Sequential Coupling between CopII and CopI Vesicle Coats in Endoplasmic Reticulum to Golgi Transport

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Abstract. COPI and COPII are vesicle coat complexes whose assembly is regulated by the ARF1 and Sar1 GTPases, respectively. We show that COPI and COPII coat complexes are recruited separately and independently to ER (COPII), pre-Golgi (COPI, COPII), and Golgi (COPI) membranes of mammalian cells. To address their individual roles in ER to Golgi transport, we used stage specific in vitro transport assays to synchronize movement of cargo to and from pre-Golgi intermediates, and GDP- and GTP-restricted forms of Sar1 and ARF1 proteins to control coat recruitment. We find that COPII is solely responsible for export from the ER, is lost rapidly following vesicle budding and mediates a vesicular step required for the build-up of pre-Golgi intermediates composed of clusters of vesicles and small tubular elements. COPI is recruited onto pre-Golgi intermediates where it initiates segregation of the anterograde transported protein vesicular stomatitis virus glycoprotein (VSV-G) from the retrograde transported protein p58, a protein which actively recycles between the ER and pre-Golgi intermediates. We propose that sequential coupling between COPII and COPI coats is essential to coordinate and direct bi-directional vesicular traffic between the ER and pre-Golgi intermediates involved in transport of protein to the Golgi complex.

NEWLY synthesized proteins are transported from the ER through the early secretory pathway via coated vesicular carriers. Two distinct protein complexes are now recognized to mediate vesicle budding from the ER and Golgi compartments. These include COPI or "coatomer" and COPII (for reviews see Nuoffer and Balch, 1994; Barlowe, 1995). Studies in vitro (Kuge et al., 1994; Peter et al., 1993, 1994) and in vivo (Guo et al., 1994; Hobbie et al., 1994; Pepperkok et al., 1993) in mammalian cells, and biochemical and genetic analyses in yeast (for review see Pryer et al., 1992), have shown that both coats are essential for the anterograde transport of protein from the ER to the Golgi stack. However, their individual specific roles in mediating the movement of cargo through early steps of the secretory pathway remains to be determined.

COPI coats are composed of α, β, β′, γ, δ, ε, and ζ-COP subunits which form a cytosolic 14-S complex (coatomer) (Waters et al., 1991). Coatomer is recruited from the cytosol after the activation of the small GTPase ARF1 (for reviews see Donaldson and Klausner, 1994; Duden et al., 1994). In vitro studies first identified a role for coatomer in the formation of Golgi-derived vesicular carriers which were suggested to be involved in the transport of cargo through successive cisternae of the Golgi stack (Malhotra et al., 1989; Orci et al., 1993*a,b,c*; Ostermann et al., 1993). Coatomer and ARF1 represent the minimal set of cytosolic proteins required for the generation of such Golgi-derived buds and vesicles (Orci et al., 1993*a*). A role for COPI coats in ER to Golgi transport in mammalian cells was demonstrated by the ability of antibodies against the β-COP subunit to inhibit export from the ER in vitro (Peter et al., 1993), and the requirement for a 19 S β-COP and Sec23 containing protein complex(es) in transport of protein from the ER to pre-Golgi intermediates (Peter et al., 1993). These results demonstrated that COPI plays a necessary, but not sufficient role in the appearance of pre-Golgi intermediates composed of clusters of vesicles and small tubules (referred to as vesicular-tubular clusters or VTCs[1]) (Peter et al., 1993). A similar block in ER to Golgi transport was observed in vivo after microinjection of β-COP antibodies (Pepperkok et al., 1993). However, in this case, cargo was found to principally accumulate in pre-Golgi intermediates which had tubular connections with the ER (Pepperkok et al., 1993). Genetic studies in mammalian cells have also demonstrated that ER to Golgi transport requires the function of ε-COP (Guo et al., 1994). Consistent with these biochemical and genetic requirements for COPI in ER to Golgi transport, morphological analyses have demonstrated that COPI coats are principally associated with pre-Golgi structures. They are

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1. *Abbreviations used in this paper*: CGN, *cis*-Golgi network; GAP, guanine nucleotide activating protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); myr, myristoylated; VSV-G, vesicular stomatitis virus glycoprotein; VTC, vesicular tubular cluster.

localized to VTCs and the more tubular/fenestrated elements found at the *cis* face of the Golgi stack (the *cis*-Golgi network or CGN [Mellman and Simon, 1992]) (Oprins et al., 1993; Pepperkok et al., 1993; Pind et al., 1994).

COPI function is evolutionarily conserved as the γ-, β-, and β′-subunits of COPI coats have homologues in yeast. Phenotypically, mutations in both Sec21p (γ-COP) and Sec 27p (β′-COP) lead to moderate accumulation of ER to Golgi vesicular carriers (Hosobuchi et al., 1992; Duden et al., 1994). In contrast, deletion of Sec26p, the yeast β-COP homologue, dramatically reduces the number of vesicles, and leads to exaggeration and tubularization of the ER (Duden et al., 1994). These proteins, along with ARF1p and ARF2p (Stearns et al., 1990), are required for ER to Golgi transport in vivo in yeast. The exact role for COPI in ER to Golgi in yeast in vitro has not been demonstrated yet. Interestingly, the mammalian α, β, β′, and γ coatomer subunits were recently shown to interact with proteins containing the ER di-lysine retention/recycling motif, KKXX (Cosson and Letourner, 1994). In yeast, sec21-1, sec21-2 ,and sec27-1 as well as ret1, a selected retrograde mutation in the α subunit of yeast coatomer, effectively abolished retrograde transport of a KKXX containing hybrid protein from the *cis*-Golgi region to the ER without affecting anterograde transport (Letourneur et al., 1995). These results have led to the recent speculative proposal that COPI coats may be exclusively involved in the retrieval of recycling proteins to the ER (Letourneur et al., 1995; Pelham, 1994).

COPII coats in yeast consist of a 400-kD Sec23p/Sec24p complex (Hicke and Schekman, 1989; Hicke et al., 1992) and a 700-kD complex which contains Sec13p and a 150-kD protein (Sec31p)(Pryer et al., 1993; Salama et al., 1993). These proteins, when recruited by activation of the small GTPase Sar1p (Barlowe et al., 1993), represent the minimal set of cytosolic proteins required for vesicle budding from yeast ER (Salama et al., 1993; Barlowe et al., 1994). The GTPase cycle of Sar1p involves components of the COPII coat. Sec23p is the GTPase–activating protein (GAP) (Yoshihisa et al., 1993) whereas a membrane-associated protein, Sec12p is the guanine nucleotide exchange factor (GEF) (Barlowe and Schekman, 1993; d'Enfert et al., 1991; Nakano et al., 1988). GEF function is required for vesicle budding (Barlowe and Schekman, 1993; Kuge et al., 1994) and GTP hydrolysis is required to promote vesicle fusion after budding (Barlowe et al., 1994). These results suggest a cycle in which the GAP component of the assembled coat complex on budded vesicles triggers coat destabilization by accelerating the intrinsic rate of hydrolysis of Sar1p. While the specific GAP activity for ARF1 is currently unknown, COPI-coated vesicles released from Golgi membranes contain bound ARF1 and can be isolated in the presence of GTP (Orci et al., 1993*b*; Ostermann et al., 1993). Therefore, COPI uncoating has been suggested to be coupled to later steps in vesicle targeting or fusion (Elazar et al., 1994).

COPII coats are also functional in export from the ER in mammalian cells. A mammalian homologue to Sec23p is present in the transitional zone between the ER and the Golgi, but does not colocalize with the distribution of COPI (Orci et al., 1991*a*). Moreover, a mutant form of Sar1a which restricts the GTPase to the GDP-bound form has been recently demonstrated to be a potent inhibitor of protein export from the ER in vivo and in vitro (Kuge et al., 1994). Mammalian homologues to Sec23p (Wadhwa et al., 1992) and Sec13p (Shaywitz et al., 1994) have been cloned. In the case of human Sec13p, it has been shown to function in yeast, and is located on transport vesicles budding from the ER (Shaywitz et al., 1994).

The requirement for COPII and COPI coats is puzzling. Both coats have been demonstrated to be involved in vesicle budding (Barlowe et al., 1994; Ostermann et al., 1993) yet, as summarized above, both COPII and COPI appear to function in ER to Golgi transport. Such a strong similarity in coat complex function clearly raises the question of their individual roles. To address this question, we now demonstrate that COPI and COPII are recruited separately and independently. To establish the relationship between this observation and their unique roles in transport, we used temperature blocks to synchronize the movement of cargo to and from pre-Golgi intermediates. In addition, we used mutants of ARF1 and Sar1 proteins which are restricted to GDP- or GTP-bound forms to selectively control COPI and COPII assembly and disassembly. We find that both coats are required to transport the same cargo molecule, in this case vesicular stomatitis virus glycoprotein (VSV-G), from the ER to the Golgi stack. COPII, but not COPI is required for vesicle budding and export of VSV-G from the ER leading to the build-up of pre-Golgi VTCs. COPII coats are lost rapidly following vesicle budding from the ER. In contrast, COPI is essential for the accumulation of ER-derived cargo in pre-Golgi intermediates (VTC). We find that efficient retrograde transport of p58, a marker for pre-Golgi elements (Plutner et al., 1992; Saraste and Svensson, 1991), and segregation from VSV-G occurs from VTCs. Segregation is mediated by COPI, but not COPII. p58 is efficiently returned to the ER by COPI where it accumulates in the absence of COPII function. These results demonstrate a key role for COPI in retrograde transport in mammalian cells. Collectively, these results suggest that COPII is responsible for mobilization of cargo from the ER in a reaction which is coupled to the segregation of anterograde and retrograde transported proteins from pre-Golgi elements via COPI. This segregation event appears to be essential to assure the proper anterograde transport of cargo to the Golgi stack.

Materials and Methods

Materials

Normal rat kidney (NRK) cells were maintained as described (Davidson and Balch, 1993).*Trans*-^{35}S-label was purchased from ICN Biomedicals, Inc. (Irvine, CA). Endoglycosidase H (endo H) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Texas red goat anti–mouse antibody was obtained from Molecular Probes Inc. (Eugene, OR) and FITC goat anti–rabbit immunoglobulin was purchased from Organon Teknika Corp. (West Chester, PA). Rat liver cytosol was prepared as described (Davidson et al., 1993).

Antibodies

An antisera specific for Sar1a (Kuge et al., 1994) and an antisera specific for ARF1 (0503) were generated in rabbits immunized with recombinant Sar1a (Kuge et al., 1994) and nonmyristoylated ARF1 (Weiss et al., 1989), respectively, as described (Harlow and Lane, 1988). A polyclonal antibody to the cytoplasmic tail of VSV-G was generated in rabbits immu-

nized with the carboxyl-terminal 16 amino acids of VSV-G (Indiana serotype) coupled to KLH (Plutner et al., 1992). All other antibodies used were generous gifts from the following laboratories: a monoclonal antibody against β-COP (M3A5) (Allan and Kreis, 1986) and a monoclonal antibody recognizing the cytoplasmic tail of VSV-G (P5D4) (Kreis, 1986) from Dr. T. Kreis, University of Geneva (Geneva, Switzerland); a polyclonal antibody against β-COP (EAGE) from Dr. M. G. Farquhar, University of California (San Diego, CA); a polyclonal antibody against p58 from Dr. J. Saraste, University of Bergen (Oslo, Norway); and a polyclonal antibody against yeast Sec23p which cross-reacts with a mammalian homologue (Orci et al., 1991*a*) from Dr. R. Schekman, University of California (Berkeley, CA).

Preparation of Recombinant Protein

Mutant ARF1(Q71L) and ARF1(T31N) in a pET3a vector (Dascher et al., 1994) were either expressed or coexpressed with *N*-myristoyltransferase in *E. coli* strain BL21 (DE3) (Novagen), and purified by DEAE-Sephacel (Pharmacia LKB Biotechnology, Piscataway, NJ) and Ultrogel AcA54 (Sepracor, Marlborough, MA) chromatography as described (Weiss et al., 1989). The Sar1a(H79G) mutation was introduced into the wild-type Sar1a using 4-primer PCR site-directed mutagenesis. His_6-tagged pET11d-Sar1a(T39N) (Kuge et al., 1994) and pET11d-Sar1a(H79G) were expressed in BL21 (DE3) *E. coli* and purified over nickel-nitrilotriacetic acid (Ni-NTA)-agarose (Quiagen) column and S100 Sepharcyl column (Pharmacia) as described (Rowe, 1995). All proteins were dialyzed against a buffer containing 25 mM Hepes-KOH (pH 7.2), 125 mM KOAc and 1 mM MgOAc before use in the in vitro assay. Protein concentration was determined by the Coomassie blue method (Bio-Rad Labs. Inc.) using bovine serum albumin (BSA) as a standard.

