruitment was most striking in the presence of GTPγS, a less substantial coat was formed in the presence of GTP and may be more representative of coats formed under physiological conditions. COPI binding was blocked by BFA or the ARF1 GDP–restricted mutant, indicating that ER-derived vesicles contain ARF1-GEF activity. While both VSV-G and COPI were both present on a prominent population of 50–70 nm vesicles bound to immunobeads, both proteins could also be detected on lower abundance structures in the size range of 80–100 nm. These, in principle, may correspond to the larger tubular elements typically associated with compact VTCs observed in vivo (Saraste and Svensson, 1991; Balch et al., 1994). It seems reasonable that a percentage of ER-derived, VSV-G–containing vesicles could begin to assemble into such structures in vitro. However, our ability to detect COPI recruitment to a population of 50–70 nm carrier vesicles suggests that coatomer can be rapidly recruited to ER-derived vesicles following the COPII-mediated budding event.

The ARF1 GDP–restricted mutant potently inhibited the delivery of VSV-G to Golgi membranes. In two stage assays, inhibition by the mutant was shown to be a consequence of its interaction with vesicular intermediates rather than Golgi membranes. The effect of the mutant in the microsome-based assay is consistent with previous results indicating that inhibition of ARF1 activation using either BFA or $ARF1_{myr}$[T31N] prevents the accumulation of p58 and VSV-G in VTCs in vitro (Aridor et al., 1995) or in vivo (Dascher and Balch, 1994). The inhibitory effect of the GTP-restricted ARF1 mutant on transport is also consistent with previous morphological studies where the activated ARF1 mutant inhibited transport of VSV-G through VTCs (Dascher and Balch, 1994; Aridor et al., 1995). These results emphasize the potential need for a complete ARF1 cycle at this step. The reduced inhibitory effect of both mutants when added to stage 2 raises the possibility that transport of VSV-G from VTCs to the Golgi stack is COPI independent.

While we were unable to demonstrate a direct requirement for ARF1 or COPI in the two-stage assay, these experiments are inconclusive because residual levels of ARF1 or coatomer remaining in the cytosol or membrane fractions may have been sufficient to drive the reaction. Alternatively, depletion of COPI from the reaction may have led to "uncoupled fusion" of ER-derived intermediates formed during stage 1 with Golgi membranes. This phenomenon has been well characterized in the case of intra-Golgi transport where the presence of low levels of coatomer or ARF1 promotes COPI-independent fusion of Golgi membranes (Elazar et al., 1994; Taylor et al., 1994).

A recent study using isolated yeast nuclei has suggested that COPI can be recruited to the ER in the context of driving a separate (COPII-independent) export pathway (Bednarek et al., 1995). Unlike vesicles generated by COPII components, those generated by COPI were devoid of known cargo proteins, leading to speculation that COPI may serve to recycle post-ER proteins required for retrograde transport. Our new results indicating that COPI is recruited to ER-derived vesicular intermediates do not exclude the possibility that separate COPI- and COPII-mediated ER export pathways may operate. One possibility is that distinct classes of coatomer complexes exist that mediate different membrane-trafficking steps (Whitney et al., 1995). Alternatively, the present experiments raise the possibility that COPI vesicles formed from the yeast nuclear envelope in vitro (Bednarek et al., 1995) bypass the normal order of events occurring in vivo, which first requires COPII for the sorting and concentration of cargo. This is supported by a large body of morphological evidence indicating that ER to Golgi intermediates rather than the ER represent the major site of COPI localization in living cells (for review see Kreis et al., 1995).