Preparation of Membranes for COPI- and COPII-binding Reactions

150-mM dishes of NRK cells were washed three times with ice-cold phosphate buffered saline (PBS) and scraped with a rubber policeman in a buffer containing 10 mM Hepes-KOH (pH 7.2) and 250 mM mannitol (buffer A). The cells were pelleted, resuspended in buffer A, and homogenized by passing the cell suspension six times through a ball-bearing homogenizer (Balch and Rothman, 1985). A postnuclear supernatant (PNS) was prepared by centrifuging the homogenate at 1,000 *g* for 10 min at 4°C.

COP-I and COPII-binding Reaction

15 μl (20–40 μg protein) of PNS was added to a binding reaction mixture containing 27.5 mM Hepes-KOH (pH 7.2), 2.75 mM MgOAc, 65 mM KOAc, 5 mM EGTA, 1.8 mM $CaCl_2$, 1 mM ATP, 5 mM creatine phosphate, 0.2 U of rabbit muscle creatine kinase (final concentrations) and 1.2–2 mg/ml of rat liver cytosol in a final volume of 200 μl on ice. Mutant proteins, GTPγS, or AlF_4– were added as indicated in the Results. The samples were incubated at 32°C for 15 min and the reaction terminated by transfer to ice. 1 ml of an ice-cold buffer containing 25 mM Hepes-KOH (pH 7.2), 2.5 mM MgOAc, and KOAc to give a final concentration of 250 mM KOAc was added. The samples were vortexed and centrifuged at 16,000 *g* for 10 min at 4°C. The supernatant was aspirated and the tubes were centrifuged for an additional 3 min at 16,000 *g* at 4°C. The residual supernatant was removed and 25 μl of a gel sample buffer (Laemmli, 1970) added. Samples were heated for 5 min at 95°C, resolved using SDS-PAGE on 12.5% or 10% gels (Laemmli, 1970), and analyzed by quantitative immunoblotting using β-COP (M3A5), Sec23, ARF1, and Sar1 specific antibodies. Binding was quantitated by densitometry using a Molecular Dynamics densitometer (Sunnyvale, CA).

Transport in Semi-intact Cells

NRK cells were infected with the tsO45 strain of VSV-G and pulse labeled with 100 μCi *Trans*-^{35}S-label at the restrictive temperature (39.5°C) as described (Plutner et al., 1992).The cells were then perforated by the swelling and scraping procedure as described (Beckers et al., 1987; Davidson and Balch, 1993). Transport from 16°C-VTCs was analyzed by pulse-labeling cells as described above. Subsequently, cells were chased for 2 h at 16°C with methionine containing α-MEM supplemented with 4% FCS in 25 mM Hepes-KOH (pH 7.2) (chase buffer) before perforation. After perforation, transport from the ER or 16°C-VTCs to the *cis*/medial-Golgi compartments was measured biochemically by following the appearance of endo H–resistant forms of VSV-G during incubation at 32°C in the presence of cytosol and ATP as described previously (Davidson and Balch, 1993; Plutner et al., 1992).

For morphological analysis of transport, cells infected as described above were permeabilized with digitonin and indirect immunofluorescence was performed as described (Plutner et al., 1992). To study transport from 16°C-VTCs, the cells were infected at 39.5°C as described above, transferred to ice, and washed twice with chase buffer. The cells were then incubated for 2 h at 16°C in chase buffer supplemented with 50 μg/ml cycloheximide to prevent VSV-G synthesis and to promote quantitative transfer of VSV-G from the ER to 16°C-VTCs. After the chase period, cells were permeabilized and transport measured using indirect immunofluorescence as described (Davidson and Balch, 1993; Plutner et al., 1992). Electron microscopy was performed as described (Bannykh and Balch, 1995).

For both biochemical and morphological transport assays, as well as for membrane-binding assays, representative experiments carried out in duplicate are presented. All experiments were repeated independently at least twice.

Results

Recruitment of COPI and COPII Coats to Microsome Membranes by Activated Sar1 and ARF1 Proteins Are Independent Events

To study the role of COPI and COPII coats in ER to Golgi transport, we first analyzed the ability of crude microsomal membranes to bind coat complexes after incubation in vitro and centrifugation to separate membrane-bound and cytosolic forms of coat complex components. COPI recruitment was measured using quantitative immunoblotting with a monoclonal antibody (M3A5) specific for β-COP (Allan and Kreis, 1986; Pepperkok et al., 1993). COPII recruitment was measured using an antibody specific for yeast Sec23p (Hicke and Schekman, 1989; Hicke et al., 1992), which cross-reacts with a mammalian homologue localized to the transitional region of the cell (Orci et al., 1991*a*; Peter et al., 1993). As shown in Fig. 1 *A* (compare *a* to *e*), both COPI and COPII coat complexes were efficiently recruited to membranes in the presence of GTPγS, a nonhydrolyzable analog of GTP. This reagent prolongs the activation of both ARF1 and Sar1p GTPases, which regulate the binding of COPI and COPII, respectively. Whereas COPI recruitment was detected in the presence of nanomolar concentrations of GTPγS (Fig. 1 *B*, *open circles*), no enhancement of COPII binding was observed. COPII binding required the addition of at least 1 μM GTPγS as evidence by the first detectable increase in the level of Sec23 bound to membranes at this concentration (Fig. 1 *B*, *closed circles*). For both coat complexes, binding was saturable (Fig. 1 *B*) and was membrane dependent (not shown). In contrast to GTPγS, addition of AlF_4–, a reagent which stabilizes ARF1 in its GTP-bound form and promotes COPI recruitment to Golgi membranes (Finazzi et al., 1994), specifically enhanced the recruitment of COPI, but not COPII, coats to total microsomal membranes (Fig. 1 *B*, *inset*).

The above results suggest that COPI and COPII coat complexes are recruited separately and independently to membranes through activation of their respective GTPases. However, GTPγS is a general activator of many GTPases and therefore only provides an indirect probe for specific recruitment of coat complexes. Therefore, we examined the recruitment of COPI and COPII coats by directly us-

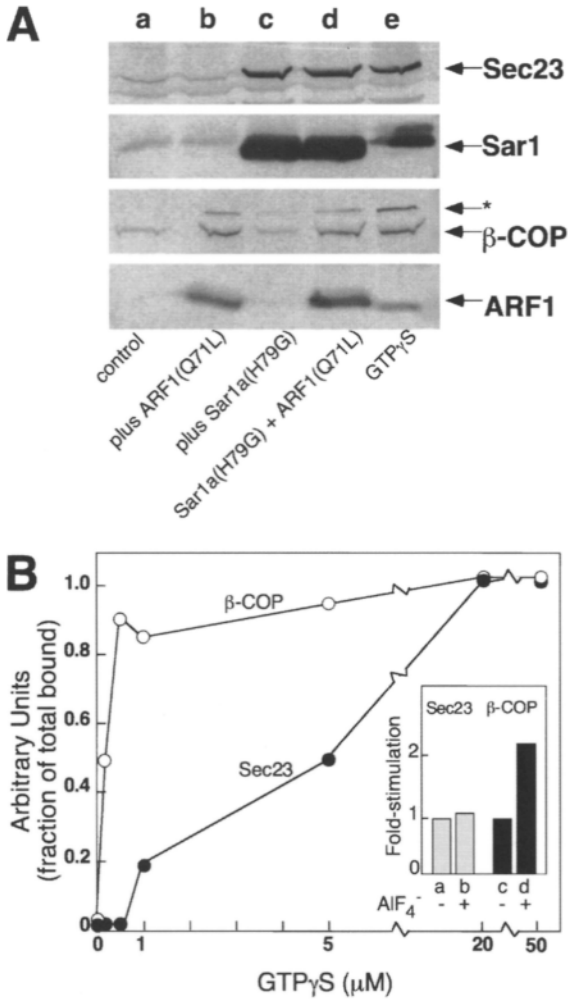

Figure 1. Recruitment of COPI and COPII coats to microsomal membranes in the presence of GTPγS, AlF_4-, and activated forms of ARF1 and Sar1a. (*A*) Microsomes were incubated (200 μl final volume) in the absence (*a*) or presence of myr-ARF1(Q71L-GTP) (13.5 μg) (*b*), Sar1(H79G-GTP)(3.5 μg) (*c*), both myr-ARF1(Q71L) and Sar1(H79G)(*d*), or 100 μM GTPγS (*e*), and the amount of Sec23, Sar1, β-COP, and ARF1 recruited to membranes determined by SDS-PAGE and immunoblotting with specific antibody as described in Materials and Methods. The asterisk indicates a band which behaves identically to β-COP and is cross-reacting with the antibody used to quantitate the recruitment of β-COP. (*B*) Microsomes were incubated in presence of the indicated levels of GTPγS. (*Inset*) AlF_4- (10 mM KF, 30 μM $AlCl_3$ [*b* and *d*] in the presence of 1 mM GTP [*a–d*]). The amount of COPI (*open circles*; based on β-COP) or COPII (*closed circles*; based on Sec23) recruited was determined by quantitative immunoblotting as described in Materials and Methods.

ing Sar1 and ARF1 mutants which remain constitutively activated and stabilize coat assembly (Dascher and Balch, 1994; Kuge et al., 1994; Oka and Nakano, 1994; Tanigawa et al., 1993; Teal et al., 1994). Incubation in the presence of the Sar1a(H79G) or myristoylated (myr) ARF1(Q71L) GTP-restricted mutants promoted the membrane-dependent recruitment of their respective coat complexes to levels equivalent to that of GTPγS (Fig. 1 *A*, compare *b* and *c* to *e*). Mutant GTPases led to specific recruitment of COPII and COPI coats to the ER and pre-Golgi intermediates composed of 60-nm vesicles and small tubular elements (referred to as VTCs), respectively, as determined morphologically (see below). These results attest to their specific roles in promoting the binding of coat complexes to only a subset of membranes present in crude microsomes.

In contrast to the specific effects of the mutant GTPases on recruitment of their respective coats, no effect on COPII binding was observed in the presence of the ARF1 GTP-restricted form, nor was there any effect on COPI binding by the addition of the activated Sar1-GTP mutant (Fig. 1 *A*, *b* and *c*). When both mutant GTPases were added to the same cocktail, the levels of recruitment observed were similar to those observed when incubated separately (Fig. 1 *A*, compare *d* to *b* and *c*). Such results demonstrate that the Sar1- and ARF1-activated mutants do not, in a dominant fashion, negatively influence the recruitment of the reciprocal coat. These results suggest that the recruitment of COPI and COPII coats are largely separate and independent events, even in a crude microsome population containing a mixture of ER, pre-Golgi VTCs, and Golgi elements. Importantly, it is evident that while Sar1 or ARF1 mutants will allow us to investigate the role of their respective coats in transport, this will be done in the context of the normal recruitment of the reciprocal coat complex.

Activated Forms of Sar1 and ARF1 Inhibit the Transport of VSV-G

We have previously used selected ARF1 and Sar1 mutants to inhibit the transport of vesicular stomatitis virus glycoprotein (VSV-G) from the ER to the Golgi stack in vivo (Dascher and Balch, 1994; Kuge et al., 1994). To address the inhibitory properties of the activated mutants of ARF1 and Sar1 on ER to Golgi transport in vitro, we examined their effects using vesicular stomatitis virus (VSV)–infected semi-intact normal rat kidney (NRK) cells, a cell population in which the plasma membrane has been perforated to expose functional ER and Golgi compartments (Beckers et al., 1987; Davidson and Balch, 1993; Plutner et al., 1992). We use the temperature-sensitive form of VSV (strain tsO45) to synchronize the export of the surface glycoprotein, VSV-G, from the ER (Balch et al., 1994; Lafay, 1974; Plutner et al., 1992). Incubation of tsO45 infected cells at the restrictive temperature (39.5°C) results in the complete retention of VSV-G in the ER (Plutner et al., 1992) (Fig. 4 *a*). Transfer of cells to the permissive temperature (32°C) results in the synchronous export of tsO45 VSV-G to the Golgi (Balch et al., 1994; Plutner et al., 1992). Cytosol and ATP-dependent transport of VSV-G from the ER to the Golgi was quantitated by following the processing of VSV-G to *cis*/medial-Golgi associated, endoglycosidase H (endo H)–resistant forms (Schwaninger et al., 1991; Davidson and Balch, 1993). As shown in Fig. 2 *A*, addition of increasing concentrations of the activated (GTP-restricted) myr-ARF1(Q71L) and Sar1a(H79G) mutants strongly inhibited the transport of VSV-G to the Golgi stack. No inhibition was observed in the case of the non-myristoylated ARF1(Q71L) mutant attesting to the specificity of the observed inhibition, nor was inhibition observed by incubation in the presence of a 10-fold excess of wild-type Sar1a (Kuge et al., 1994; not shown). Thus, while

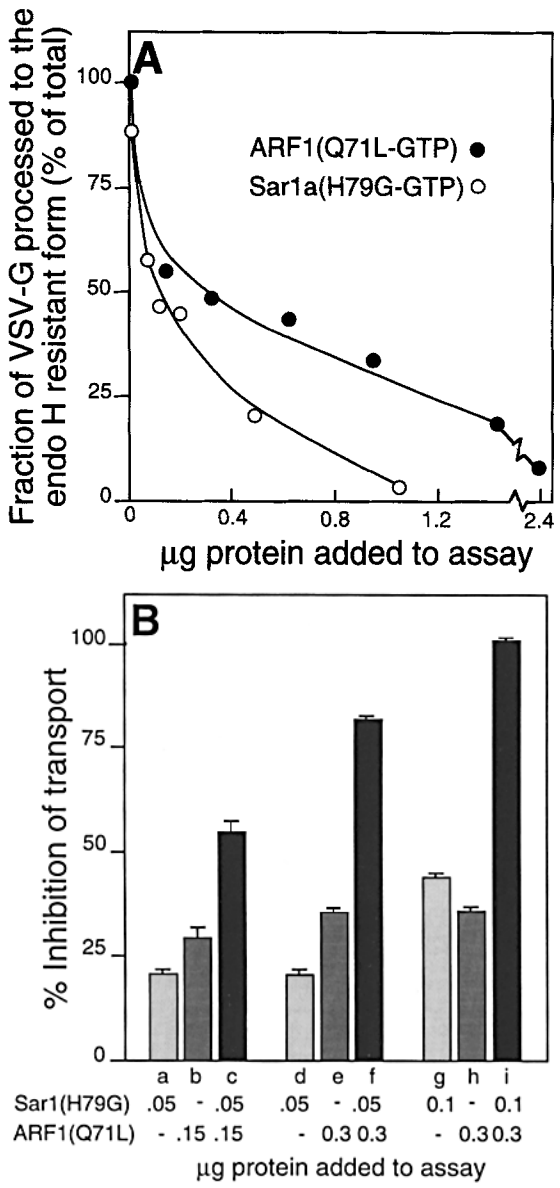

Figure 2. Activated forms of ARF1 and Sar1a inhibit ER to Golgi transport. (*A*) The transport of VSV-G in vitro in semi-intact NRK cells from the ER to the Golgi was measured in the presence of the indicated concentration of the myr-ARF1(Q71L-GTP) (*closed circles*) or the Sar1a(H79G-GTP) mutant (*open circles*) in a final volume of 40 µl as described in the Materials and Methods. The fraction of VSV-G processed to endo H–resistant forms was determined as described in Materials and Methods. (*B*) Semi-intact cells were incubated in the presence of the indicated concentrations of activated Sar1a and ARF1 mutants for 90 min at 32°C. The fraction of VSV-G processed to the endo H–resistant forms was determined and the % inhibition relative to the control (no inhibitor) reported.

it is apparent that Sar1 and ARF1 only dictate the recruitment of their cognate coat components (Fig. 1), they both inhibited the transport of VSV-G when added to the assay.

To address whether potential interactions occurred which were not detectable in the binding assay, we examined whether incubation in the presence of both mutants led to additive or synergistic effects on VSV-G transport. As shown in Fig. 2 *B*, at low concentrations of ARF1(Q71L) and Sar1(H79G), the two activated proteins inhibited transport in an additive manner (Fig. 2 *B*, *a–c*). In contrast, higher concentrations of either mutant led to reproducible, statistically significant synergistic effects (Fig. 2 *B*, *d–i*). For example, in Fig. 2 *B* (*g–i*), transport was inhibited by 44% with ARF1(Q71L) and 36% with Sar1(H79G) when added separately. Transport was inhibited nearly 100% by incubation in the presence of both mutants (Fig. 2 *B*, *i*). Thus, at low concentrations of the ARF1- and Sar1-activated mutants, each protein separately interferes with different steps in the overall pathway, a result consistent with their independent effects on COPI or COPII recruitment, respectively. However, when either wild-type Sar1 or wild-type ARF1 function becomes limiting due to displacement by the actions of their respective mutant, retention of coat components interferes in a dominant, synergistic fashion with the overall functionality of the ER to Golgi transport machinery. The combined data provide evidence that COPII and COPI may be involved in sequential events along the same pathway.

Use of Stage Specific Assays: COPII and COPI Function Precedes a Late Ca^{2+}-dependent Fusion Step

Given the ability of activated ARF1 to recruit COPI to the

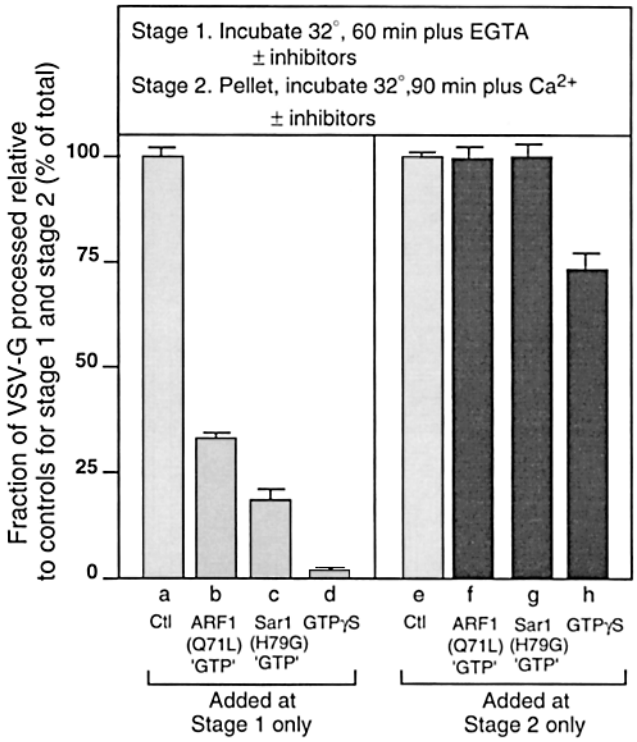

Figure 3. Inhibition by activated ARF1 and Sar1 mutants precedes a late Ca^{2+}-dependent fusion step. Semi-intact NRK cells were incubated (40 µl final volume) in a first stage (Stage 1) for 60 min at 32°C in the presence EGTA and in the absence (*a*, *e–h*) or presence (*b–d*) of the indicated inhibitor myr-ARF1(Q71L-GTP) (4 µg); Sar1(H79G-GTP) (1 µg); GTPγS (100 µM)]). After a brief pelleting (4 min, 500 *g*), semi-intact cells were resuspended in complete transport cocktail lacking further additions (*a–e*), or supplemented with the indicated inhibitor (*f–h*) and incubated for an additional 90 min at 32°C. The fraction of VSV-G processed to endo H–resistant forms was determined as described in Materials and Methods.

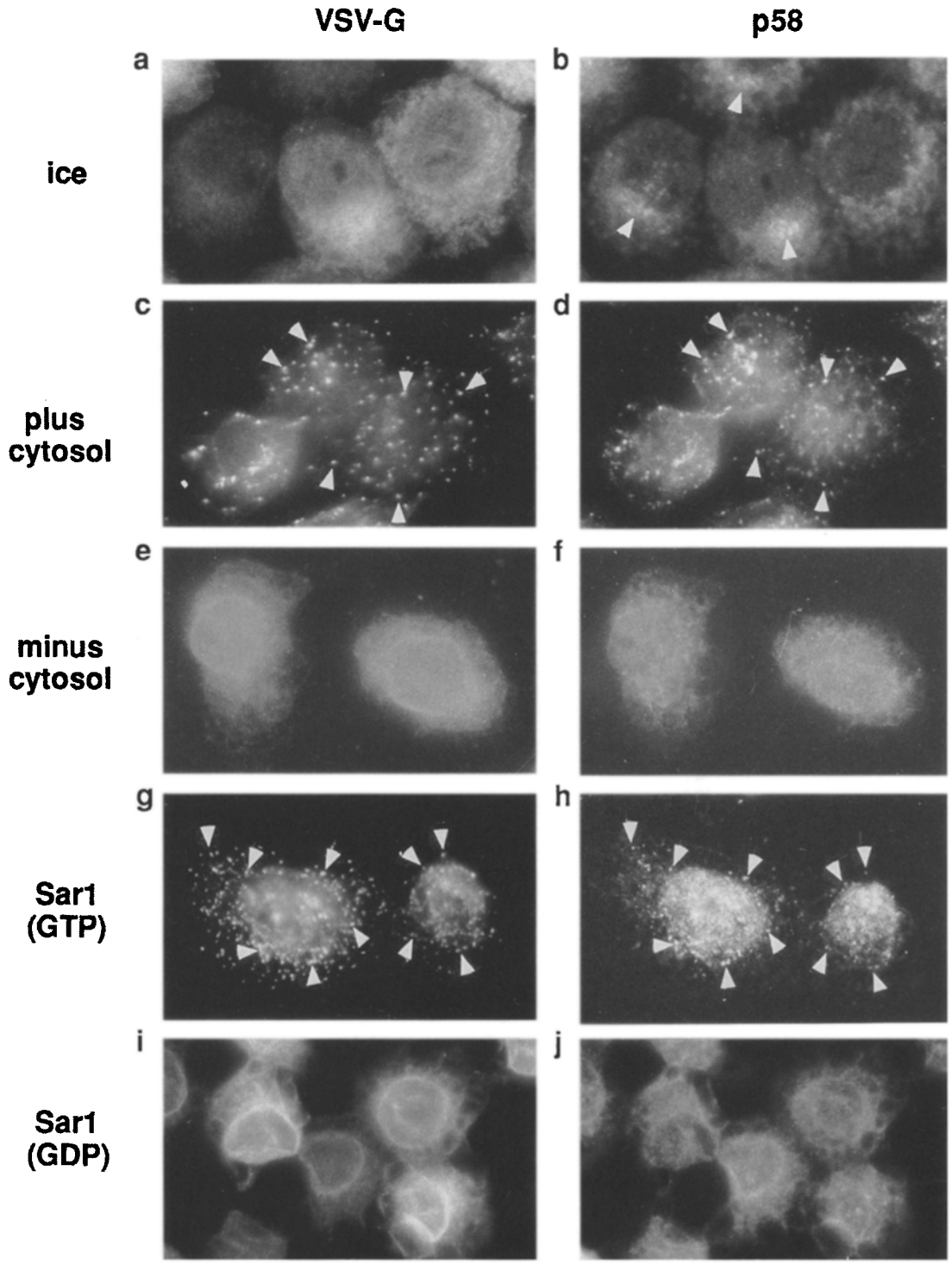

Figure 4. Accumulation of VSV-G and p58 in pre-Golgi VTCs requires COPII. (*a–j*) Digitonin-permeabilized NRK cells were incubated (200 μl final volume) in the absence (*e* and *f*) or presence of cytosol (*a–d*, *g–j*) for 4 h at reduced temperature (16°C) (*c–j*) in vitro with the indicated supplements ([*g* and *h*] Sar1(H79G-GTP) [7.3 μg]; [*i* and *j*] Sar1(T39N-GDP) [4.7 μg]). The distribution of VSV-G and p58, a marker for pre-Golgi intermediates (Saraste et al., 1987), was determined by indirect immunofluorescence using P5D4 (VSV-G) and a polyclonal antibody specific for p58 as described in Materials and Methods.

Golgi region (Tanigawa et al., 1993; Teal et al., 1994), we were concerned that the transport block observed above by the GTP-restricted ARF1(Q71L) mutant was possibly a consequence of inhibition of the acceptor function of Golgi compartments. To examine this possibility, we conducted a two stage in vitro transport reaction taking advantage of the fact that Ca^{2+} is required at a late step preceding fusion of VTCs to the Golgi stack, but not for vesicle budding from the ER (Balch et al., 1994; Beckers and Balch, 1989; Pind et al., 1994). In these experiments,

semi-intact NRK cells, cytosol, and ATP were incubated in the absence of Ca^{2+} (by addition of EGTA) for 60 min to accumulate VSV-G in clusters of 60–70 nm vesicular carriers and small tubular elements (referred to as EGTA-VTCs) which are largely indistinguishable from normal VTCs (Balch et al., 1994; Pind et al., 1994) (Fig. 3, stage 1). Subsequently, cells were pelleted and resuspended in a cocktail which was supplemented with Ca^{2+}, and incubated for an additional 90 min to promote transport of VSV-G to the Golgi stack (Fig. 3, stage 2). Addition of GTPγS, or activated ARF1 and Sar1 mutants in the first stage of the two-stage incubation, significantly blocked transport during subsequent incubation in stage 2 in their absence (Fig. 3, *a–d*). In contrast, addition of either GTPγS or the activated mutants to stage 2 after accumulation in EGTA-VTCs had little or no effect on transport in the presence of Ca^{2+} (Fig. 3, *e–h*). Since EGTA per se does not interfere with COPI recruitment (see below), these results provide strong evidence that the activated forms of Sar1 or ARF1 do not inhibit transport by blocking acceptor (Golgi) function, rather their sites of action are on earlier steps in the transport of VSV-G from the ER through pre-Golgi intermediates.