Working Model for Coupled COPII and COPI Function in Segregation of Cargo in VTCs

We have previously demonstrated a coupling between COPII and COPI function during ER to Golgi transport in semiintact cells (Aridor et al., 1995). Results obtained in the present study now provide the basis for a more refined model for these events. This working model, incorporating COPII/COPI coat exchange on ER-derived vesicles in transit between the ER and pre-Golgi VTCs, is illustrated in Fig. 11. Since COPII coats are inherently unstable (Barlowe et al., 1994), they are likely to be shed rapidly after budding. ARF1 activation may lead to COPI recruitment directly to uncoated ER-derived vesicles and this is likely to be the step blocked by BFA or the ARF1 GDP–bound mutant. One can speculate that coatomer binding may serve to "tag" proteins bearing the di-lysine retrieval signal for subsequent recycling from VTCs. After the assembly of ER-derived vesicles into larger elements typically associated with VTCs, assembly of coatomer-tagged proteins in these tubular elements would lead to the formation of COPI-coated vesicles, leading to their selective retrieval to the ER. While only a working model, this view is consistent with increasing evidence indicating that the binding of COPI is essential for the segregation of anterograde and retrograde transported proteins from VTCs. This model can explain the effects of specific mutations in coatomer subunits that differentially perturb anterograde or retrograde transport (Duden et al., 1994; Letourneur et al., 1995; Cosson et al., 1996). The phenotype of a particular mutation in a given COPI subunit would be dependent on its role(s) in an early tagging step and/or later events involved in segregation and vesicle fission. Thus, the exchange of COPII for COPI after budding from the ER provides a plausible mechanism to balance anterograde and retrograde vesicular traffic by linking COPI vesicle assembly to components of ER-derived COPII carriers.

We thank Dr. M.G. Farquhar, University of California San Diego, for her helpful comments concerning the electron microscopy. Antibodies to

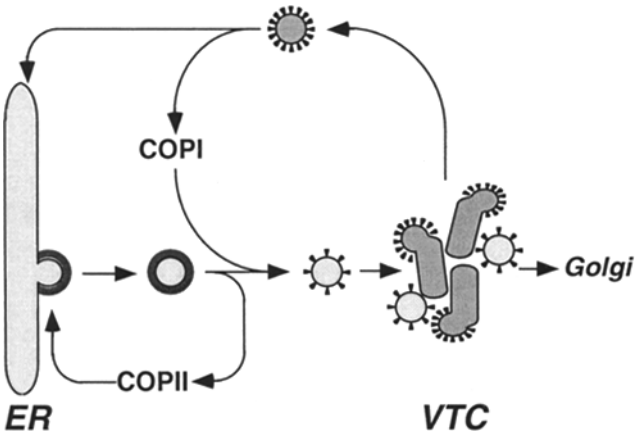


Figure 11. Working model describing the role of COPII/COPI coat exchange on ER-derived vesicles. The COPII coat (*gray curve*) drives export from the ER and is lost rapidly after vesicle budding in response to GTP hydrolysis by Sar1. The initial recruitment of COPI (*broken curve*) in response to ARF1 activation may occur by interaction with proteins in vesicles that contain dilysine ER retention/recycling motifs. This "tagging" identifies components for subsequent recycling from VTCs via COPI vesicular carriers.

β-COP (EAGE epitope) and calnexin were generously provided by Dr. M. Jackson, Johnson and Johnson, Inc. TSRI manuscript # 9558-CB.

This work was supported by grants from the National Institutes of Health (GM 42336 and CA586689) to W.E. Balch and postdoctoral fellowships from The Human Frontier Science Program Organization (HFSPO), the Muscular Dystrophy Association (T. Rowe), and the European Molecular Biology Organization and HFSPO (M. Aridor). The electron microscopy made extensive use of Core B in CA 586689.

Received for publication 15 April 1996 and in revised form 19 August 1996.

References

Arar, C., V. Carpentier, J.-P. Le Caer, M. Monsigny, A. Legrand, and A.-C. Roche. 1995. ERGIC-53, a membrane protein of the endoplasmic reticulum-Golgi intermediate compartment is identical to MR60, and intracellular mannose-specific lectin of myelomonocytic cells. *J. Biol. Chem.* 270:3551–3553.

Aridor, M., and W.E. Balch. 1996. Principles of selective transport: coat complexes hold the key. *Trends Cell Biol.* 6:315–320.