COPII but Not COPI Is Required for ER Export

The independent recruitment of COPII and COPI coats, combined with the complete inhibitory effects of activated Sar1 and ARF1 mutants on transport to EGTA-VTCs, suggested that a multi-step transport process is responsible for the delivery of VSV-G from the ER through pre-Golgi intermediates to the Golgi stack (Pind et al., 1994). Since these sequential steps are not evident in our biochemical assay, we followed VSV-G transport from the ER to the Golgi stack morphologically using indirect immunofluorescence. We took advantage of the fact that incubation of cells at reduced temperature (16°C) results in the accumulation of anterograde transported proteins such as VSV-G and recycling proteins such as p58 (a frequently used marker for pre-Golgi intermediates [Saraste et al., 1987; Saraste and Svensson, 1991]) in numerous pre-Golgi VTCs which reside adjacent to budding sites of the ER scattered throughout the peripheral cytoplasm and adjacent to the *cis* face of the peri-nuclear Golgi stack (Balch et al., 1994; Lotti et al., 1992; Oprins et al., 1993; Plutner et al., 1992). These structures show no continuity with the ER and lack ER marker proteins such as calnexin (Saraste and Svensson, 1991; not shown). While incubation at 32°C results in movement of protein to and from pre-Golgi intermediates and the Golgi stack in a rapid and highly dynamic fashion, incubation in vitro at 16°C allows us to isolate the first step in delivery of cargo to VTCs from other steps of the early secretory pathway.

Accumulation of VSV-G in pre-Golgi intermediates in

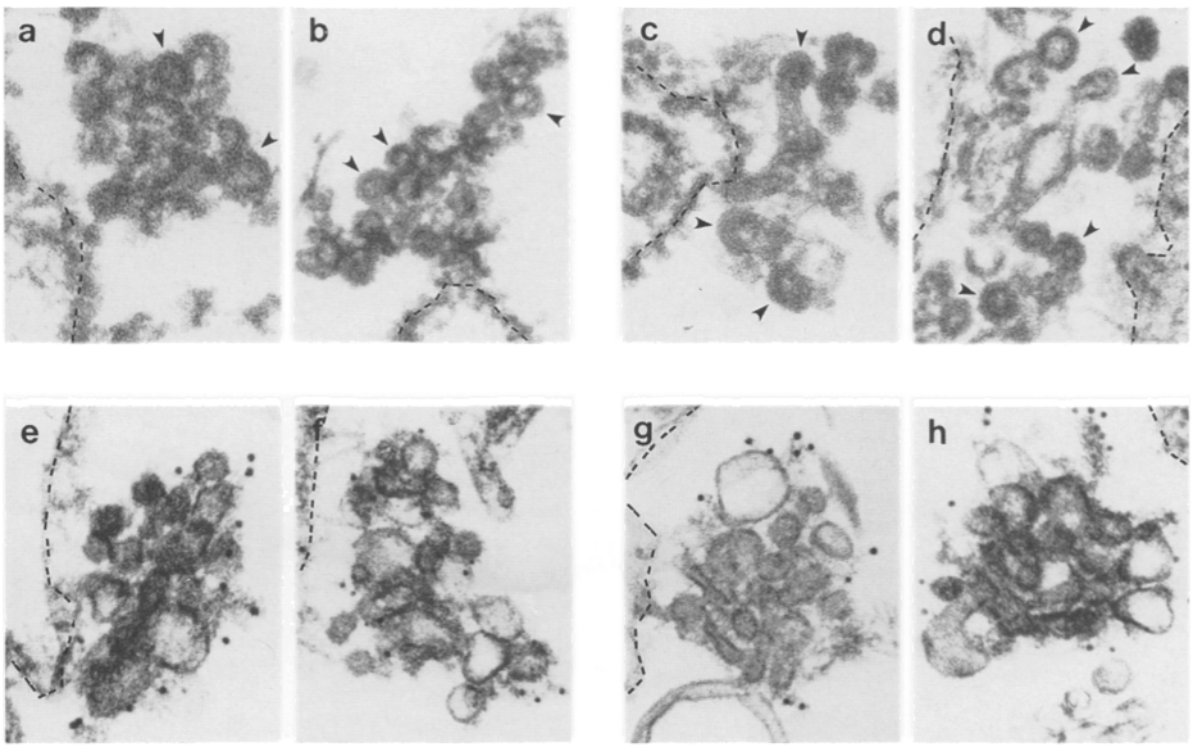

Figure 5. VSV-G accumulates in coated VTCs during incubation at reduced temperature.(*a–d*) Permeabilized cells were incubated (200 μl final volume) for 4 h at 16°C before preparation for electron microscopy as described in Materials and Methods in the presence of Sar1(H79G-GTP) (7.3 μg) (*a* and *b*) and ARF1(Q71-GTP) (25 μg) (*c* and *d*). Arrowheads indicate the distribution of coats on typical 16°C-VTCs consisting of clusters of 60-nm vesicles and small tubular elements. (*e–h*) VSV-G was accumulated in 16°C-VTCs for 2 h in vivo, cells were permeabilized and further incubated for 30 min at 32°C in the presence of Sar(H79G-GTP) (*e* and *f*) or ARF1(Q71L-GTP) (*g* and *h*) as described in Results. The distribution of VSV-G was determined using immunoelectron microscopy as described in Materials and Methods. The dotted lines indicate the ER membrane not evident in the cropped image.

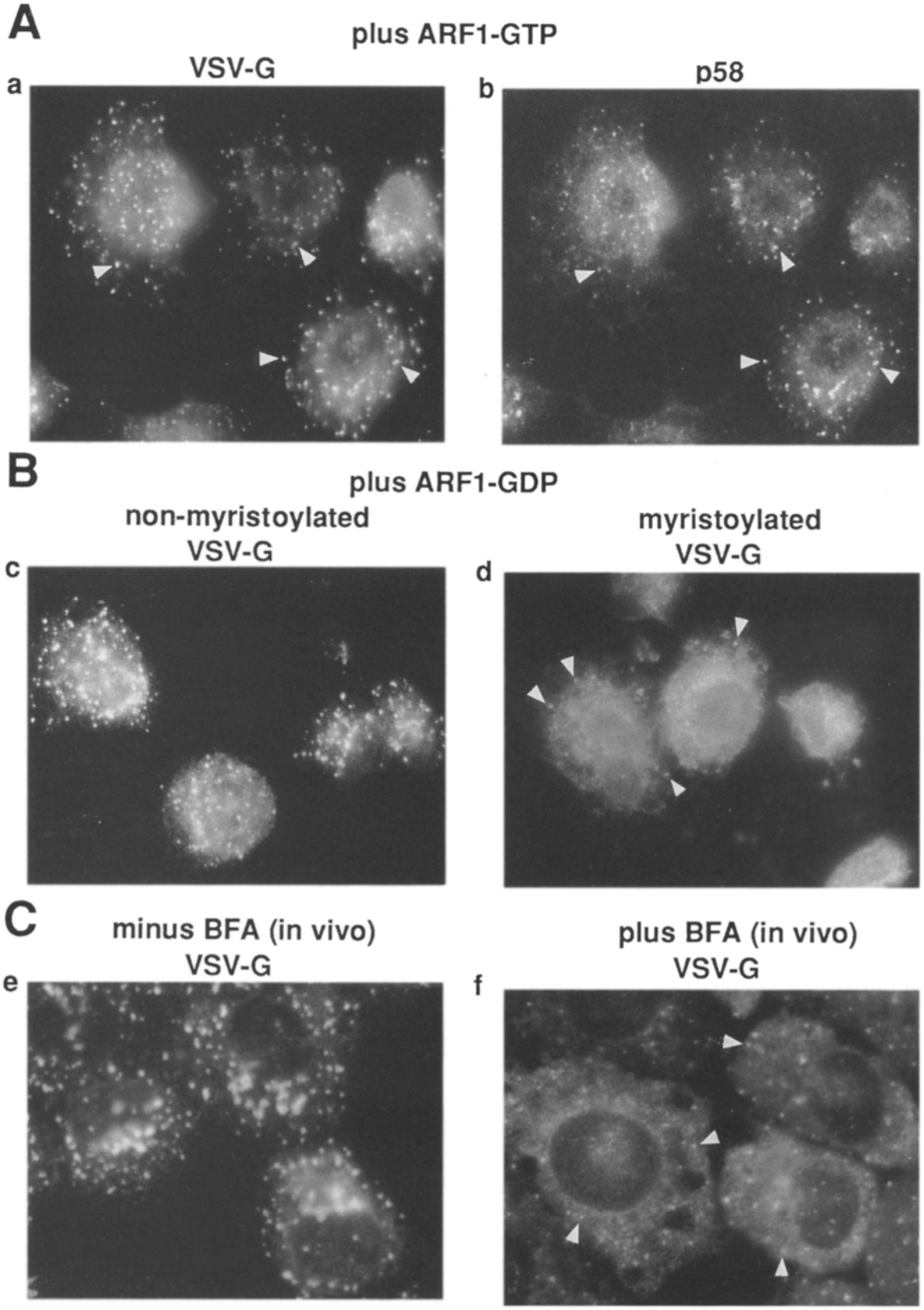

Figure 6. Effects of ARF1 mutants on export of VSV-G from the ER at reduced temperature. (*A* and *B*) Semi-intact cells were incubated (200 μl final volume) at reduced temperature (16°C) for 4 h in the presence of myr-ARF1(Q71L-GTP) (25 μg) (*a* and *b*) or the nonmyristoylated (*c*) and myristoylated (*d*) forms ARF1(T31N) (25 μg). The distribution of VSV-G (*a*, *c*, and *d*) and p58 (*b*) was determined using indirect immunofluorescence as described in Materials and Methods. (*C*) Intact NRK cells were incubated at 16°C in the absence (*e*) or presence (*f*) of BFA (15 μg/ml). The distribution of VSV-G (*e* and *f*) was determined using indirect immunofluorescence as described in Materials and Methods.

vivo normally requires incubation for 90–180 min at reduced temperature (Beckers and Balch, 1989; Beckers et al., 1989, 1990; Plutner et al., 1992; Saraste and Svensson, 1991). In vitro, we found that VSV-G and p58 first appeared in VTCs at 30–60 min and continued to accumulate in pre-Golgi VTCs for up to 4 h (not shown), reflecting the reduced kinetics of transport in permeabilized cells (~2–3-fold) (Davidson and Balch, 1993; Plutner et al., 1992). In the presence of cytosol, VSV-G accumulated in 16°C-VTCs as indicated by the strong overlap in the distribution of VSV-G and p58 (Fig. 4, *c* and *d*, *arrowheads*). Transport of VSV-G to VTCs was ATP (not shown) and cytosol dependent (Fig. 4, compare *e* to *c*). In the absence of cytosol, the distribution of p58, which at the beginning of the transport reaction can be readily detected in the perinuclear *cis*-Golgi region (Fig. 4 *b*, *arrowhead*) (Plutner et al., 1992; Schwaninger et al., 1992), redistributed to a more diffuse ER-like pattern (Fig. 4, compare *f* to *d*). In this particular case, the absence of cytosolic COPI may lead to some retrograde fusion of Golgi compartments to the ER similar to the effects of brefeldin A (BFA) in vivo (for a review see Lippincott-Schwartz, 1993).

To define the role of COPII and COPI in the transport of VSV-G to VTCs, we incubated permeabilized cells at 16°C in the presence of the activated ARF1 and Sar1 mutants. When cells were incubated with the activated form of Sar1 (Sar1[H79G]) to stabilize newly recruited COPII coats, VSV-G was exported from the ER and concentrated in structures containing p58 (Fig. 4, *g* [VSV-G] and *h* [p58], *arrowheads*). However, these VSV-G–containing sites were more numerous and had a weak punctate appearance distinct from the control incubation in the same experiment (compare Fig. 4 *g* to *c*) suggesting a structure different from that of normal VTCs. When examined, using transmission electron microscopy (TEM) (Fig. 5, *a* and *b*), punctate structures accumulated in the presence of Sar1(H79G) were typically a collection of 60-nm vesicles which were organized as "clusters of grapes", were frequently found to be associated with budding structures on the ER and lacked the small tubular, pleomorphic elements characteristic of normal VTCs. These structures had a distinctive coat (Fig. 5, *a* and *b*, *arrowheads*), presumably COPII, and contained VSV-G when examined, using immunoelectron microscopy (Balch et al., 1994; not shown).

In contrast to the effects of the GTP-restricted Sar1 mutant, addition of the Sar1 mutant restricted to the GDP-bound form (Sar1[T39N]) which blocks recruitment of wild-type Sar1 by interfering with GEF function (Kuge et al., 1994) during incubation at 16°C completely abolished the accumulation of VSV-G in 16°C-VTCs (Fig. 4 *i*) as reported previously for 32°C (Kuge et al., 1994). This was associated with a striking redistribution of p58 to the ER (Fig. 4 *j*) compared to normal incubation conditions (Fig. 4 *d*), and a near complete absence of pre-Golgi VTCs in semi-intact cells when examined using TEM (not shown). The retention of p58 in the ER suggests that the export of both p58 and VSV-G from the ER is COPII dependent whereas p58 recycling may be COPII independent.

COPI Is Required to Stabilize Pre-Golgi Intermediates

While the Sar1-GTP–restricted mutant prevented the accumulation of larger more pleomorphic VTCs, incubation in the presence of the activated ARF1(Q71L) mutant did not inhibit the accumulation of either VSV-G or p58 in VTCs. Indeed, their level of overlap was striking (>80%) (Fig. 6, *a* and *b*, *arrowheads*). The stabilization of these structures by COPI is in agreement with previous results which have demonstrated that pre-Golgi intermediates are one of the principle sites of COPI binding in vivo and in vitro (Duden et al., 1991; Oprins et al., 1993; Pepperkok et al., 1993; Peter et al., 1993; Pind et al., 1994). Examination using TEM revealed that typical VSV-G–containing VTCs accumulated in the presence of ARF1(Q71L) have regions of dense cytoplasmic coats and tubular elements diagnostic of normal VTCs (Fig. 5, *c* and *d*, *arrowheads*).

The ARF1(T31N) mutant, which is restricted to the GDP-bound form and inhibits ARF1 activation and COPI recruitment (Dascher and Balch, 1994), also blocked transport in vitro by ~90% as measured by inhibition of processing of VSV-G to the Golgi associated endo H–resistant forms (Fig. 7). ARF1(T31N) did not, however, block ER export when examined using indirect immunofluorescence, although accumulation of VSV-G in 16°C-VTCs was inefficient (Fig. 6 *d*, *arrowheads*) compared to the nonmyristoylated ARF1(T31N) control (Fig. 6, compare *d* to *c*). This result was similar to the effects of BFA on the accumulation of VSV-G in punctate VTCs in vivo at 16°C (Fig. 6, *e* [minus BFA] and *f* [plus BFA]). A number of lines of evidence suggest that both BFA and the ARF1 GDP-restricted mutant function by preventing COPI coat assembly (Dascher and Balch, 1994; Elazar et al., 1994; Lippincott-Schwartz, 1993). Thus, it is apparent that at least one function of COPI is to stabilize the formation of VTCs from ER-derived COPII vesicles.

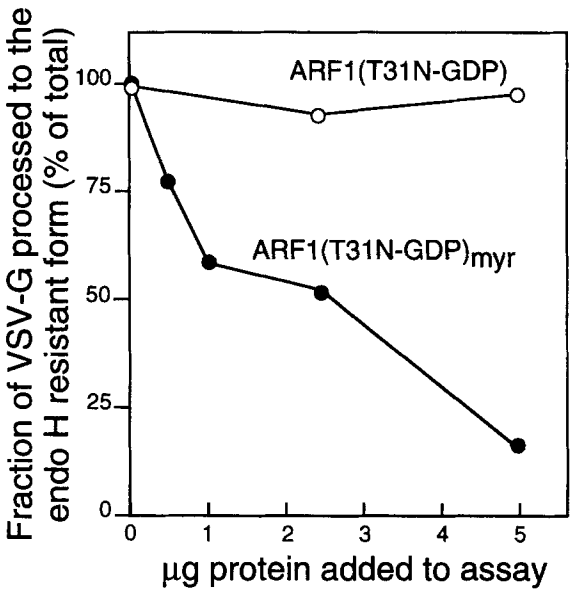

Figure 7. Effects of GDP-restricted ARF1(T31N-GDP) on transport of VSV-G to the Golgi. Semi-intact cells were incubated (40 µl final volume) at 32°C for 90 min in the presence of the indicated concentration of the nonmyristoylated (*open circles*) or myristoylated (*closed circles*) ARF1(T31N-GDP). The fraction of VSV-G processed to endo H–resistant forms was determined as described in Materials and Methods.

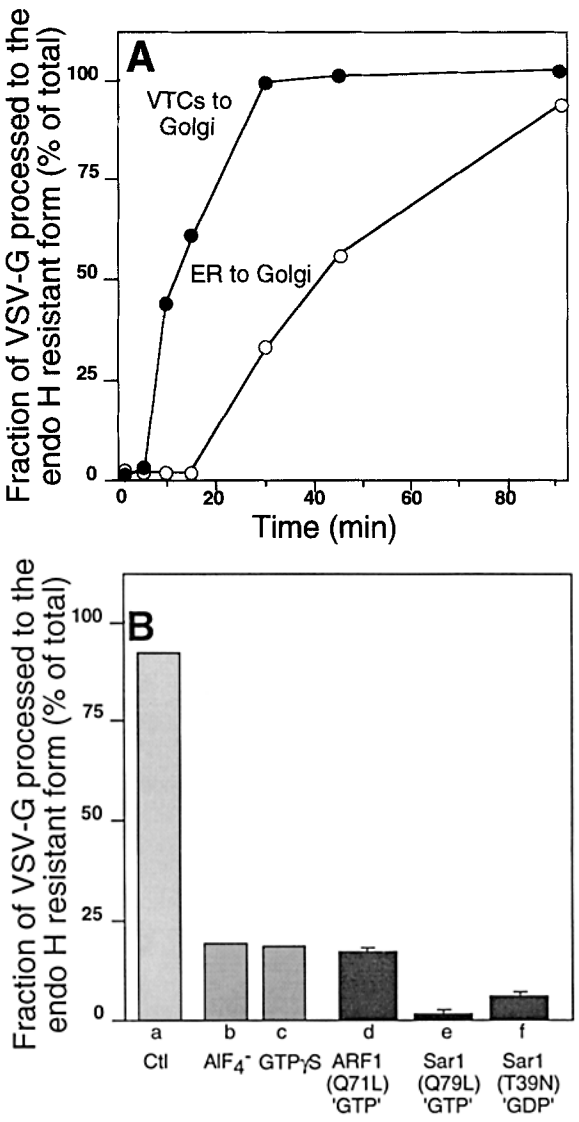

Figure 8. Transport from 16°C-VTCs to the Golgi stack is sensitive to GTPγS, AlF_4-, and Sar1a and ARF1 trans dominant mutants. (*A*) (*Open circles*) After infection at the restrictive temperature (4.5 h), semi-intact cells were pulse-labeled at 39.5°C, transferred to ice, perforated, and incubated in vitro at 32°C for the indicated time. The fraction of VSV-G processed to endo H–resistant forms was determined as described in Materials and Methods. (*closed circles*) After infection at the restrictive temperature (4.5 h), cells were pulse-labeled at 39.5°C, and then rapidly equilibrated to 16°C and incubated for a total time of 2 h to mobilize VSV-G to 16°C-VTCs before preparation of semi-intact cells as described in Materials and Methods. Semi-intact cells were subsequently incubated in vitro for the indicated time at 32°C (*closed circles*). The fraction of VSV-G processed to endo H–resistant forms was determined as described in Materials and Methods. (*B*) Semi-intact cells prepared from cells preincubated in vivo for 2 h at 16°C were subsequently incubated (40 μl final volume) at 32°C for 30 min in vitro in a cocktail supplemented with the indicated inhibitors: (*a*) none, (*b*) 10 mM KF, 30 μM $AlCl_3$, (*c*) 100 μM GTPγS, (*d*) 2.7 μg ARF1(Q71L-GTP), (*e*) 1 μg Sar1(H79G-GTP), and (*f*) 1 μg Sar1(T39N-GDP). The results are reported as fraction of total VSV-G processed to endo H–resistant forms as described in Materials and Methods.

The Effect of ARF1 and Sar1 Mutants on Transport of VSV-G from 16°C-VTCs to the Golgi Stack

To address more directly the role of COPI in transport from pre-Golgi intermediates, cells were incubated for 2 h in vivo at reduced temperature to accumulate VSV-G and p58 in 16°C-VTCs before perforation. VSV-G accumulated in VTCs at 16°C was present in the endo H–sensitive form before incubation in vitro (Fig. 8 *A*, 0 min, *closed circles*) attesting to the efficiency of the low temperature block. However, when transport was initiated by incubation at 32°C in the presence of cytosol and ATP, VSV-G was rapidly transported to the Golgi stack as indicated by the short lag period (~5 min) (Fig. 8 *A*, *closed circles*) compared to the much longer lag (15–20 min) required for export from the ER (Fig. 8 *A*, *open circles*) in the same experiment (Davidson et al., 1992; Plutner et al., 1992). Moreover, transport to the Golgi stack from 16°C-VTCs was complete within 30 min (Fig. 8 *A*, *closed circles*) as opposed to the 90-min incubation period required for transport from the ER (Fig. 8 *A*, *open circles*). We therefore used 30-min incubations at 32°C in subsequent experiments to focus on transport from 16°C-VTCs to the Golgi stack.

Transport from 16°C-VTCs was cytosol and ATP dependent (Beckers and Balch, 1989; Beckers et al., 1990) and was insensitive to incubation at the restrictive temperature (39.5°C), a temperature which inhibits export from the ER in vitro (not shown). Transport was also sensitive to AlF_4- (Fig. 8, *B b*) and GTPγS (Fig. 8, *B c*) (Beckers and Balch, 1989; Beckers et al., 1990). As expected, addition of the activated ARF1 mutant potently inhibited transport (Fig. 8, *B d*). Surprisingly, the addition of the activated Sar1(H79G) mutant, as well as the GDP-bound Sar1(T39N) mutant also inhibited transport to the Golgi (Fig. 8, *B*, *e–f*). Thus, unlike EGTA-VTCs, which are independent of COPII and COPI function, 16°C-VTCs are a novel intermediate in the pathway in transition from a COPII to a COPI-dependent state. The effects of both the Sar1 and ARF1 mutants on this step suggest that 16°C-VTCs are immature intermediates which may still retain receptors for components of both coat complexes.

Morphological Analysis of Sar1 and ARF1-dependent Transport of VSV-G and p58 from 16°C-VTCs. Role for COPI in p58 Recycling

To explore morphologically the potential roles of Sar1 and ARF1 on transport from pre-Golgi intermediates, cells were incubated for 2 h at 16°C before permeabilization. Accumulation of VSV-G and p58 in 16°C-VTCs before preparation of semi-intact cells allows us to focus not only on the anterograde transport of VSV-G from this step to the Golgi stack, but, in addition, the retrograde transport of p58 to the ER as noted previously (Fig. 4 *j*). Before incubation in vitro, the distribution of VSV-G and p58 nearly completely overlapped in VTCs which appeared as scattered punctate elements distributed throughout the cytoplasm (Fig. 9, *a* [VSV-G] and *b* [p58]). Incubation in vitro for 30 min at 32°C led to the delivery of VSV-G to perinuclear Golgi elements (Fig. 9 *c*) which overlapped with the distribution of α-1,2-mannosidase II (Man II) (not shown). During incubation, overlap between p58 and VSV-G in the peripheral cytoplasm was markedly decreased (Fig. 9,