Aridor, M., S.I. Bannykh, T. Rowe, and W.E. Balch. 1995. Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi transport. *J. Cell Biol.* 131:875–893.

Balch, W.E., and J.E. Rothman. 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. Biophysics.* 240:413–425.

Balch, W.E., R.A. Kahn, and R. Schwaninger. 1992. ADP-ribosylation factor (ARF) is required for vesicular trafficking between the endoplasmic reticulum (ER) and the cis Golgi compartment. *J. Biol. Chem.* 267:13053–13061.

Balch, W.E., J.M. McCaffery, H. Plutner, and M.G. Farquhar. 1994. Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum. *Cell.* 76:841–852.

Barlowe, C., C. d'Enfert, and R. Schekman. 1993. Purification and characterization of SAR1p, a small GTP-binding protein required for transport vesicle formation from the endoplasmic reticulum. *J. Biol. Chem.* 268:873–879.

Barlowe, C., L. Orci, T. Yeung, M. Hosobuchi, S. Hamamoto, N. Salama, M.F. Rexach, M. Ravazzola, M. Amherdt, and R. Schekman. 1994. COPII: a membrane coat formed by sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell.* 77:895–907.

Bednarek, S.Y., M. Ravazzola, M. Hosobuchi, M. Amherdt, A. Perrelet, R. Schekman, and L. Orci. 1995. COPI- and COPII-coated vesicles bud directly from the endoplasmic reticulum in yeast. *Cell.* 83:1183–1196.

Cosson, P., and F. Letourner. 1994. Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. *Science (Wash. DC).* 263:1629–1631.

Cosson, P., C. Demolliere, S. Hennecke, R. Duden, and F. Letourneur. 1996. δ- and ζ-COP, two coatomer subunits homologous to clathrin-associated proteins, are involved in ER retrieval. *EMBO (Eur. Mol. Biol. Organ.) J.* 15: 1792–1798.

d'Enfert, C., L.J. Wuestehube, T. Lila, and R. Schekman. 1991. Sec12p-dependent membrane binding of the small GTP-binding protein Sar1p promotes formation of transport vesicles from the ER. *J. Cell Biol.* 114:663–670.

Dascher, C., and W.E. Balch. 1994. Dominant inhibitory mutants of ARF1 inhibit ER to Golgi transport and trigger the disassembly of the Golgi apparatus. *J. Biol. Chem.* 269:1437–1448.

Davidson, H.W., and W.E. Balch. 1993. Differential inhibition of multiple vesicular transport steps between the endoplasmic reticulum and trans Golgi network. *J. Biol. Chem.* 268:4216–4226.

Doms, R.W., D.S. Keller, A. Helenius, and W.E. Balch. 1987. Role for adenosine triphosphate in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. *J. Cell Biol.* 105:1957–1969.

Donaldson, J.G., J. Lippincott-Schwartz, and R.D. Klausner. 1990. Guanine nucleotides modulate the effects of brefeldin A in semipermeable 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., D. Cassel, R. Kahn, and R.D. Klausner. 1992*a*. ADP-ribosylation factor, a small GTP-binding protein, is required for binding of the coatomer protein β-COP to Golgi membranes. *Proc. Natl. Acad. Sci. USA.* 89:6408–6422.

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

Duden, R., M. Hosobuchi, S. Hamamoto, M. Winey, B. Byers, and R. Schekman. 1994. Yeast β- and β′-coat proteins (COP). *J. Biol. Chem.* 269:24486–24495.

Elazar, Z., L. Orci, J. Ostermann, M. Amherdt, G. Tanigawa, and J.E. Rothman. 1994. ADP-ribosylation factor and coatomer couple fusion to vesicle budding. *J. Cell Biol.* 124:415–424.

Griffiths, G., R. Pepperkok, J. Krijnse-Locker, and T.E. Kreis. 1995. Immunocytochemical localization of β-COP to the ER-Golgi boundary and the TGN. *J. Cell Sci.* 108:2839–2856.