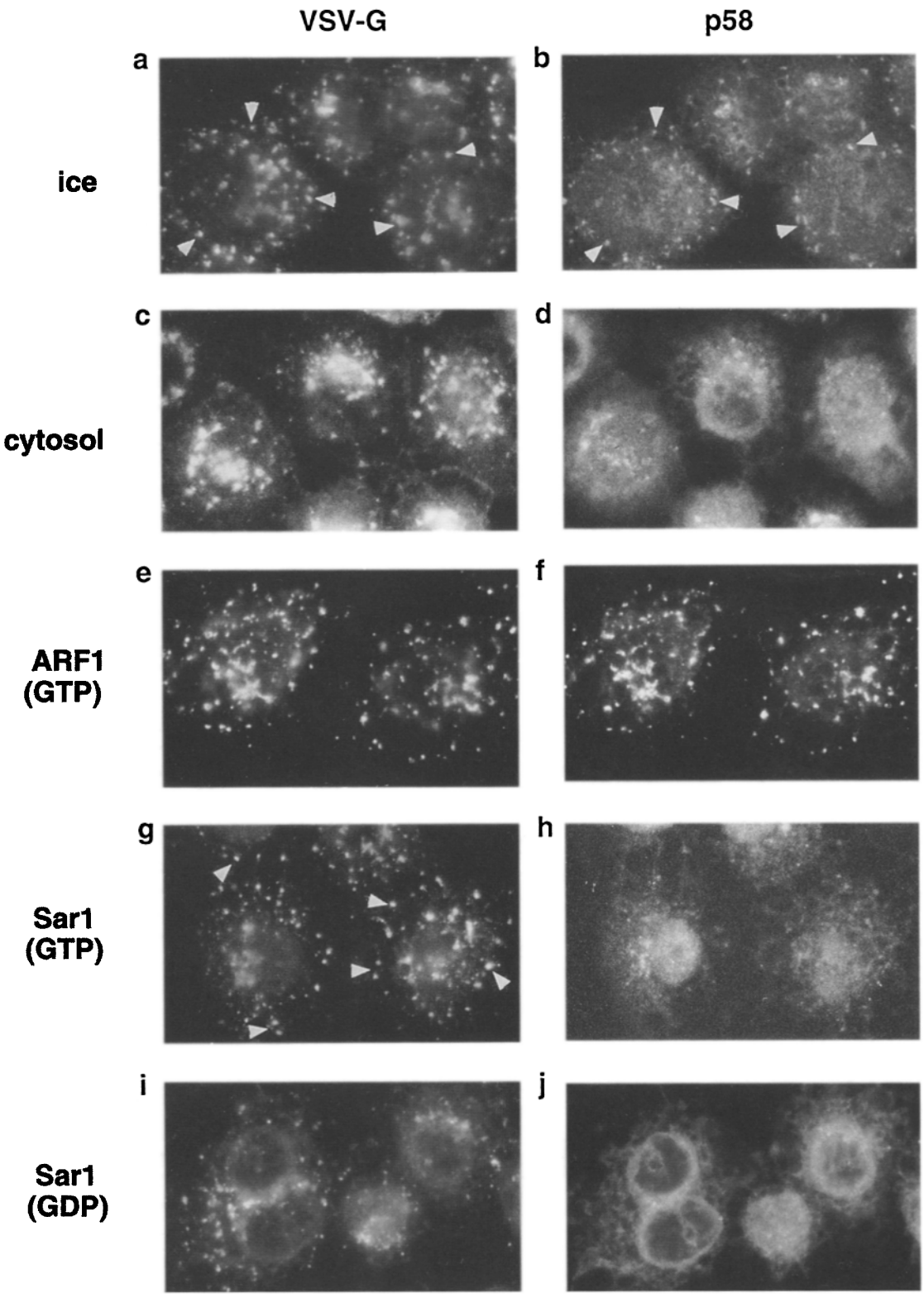

Figure 9. p58 is segregated from VSV-G in VTCs via a COPI dependent/COPII-independent pathway. (*a–j*) NRK cells were preincubated for 2 h at reduced temperature (16°C) to accumulate VSV-G in VTCs before permeabilization as described in Materials and Methods. Cells were incubated (200 μl) in vitro in the presence of cytosol at 32°C (*c–j*) with the indicated supplements (ARF1(Q71L-GTP) (25 μg) [*e* and *f*]; Sar1(H79G-GTP) (7.3 μg) [*g* and *h*]; Sar1(T39N-GDP) (4.7 μg) [*i* and *j*]) and the distribution of VSV-G and p58 determined by indirect immunofluorescence as described in Materials and Methods. Arrowheads indicated the distribution of punctate elements which contain both VSV-G and p58 (*a* and *b*) or lack of colocalization of VSV-G with p58 (*g* and *h*).

c and *d*) suggesting that segregation of p58 and VSV-G was in progress. Addition of the activated ARF1(Q71L) mutant resulted in complete stabilization of the overlap of VSV-G and p58 during the 30-min incubation at 32°C in the presence of cytosol and ATP (Fig. 9, *e* [VSV-G] and *f* [p58]). This observation was confirmed using immunoelectron microscopy where VSV-G could be readily detected in VTCs (Fig. 5, *g* and *h*), but not the Golgi stack (not shown).

While the activated ARF1 mutant prevented the export of both p58 and VSV-G from 16°C-VTCs, the Sar1 GDP-restricted mutant did not interfere with the exit of p58 from these pre-Golgi intermediates and led to its redistribution to the ER (Fig. 9, compare *j* to *b*). These results reinforce the observation that retrograde transport is COPII independent. In contrast, VSV-G was principally retained in numerous punctate sites in the presence of the Sar1-GDP restricted mutant (Fig. 9 *i*), consistent with the effects of this mutant on inhibiting processing to the endo H–resistant form (Fig. 8 *B, f*), but suggesting that COPII function in regards to anterograde transport was incomplete. Incubation in the presence of the activated Sar1(H79G) also resulted in the retention of VSV-G in VTCs (Fig. 9 *g*) as indicated by their vesicular-tubular structure using immunoelectron microscopy (Fig. 5, *e* and *f*). Although segregation of p58 from VSV-G was readily detected (Fig. 9, *g* [VSV-G] and *h* [p58]), distribution of p58 in VTCs was not inhibited to the same extent observed during incubation in the presence of the Sar1-GDP mutant (Fig. 9, compare *h* [Sar1-GTP] to *i* [Sar1-GDP]). This would be expected if p58 was recycled to the ER, but re-exported to VTCs through the recruitment and stabilization of COPII coats by the GTP-restricted mutant.

Thus, it is apparent that we have identified conditions which dissect retrograde recycling to the ER from anterograde transport to the Golgi. Since the general recruitment of COPI coats is unaffected by the Sar1-GDP mutant (see below), these results suggest that pre-Golgi intermediates are the first site of retrograde transport and suggest that recycling of p58 is mediated by a sorting event involving COPI.

Localization of COPI and COPII on ER to Golgi Transport Intermediates

To gain further insight into the role of COPI and COPII coats in transport, we analyzed the intracellular localization of β-COP (COPI) and Sec23 (COPII) in semi-intact NRK cells using indirect immunofluorescence. At steady-state, Sec23 is localized to the ER and β-COP to pre-Golgi intermediates in the perinuclear and peripheral cytoplasm (not shown) (Orci et al., 1991*a*; Peter et al., 1993; Kuge et al. 1994). When VSV-G was accumulated in 16°C-VTCs in vitro, its distribution was found to overlap with the distribution of β-COP in punctate intermediates scattered throughout the peripheral cytoplasm (Fig. 10, *a* and *b*). In contrast, Sec23 was largely absent from punctate VSV-G containing VTCs and distributed principally in a weak ER/nuclear envelope reticular-staining pattern (Fig. 10, *c* and *d*). When cells containing VSV-G in 16°C-VTCs were incubated in vitro at 32°C in the presence of activated Sar1(H79G) mutant, we noted a striking recruitment of Sec23 to punctate elements which overlapped with the distribution with VSV-G (Fig. 10, *g* and *h*). Under these conditions, colocalization with p58 was completely lost (Fig. 9, *g* and *h*). These findings support our previous observations that the addition of Sar1(H79G) strongly promoted the binding of Sec23 to microsomes (Fig. 1) and inhibited transport from 16°C-VTCs to the Golgi stack (Fig. 8 *B* and Fig. 9 *g*). Thus, VTCs accumulated at reduced temperature still contain functional receptors for Sar1 and/or COPII components.

In contrast to the effects of GTP-restricted Sar1 mutant on β-COP and Sec23 recruitment, incubation of 16°C-VTCs with the activated ARF1(Q71L) mutant resulted in a marked overlap of both p58 (Fig. 9, *e* and *f*) and β-COP (Fig. 10, *e* and *f*). No accumulation of Sec23 was detected under these circumstances (not shown). The recruitment of β-COP to VTCs and to the pre-Golgi region in the presence of ARF1-GTP confirms our previous observation that addition of activated ARF1 strongly promotes the binding of β-COP to microsomes (Fig. 1 *A*). Since the accumulation of Sec23 on 16°C-VTCs cannot be observed unless the Sar1p GTPase activity is blocked (either by activated Sar1 or GTPγS [not shown]), these results suggest that under normal transport conditions, COPII coats are rapidly lost following vesicle budding, but that VTCs still retain Sar1/COPII receptors. This result is consistent with the fact that incubation of semi-intact cells at 32°C in the presence of GTPγS without preincubation at reduced temperature also leads to a strong overlap in the distribution of β-COP and Sec23 (not shown).

To directly examine the sequential order of action of COPII and COPI coats in transport, both the Sar1-GDP–restricted form and the ARF1-GTP–restricted form were added to the same assay cocktail in the presence of semi-intact cells which contained VSV-G in the ER. Incubation at 32°C in the presence of both mutants resulted in the complete retention of VSV-G in ER (Fig. 11 *c*), similar to the effects of Sar1-GDP–restricted form alone (Fig. 11 *a*). While the ARF1-GTP led to striking recruitment of COPI to the Golgi region (Fig. 11 *d*), as observed in the presence of ARF1-GTP alone to 16°C-VTCs (Fig. 10 *f*), this result was not observed in the presence of Sar1-GDP alone (Fig. 11 *b*). Thus, the GTP-restricted form of ARF1 stabilizes pre-existing VTCs while Sar1-GDP prevented COPII vesicle formation and export of VSV-G from the ER. These results establish that COPII temporally precedes COPI function in ER to Golgi transport.

EGTA-VTCs Are Mature VTCs which Have Already Segregated p58

While transport of VSV-G from 16°C-VTCs is sensitive to both activated ARF1 and Sar1 mutants (Fig. 8 *B*), migration to the Golgi stack from EGTA-VTCs was insensitive to these mutants (Fig. 3). Therefore, we examined whether COPI binding could still be detected during the generation of EGTA-VTCs and whether p58 segregation can occur in the absence of Ca^{2+}. We noted using indirect immunofluorescence that VTCs which have accumulated VSV-G in the presence of EGTA were largely deficient in p58 staining (not shown), suggesting that recycling had occurred. To visualize this segregation event, VSV-G and p58 were

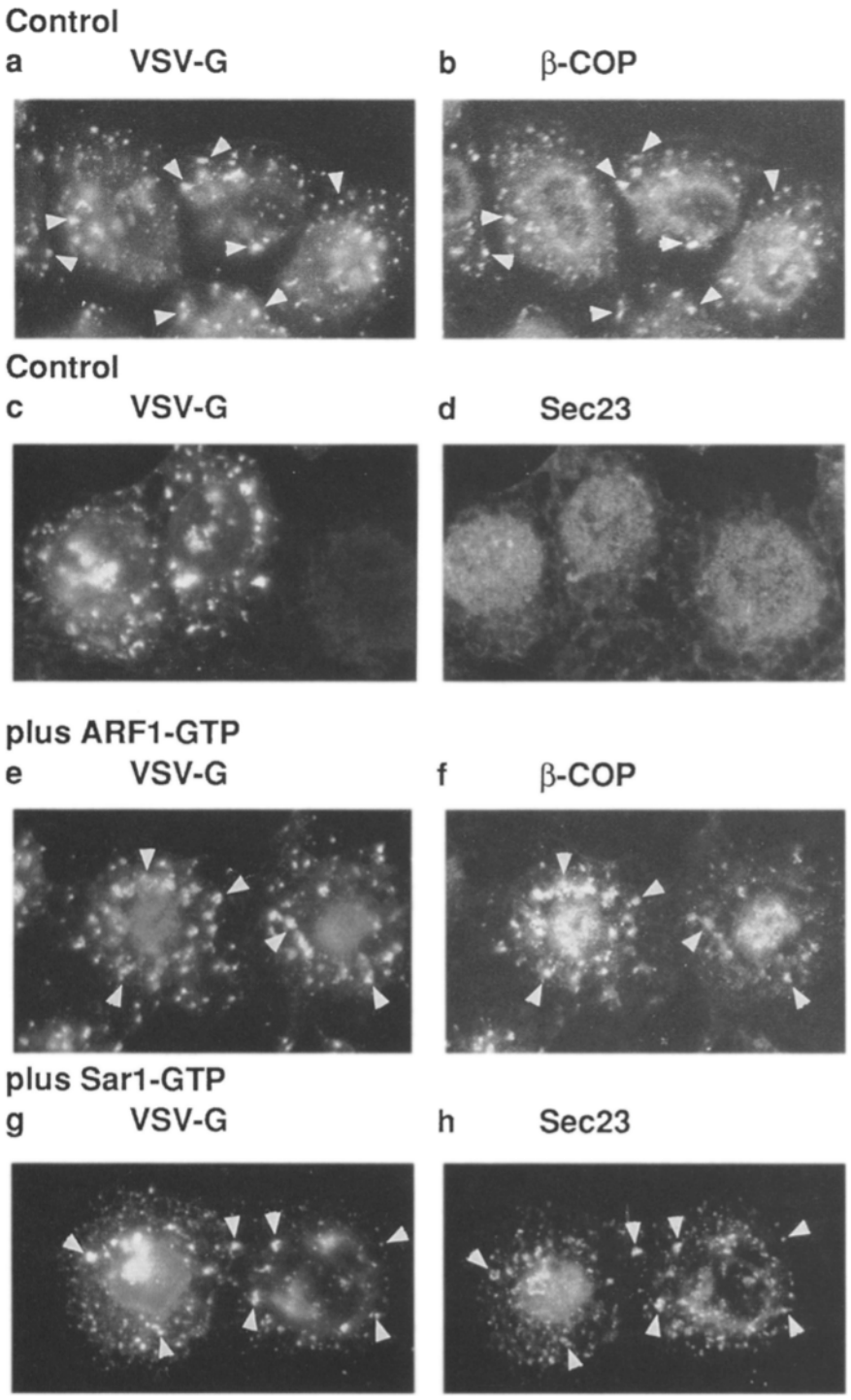

Figure 10. The effect of ARF1(Q71L-GTP) and Sar1(H79G-GTP) on the recruitment of COP coats to 16°C-VTCs. NRK cells were preincubated for 2 h at reduced temperature (16°C) to accumulate VSV-G in VTCs in vivo before permeabilization. Semi-intact cells were subsequently either held on ice (*a–d*) or incubated at 32°C (*e–h*) for an additional 30 min in the presence of ARF1(Q71L-GTP) (25 μg) (*e* and *f*) or Sar1 (H79G-GTP) (7.3 μg) (*g* and *h*) as described in Materials and Methods. The distributions of β-COP (COPI), Sec23 (COPII), and VSV-G were determined by indirect immunofluorescence using M3A5 (β-COP), or polyclonal antibodies specific for Sec23 and the VSV-tail as described in Materials and Methods.

first accumulated in 16°C-VTCs in vivo as described above. After permeabilization, semi-intact cells were incubated at 32°C in the presence of EGTA. Before incubation, the distribution of VSV-G strongly overlapped with the distribution of both β-COP and p58 in VTCs in the peripheral cytoplasm and the Golgi region (Fig. 12, *a* [VSV-G], *b* [β-COP], *e* [VSV-G], and *f* [p58]). After 30 min incubation in the presence of EGTA, VSV-G remained in punctate elements (Fig. 12 *c*) which retained reduced yet significant overlap with β-COP (Fig. 12 *d*). In contrast, the strong overlap between VSV-G and p58 observed before incubation (Fig. 12, *e* and *f*) was nearly completely lost (Fig. 12, *g* and *h*). These results suggest that COPI mediated retrograde retrieval occurs in the absence of Ca^{2+} and before the Ca^{2+}-sensitive step preceding delivery of cargo from VTCs to the Golgi stack.

Discussion

We have demonstrated for the first time the sequential roles of COPII and COPI coats, and their sites of action in ER to Golgi traffic in mammalian cells. A model summa-

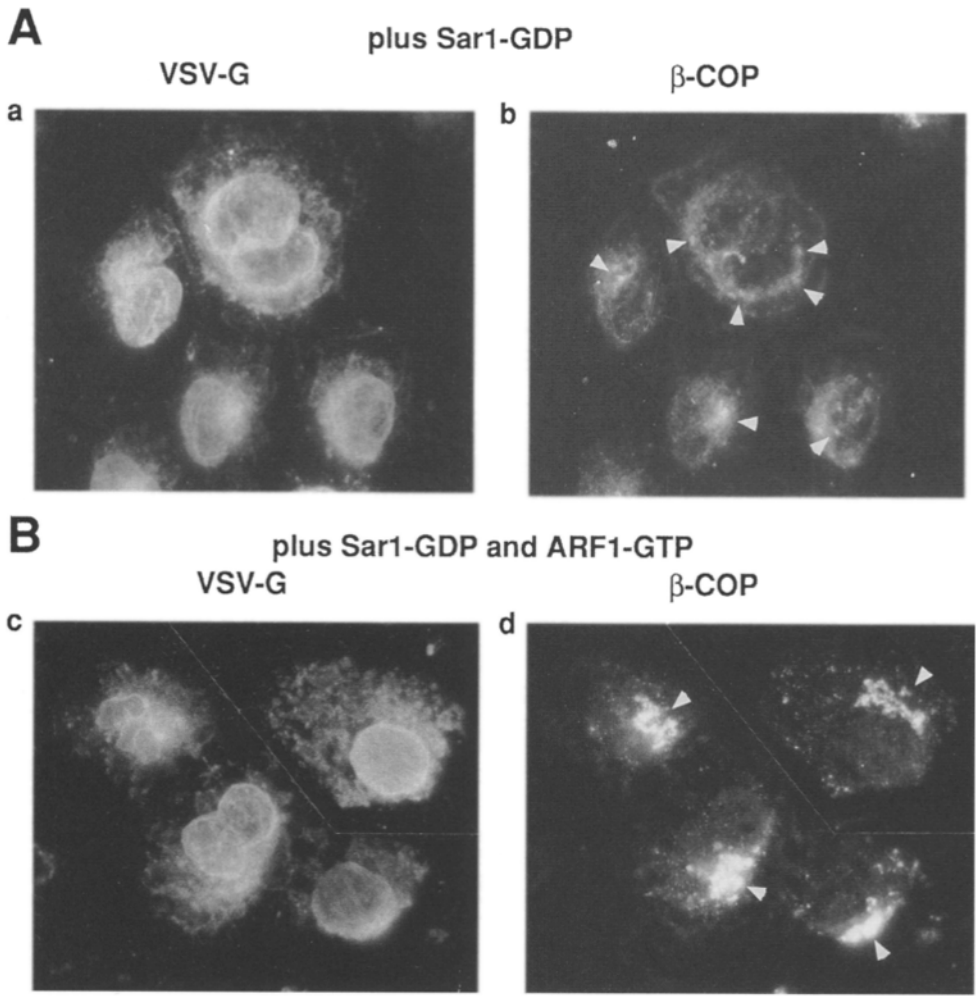

Figure 11. ARF1 (Q71L-GTP) does not promote export from the ER in the presence of Sar1(T39N-GDP). Semi-intact cells were incubated (200 μl final volume) at 32°C for 45 min in the presence of either Sar1-(T39N-GDP) (4.7 μg) (*A*), or both Sar1(T39N-GDP) and ARF1(Q71L-GTP) (25 μg) (*B*). The distribution of VSV-G (*a* and *c*) and β-COP (COPI) was determined by indirect immunofluorescence using the P5D4 antibody (VSV-G) and a polyclonal antibody directed against β-COP (EAGE) as described in Materials and Methods.

rizing our results is shown in Fig. 13. First, we find that both coats are required to promote ER to Golgi transport suggesting a close coupling between their respective functions. Second, while COPII appears to be the fundamental basis for vesicular export from the ER, COPI coats are critical for stabilization of pre-Golgi intermediates and recycling of p58. Third, it is evident that VTCs generated from ER-derived vesicles are very dynamic structures which undergo continuous maturation during the transport of cargo to the Golgi complex. They play a critical function in the secretory pathway as they are the first site of segregation of retrograde transported protein, an event which appears to be coupled to the anterograde transport of cargo (Fig. 13).

Role of COPII in Export from the ER

Previous pioneering work by Schekman and colleagues (Barlowe et al., 1993, 1994; Barlowe and Schekman, 1993; Hicke and Schekman, 1989; Hicke et al., 1992; Pryer et al., 1993; Rexach et al., 1994; Salama et al., 1993) established in yeast a role for the COPII machinery in the formation of ER-derived vesicles and in the export of cargo from the ER. We have now demonstrated that the principle mechanism of export of VSV-G is also via a GTP-dependent recruitment of COPII vesicle coats (Fig. 13). A similar mechanism promotes the export of the recycling protein p58 from the ER and is the basis for the formation of pre-Golgi intermediates (Fig. 13). These conclusions are derived from previous studies (Kuge et al., 1994) and current studies which demonstrate that the Sar1(T39N) GDP-restricted mutant prevents COPII recruitment, blocks vesicle budding and cargo mobilization from the ER, and prevents VTC build-up. The requirement for COPII in budding is also consistent with the effect of the activated Sar1(H79G) mutant which led to the accumulation of p58 and VSV-G in pre-Golgi clusters containing numerous 60-nm vesicles. Since VSV-G is concentrated in the presence of GTPγS (Pind et al., 1994), presumably reflecting COPII-coated vesicles, and in vesicles or VTCs which accumulate in the presence of activated ARF1 and Sar1 mutants (Bannykh, S., and W. E. Balch, manuscript in preparation), it is now apparent that COPII coats participate either directly or indirectly in the sorting and concentration of cargo (Balch et al., 1994). Interestingly, the EMP24 protein, a component of ER-derived COPII-coated vesicles, was recently demonstrated to cause a defect in transport of selected proteins from the ER to the Golgi (Schimmoller et al., 1995). Hence, at least one component of COPII vesicles appears to be required for sorting. It remains to be determined if such molecular sorting events regulate the actual recruitment of COPII components during ER budding.

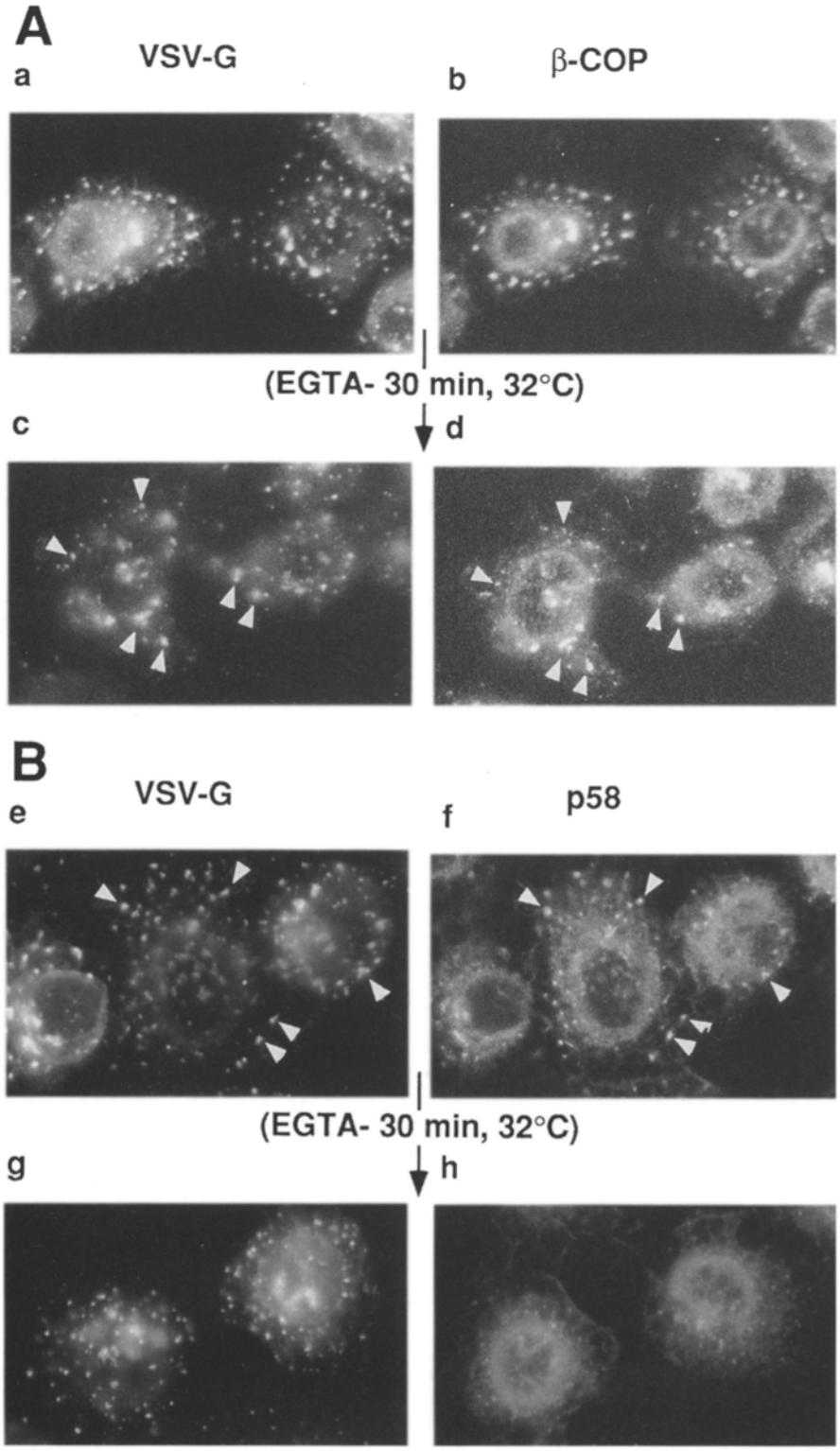

Figure 12. EGTA-VTCs retain the capacity to bind COPI. (*a–h*) NRK cells were preincubated for 2 h at reduced temperature (16°C) to accumulate VSV-G in VTCs before permeabilization as described in Materials and Methods. Cells were either held on ice (*a*, *b*, *e*, and *f*) or incubated for 30 min at 32°C (*c*, *d*, *g*, and *h*) and the distribution of VSV-G (*a*, *c*, *e*, and *g*), β-COP (*b* and *d*) and p58 (*f* and *h*) determined by indirect immunofluorescence using P4D5 antibody (VSV-G) and EAGE antibody (β-COP) as described (Plutner et al., 1992).