Hammond, C., and A. Helenius. 1994*a*. Folding of VSV G protein: sequential interaction with BiP and calnexin. *Science (Wash. DC).* 266:456–458.

Hammond, C., and A. Helenius. 1994*b*. Quality control in the secretory pathway: retention of a misfolded viral membrane glycoprotein involves cycling between the ER, intermediate compartment, and Golgi apparatus. *J. Cell Biol.* 126:41–52.

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

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

Kahn, R.A., P. Randazzo, T. Serafini, O. Weiss, C. Rulka, J. Clark, M. Amherdt, P. Roller, L. Orci, and J.E. Rothman. 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.

Kreis, T.E. 1986. Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:931–941.

Kreis, T.E., M. Lowe, and R. Pepperkok. 1995. COPs regulating membrane traffic. *Annu. Rev. Cell Biol.* 11:677–706.

Kuge, O., C. Dascher, L. Orci, T. Rowe, M. Amherdt, H. Plutner, M. Ravazzola, G. Tanigawa, J.E. Rothman, and W.E. Balch. 1994. Sar1 promotes vesicle budding from the endoplasmic reticulum but not Golgi compartments. *J. Cell Biol.* 125:51–65.

Kuge, W., S. Hara-Kuge, L. Orci, M. Ravazzola, M. Amherdt, G. Tanigawa, F.T. Wieland, and J.E. Rothman. 1993. ζ-COP, a subunit of coatomer, is required for COP-coated vesicle assembly. *J. Cell Biol.* 123:1727–1734.

Lafay, F. 1974. Envelope viruses of vesicular stomatitis virus: effect of temperature-sensitive mutations in complementation groups III and V. *J. Virol.* 14: 1220–1228.

Letourneur, F., E.C. Gaynor, S. Hennecke, C. Demolliere, R. Duden, S.D. Emr, H. Riezman, and P. Cosson. 1995. Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell.* 79:1199–1207.

Lippincott-Schwartz, J. 1993. Bidirectional membrane traffic between the endoplasmic reticulum and Golgi apparatus. *Trends Cell Biol.* 3:81–88.

Lippincott-Schwartz, J., L.C. Yuan, J.S. Bonifacino, and R.D. Klausner. 1989. Rapid redistribution of Golgi proteins into the endoplasmic reticulum in cells treated with Brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell.* 54:209–220.

Lucian, S., and G.E. Palade. 1994. Membrane and secretory proteins are transported from the Golgi complex to the sinusoidal plasmalemma of hepatocytes by distinct vesicular carriers. *J. Cell Biol.* 125:733–741.

Malhotra, V., T. Serafini, L. Orci, J.C. Shepherd, and J.E. Rothman. 1989. Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell.* 58:329–336.

Oka, T., and A. Nakano. 1994. Inhibition of GTP hydrolysis by Sar1p causes accumulation of vesicles that are a functional intermediate of the ER-to-Golgi transport in yeast. *J. Cell Biol.* 124:425–434.

Oprins, A., R. Duden, T.E. Kreis, H.J. Geuze, and J.W. Slot. 1993. β-COP lo-

calizes mainly to the *cis*-Golgi side in exocrine pancreas. *J. Cell Biol.* 121:49–59.
Orci, L., B.S. Glick, and J.E. Rothman. 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., D.J. Palmer, M. Amherdt, and J.E. Rothman. 1993*a*. Coated vesicle assembly in the Golgi requires only coatomer and ARF proteins from the cytosol. *Nature (Lond.).* 364:732–734.
Orci, L., D.J. Palmer, M. Ravazzola, A. Perrelet, and J.E. Rothman. 1993*b*. Budding from Golgi membranes requires the coatomer complex of non-clathrin proteins. *Nature (Lond.).* 362:648–652.
Ostermann, J., L. Orci, K. Tani, M. Amherdt, M. Ravazzola, Z. Elazar, and J.E. Rothman. 1993. Stepwise assembly of functionally active transport vesicles. *Cell.* 75:1015–1025.
Ou, W.-J., P.H. Cameron, D.Y. Thomas, and J.J.M. Bergeron. 1993. Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature (Lond.).* 364:771–776.
Pelham, H.R.B. 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:913–918.
Pelham, H.R.B. 1991. Recycling of proteins between the endoplasmic reticulum and Golgi complex. *Curr. Opin. Cell Biol.* 3:585–591.
Pelham, H.R.B. 1994. About turn for the COPs? *Cell.* 79:1125–1127.
Peter, F., H. Plutner, T. Kreis, and W.E. Balch. 1993. β-COP is essential for transport of protein from the endoplasmic reticulum to the Golgi in vitro. *J. Cell Biol.* 122:1155–1168.
Pind, S., C. Nuoffer, J.M. McCaffery, H. Plutner, H.W. Davidson, M.G. Farquhar, and W.E. Balch. 1994. Rab1 and Ca^{2+} are required for the fusion of carrier vesicles mediating endoplasmic reticulum to Golgi transport. *J. Cell Biol.* 125:239–252.
Plutner, H., A.D. Cox, S. Pind, R. Khosravi-Far, J.R. Bourne, R. Schwaninger, C.J. Der, and W.E. Balch. 1991. Rab1b regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments. *J. Cell Biol.* 115:31–43.
Plutner, H., H.W. Davidson, J. Saraste, and W.E. Balch. 1992. Morphological analysis of protein transport from the endoplasmic reticulum to Golgi membranes in digitonin-permeabilized cells: role of the p58 containing compartment. *J. Cell Biol.* 119:1097–1116.
Rothman, J.E. 1994. Mechanisms of intracellular protein transport. *Nature (Lond.).* 372:55–63.
Rowe, T., and W.E. Balch. 1995. Expression and purification of mammalian Sar1. *Methods Enzymol.* 257:49–53.
Saraste, J., and E. Kuismanen. 1992. Pathways of protein sorting and membrane traffic between the rough endoplasmic reticulum and the Golgi complex. *Semin. Cell Biol.* 3:343–355.
Saraste, J., and K. Svensson. 1991. Distribution of the intermediate elements operating in ER to Golgi transport. *J. Cell Sci.* 100:415–430.
Schekman, R., and L. Orci. 1996. Coat proteins and vesicle budding. *Science (Wash. DC).* 271:1526–1533.
Schwaninger, R., C.J.M. Beckers, and W.E. Balch. 1991. Sequential transport of protein between the endoplasmic reticulum and successive Golgi compartments in semi-intact cells. *J. Biol. Chem.* 266:13055–13063.
Tang, B.L., S.H. Wong, X.L. Qi, S.H. Low, and W. Hong. 1993. Molecular cloning, characterization, subcellular localization and dynamics of p23, the mammalian KDEL receptor. *J Cell Biol.* 120:325–338.
Tang, B.L., S.H. Low, H.-P. Hauri, and W. Hong. 1995. Segregation of ERGIC53 and the mammalian KDEL receptor upon exit from the 15°C compartment. *Eur. J. Cell Biol.* 68:397–410.
Tanigawa, G., L. Orci, M. Amherdt, M. Ravazzola, J.B. Helms, and J.E. Rothman. 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., M. Kanstein, P. Weidman, and P. Melancon. 1994. Cytosolic ARF's are required for vesicle formation but not for cell-free intra-Golgi transport: evidence for coated vesicle-independent transport. *Mol. Biol. Cell.* 5:237–252.
Waters, M.G., C.J.M. Beckers, and J.E. Rothman. 1992. Purification of coat protomers. *Methods Enzymol.* 219:331–337.
Weiss, O., J. Holden, C. Rulka, and R.A. Kahn. 1989. Nucleotide binding and cofactor activities of purified bovine brain and bacterially expressed ADP-ribosylation factor. *J. Biol. Chem.* 264:21066–21072.
Whitney, J.A., M. Gomez, D. Sheff, T.E. Kreis, and I. Mellman. 1995. Cytoplasmic coat proteins involved in endosome fusion. *Cell.* 83:703–713.