In contrast to the requirement for COPII in vesicle budding, COPI recruitment was not found to be essential. VSV-G mobilization from the ER, while reduced, was not blocked in vivo by the drug BFA or in vitro by the GDP-bound form of ARF1. Both of these reagents would be expected to prevent COPI coat recruitment (Dascher and Balch, 1994; Elazar et al., 1994; Helms and Rothman, 1992; Lippincott-Schwartz, 1993; Orci et al., 1991*b*). These results are in agreement with our previous observation that purified coatomer (COPI) will not support export of VSV-G from the ER (Peter et al., 1993).

A striking feature of ER export is the rapid loss of the COPII coat after vesicle budding. Under normal incubation conditions, the distribution of VSV-G and β-COP (COPI) strongly overlap in pre-Golgi intermediates, whereas Sec23 (COPII) is absent. Incubation of semi-intact cells at

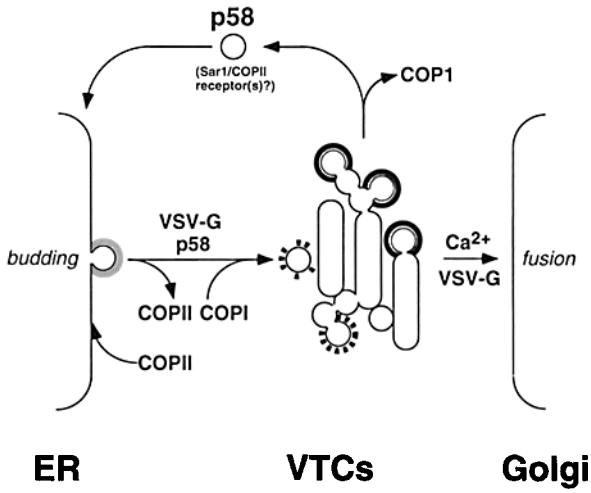

Figure 13. Model summarizing the role of COPI and COPII in the anterograde and retrograde transport of protein between the ER and the Golgi stack. The cartoon summarizes the putative roles of COPII (*gray*) and COPI (*black*) coats in anterograde and retrograde transport pathways of VSV-G and p58 in ER to Golgi transport. COPII coats are required for export from the ER and are lost rapidly after budding. This is followed by the recruitment (*dotted marks*) of COPI components which leads to the assembly of COPI coats involved in retrograde transport of p58 back to the ER from VTCs. See text of Discussion.

16° also leads to the rapid loss of the COPII as only COPI coats can be detected on 16°C-VTCs. A similar result has been observed in yeast as COPII coats present on purified ER-derived vesicles are very unstable (Rexach et al., 1994). However, it is now clear that the receptor(s) for Sar1/COPII components remain to be segregated from VSV-G in 16°C-VTCs which accumulate at reduced temperature. This conclusion is based on the observation that the addition of the activated Sar1(H79G) mutant led to extensive recruitment of Sec23 to these intermediates after a shift to 32°C. In yeast, COPII-coated vesicles formed in the presence of GTPγS do contain Sar1p (Barlowe et al., 1994; Oka and Nakano, 1994). Therefore, it is likely that a receptor or docking protein for Sar1p is exported from the ER in yeast as well. The lack of COPII colocalization with the exported cargo in VTCs highlights the role of the coat component Sec23 as a Sar1 GAP (Yoshihisa et al., 1993) which must rapidly promote Sar1 hydrolysis and loss of COPII coats after vesicle budding.

Sequential Coupling between COPII and COPI Coats Is Required for Efficient ER to Golgi Transport

While COPII appears to be the principle coat responsible for export of cargo from the ER, the inability to recruit COPI coats had a significant effect on the appearance of VSV-G in pre-Golgi intermediates. What, then, is the role of COPI in this step? Previous biochemical studies exploring the role of COPI in ER to Golgi transport in vitro led to the suggestion that it is involved in ER budding (Peter et al., 1993). In these experiments, an antibody specific to β-COP largely prevented mobilization of VSV-G from the ER to punctate p58 containing VTCs (Peter et al., 1993). When the antibody was microinjected into living cells, VSV-G accumulated in VTCs which had extended tubular linkages to the ER (Pepperkok et al., 1993). In the latter case, it was concluded that membrane synthesis continued, but that budding was blocked (Pepperkok et al., 1993).

The current studies have allowed us to focus on the above events and to now clarify these observations. We found that by blocking the recruitment of COPI in vivo by BFA, or in vitro by the ARF1-GDP restricted mutant, we markedly suppressed the appearance of VTCs. Since vesicle formation via COPII would be expected to be normal under these conditions, it is now apparent that these vesicles were not detected previously using indirect immunofluorescence due to their failure to efficiently accumulate into compact VTCs (Peter et al., 1993). In addition, we cannot presently rule out the possibility that in the absence of COPI, ER-derived vesicles may be unstable and fuse back to the ER via an uncoupled mechanism, similar to the effects of BFA on the Golgi stack in vivo (Lippincott-Schwartz, 1993) and in vitro (Elazar et al., 1994). A partial reduction in COPI function induced by microinjection of β-COP specific antibody may also contribute to the instability of VTCs and account for the BFA-like tubular connections connecting VTCs to the ER in vivo (Pepperkok et al., 1993). Thus, while COPI is not critical for export from the ER, it plays an essential role in post-ER events. Insight from the current studies now clarifies our previous observation that while purified coatomer complex failed to support ER export, a 19 S cytosolic fraction which contained both COPI and COPII (as judged by the presence of Sec23 and β-COP) (Peter et al., 1993), efficiently supported ER export in vitro. In this case, the combined action of COPII and COPI components functioned in concert to promote vesicle budding and intermediate stability.

The rapid, sequential recruitment of COPI components after disassembly of COPII coats is similar to biochemical events associated with budding of vesicles from yeast ER membranes. While COPI is absent from yeast vesicles generated using purified Sar1 and COPII components (Barlowe et al., 1994), yeast ER-derived vesicular carriers generated in vitro in the presence of cytosol are significantly enriched in COPI after fractionation through a number of density gradients (Rexach et al., 1994) and therefore COPI is apparently a component of these vesicles. One interpretation of these results is that COPI recruitment follows budding from the ER in both yeast and mammalian cells (Fig. 13). Genetic studies reinforce this concept. A null mutant lacking β-COP (Sec26) displays an ER to Golgi transport block leading to the accumulation of ER membranes (Duden et al., 1994). In this case, recruitment of COPI after budding may also be required to stabilize yeast intermediates in vivo. Since we observed a modest synergism in the effects of the Sar1 and ARF1 mutants on transport, we suggest there is a close temporal coupling in the exchange of coats after budding (Fig. 13). The purpose of this sequential recruitment may be to set the stage for subsequent recycling of components from pre-Golgi intermediates to the ER (see below).

The rapid recruitment of COPI after budding from the ER is reflected in the steady state localization of these coat proteins. In mammalian cells, both Sec23 (Orci et al., 1991*a*) and Sec13 (Shaywitz et al., 1994) are localized to ER transitional elements. β-COP is also localized to VTCs

adjacent to budding sites in vivo and in vitro (Balch et al., 1994; Oprins et al., 1993; Pepperkok et al., 1993; Pind et al., 1994). Such a close overlap is particularly exaggerated in the presence of GTPγS where both COPI and COPII can be found in the same punctate VTCs. An important issue which still remains to be resolved is whether both COPII and COPI coats can occupy the same vesicle or tubule within VTCs. Related to this issue was the surprising observation that anterograde transport from 16°C-VTCs is sensitive to Sar1 mutants despite the fact that these structures are not in continuity with the ER (Balch et al., 1994; Saraste and Svensson, 1991). Although we cannot rule out the possibility that COPII mediates a second round of vesicle budding from VTCs, we feel that inhibition by Sar1 mutants is likely to reflect their interference with a requirement for sorting of retrograde transported proteins such as COPII receptors from pre-Golgi intermediates. This sorting step is incomplete at reduced temperature, but is required for normal maturation of VTCs. We suggest that 16°C-VTCs are intermediates in transition between a COPII- and COPI-dependent state, reflecting the possible need for segregation of recycling components by COPI (see below) to insure further anterograde transport of specific cargo through the secretory pathway.

Role of COPI in Recycling

Our results indicate that when COPI is retained on membranes by activated ARF1, retrograde transport of p58 from VTCs to the ER is blocked. In contrast, inhibiting COPII function by the Sar1-GDP–restricted mutant, a condition which maintains normal COPI function, did not affect retrograde transport of p58. The combined results provide evidence for the first time that COPI is involved in the retrograde recycling of p58 from pre-Golgi intermediates to the ER (Fig. 13). This conclusion is in agreement with the proposed role of COPI in retrograde transport in yeast (Cosson and Letourneur, 1994; Letourneur et al., 1995). Both p58 and its human homologue p53 (Schindler et al., 1993) carry di-lysine ER retrieval motifs at their cytoplasmic carboxyl-termini which bind COPI in vitro (Cosson and Letourner, 1994). p58 and other di-lysine motif containing proteins present in ER-derived vesicles and newly formed VTCs may be responsible for the recruitment of COPI to membranes after vesicle release from the ER. Thus, a key event in the functional maturation of VTCs may be the segregation of p58 and in particular, Sar1/COPII receptors, which need to be recycled for reuse in subsequent rounds of vesicle budding from the ER. Such a sequential, coupled requirement for coat assembly mediated by the Sar1 and ARF1 GTPases would confer vectorial progression linking recycling to anterograde transport. Sequential coupling mechanisms are also found for different classes of GTP-binding proteins, those involved in protein synthesis. Both Ef-Tu and Ef-G are required to assure the proper selection and incorporation of amino acids into newly synthesized proteins. Sequential coupling mechanisms may therefore serve as a general paradigm for the interaction of multiple GTPases to confer vectorial progression in a variety of complex cellular processes.

Is COPI also involved in the anterograde transport of VSV-G from VTCs to the Golgi stack? Isolated COPI-coated vesicles have been suggested to be anterograde carriers of VSV-G between *cis*- and medial-Golgi compartments (Ostermann et al., 1993). We found that the GTP-restricted form of ARF1 prevented both p58 recycling and the anterograde transport of VSV-G from 16°C-VTCs. However, mutations in subunits of coatomer blocked retrograde but not anterograde transport in yeast (Letourneur et al., 1995), a result which led to the suggestion that COPI is exclusively involved in recycling (Pelham, 1994). Whether different adaptors are required to direct COPI function, or whether anterograde transport is coupled to COPI-mediated retrograde transport remains to be clarified.

Role of Pre-Golgi Intermediates in ER to Golgi Transport

The sequential coupling between COPII and COPI coats after budding from the ER is consistent with the dynamic character of pre-Golgi intermediates. Indeed, the function and composition of these intermediates have been the topic of considerable controversy due to their highly variable vesicular-tubular morphology found in different cell types and under different incubation conditions. Pre-Golgi intermediates have been referred to as the salvage compartment (Warren, 1987), the ER to Golgi intermediate compartment (ERGIC) (Schweizer et al., 1990), the Rubella virus accumulating compartment (Hobman et al., 1993), the budding compartment for coronavirus (Krijnse-Locker et al., 1994; Tooze et al., 1988), the CGN (Mellman and Simon, 1992), and VTCs (Balch et al., 1994). With one exception (Krijnse-Locker et al., 1994), most studies recognize that pre-Golgi elements are distinct from the ER and compartments comprising the Golgi stack. The apparent continuity of VTCs with the ER as suggested (Krijnse-Locker et al., 1994) may be due to the fact that permeabilized cells were incubated in the absence of cytosol, a condition which mimics a COPI deficiency and BFA-like phenotype.

Given the dynamic character of the pre-Golgi intermediates, we would like to suggest that previous morphological/biochemical descriptions represent different steps in their formation and maturation. Each of these steps is likely to involve specific membrane-associated and cytosolic factors. These may be differentially rate-limiting in cells of diverse origin and under different incubation conditions. However, one fact evident from the present studies is the apparent similarity of pre-Golgi elements to endosomes involved in the segregation and recycling of proteins derived from the cell surface. Endosomes are dynamic structures, of variable morphology and function to segregate proteins to different cellular destinations following input from the plasma membrane. Given this analogy, we propose that the term "exosome" may provide a more useful designation for pre-Golgi intermediates (Beckers et al., 1990; Lippincott-Schwartz et al., 1989). It demarks their biochemical role in recycling and emphasizes the importance of segregation for anterograde transport of cargo through the secretory pathway.

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