COPI-independent Anterograde Transport: Cargo-selective ER to Golgi Protein Transport in Yeast COPI Mutants

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Abstract. The coatomer (COPI) complex mediates Golgi to ER recycling of membrane proteins containing a dilysine retrieval motif. However, COPI was initially characterized as an anterograde-acting coat complex. To investigate the direct and primary role(s) of COPI in ER/Golgi transport and in the secretory pathway in general, we used PCR-based mutagenesis to generate new temperature-conditional mutant alleles of one COPI gene in Saccharomyces cerevisiae, SEC21 (γ-COP). Unexpectedly, all of the new sec21 ts mutants exhibited striking, cargo-selective ER to Golgi transport defects. In these mutants, several proteins (i.e., CPY and α-factor) were completely blocked in the ER at nonpermissive temperature; however, other proteins (i.e., invertase and HSP150) in these and other COPI mutants were secreted normally. Nearly identical cargo-specific ER to Golgi transport defects were also induced by Brefeldin A. In contrast, all proteins tested required COPII (ER to Golgi coat complex), Sec18p (NSF), and Sec22p (v-SNARE) for ER to Golgi transport. Together, these data suggest that COPI plays a critical but indirect role in anterograde transport, perhaps by directing retrieval of transport factors required for packaging of certain cargo into ER to Golgi COPII vesicles. Interestingly, CPY–invertase hybrid proteins, like invertase but unlike CPY, escaped the sec21 ts mutant ER block, suggesting that packaging into COPII vesicles may be mediated by cis-acting sorting determinants in the cargo proteins themselves. These hybrid proteins were efficiently targeted to the vacuole, indicating that COPI is also not directly required for regulated Golgi to vacuole transport. Additionally, the sec21 mutants exhibited early Golgi-specific glycosylation defects and structural aberrations in early but not late Golgi compartments at nonpermissive temperature. Together, these studies demonstrate that although COPI plays an important and most likely direct role both in Golgi–ER retrieval and in maintenance/function of the cis-Golgi, COPI does not appear to be directly required for anterograde transport through the secretory pathway.

In the secretory pathway of eukaryotic cells, transport between organelles is mediated by coated vesicles that must efficiently capture and package cargo molecules, and then target, dock, and fuse with an appropriate acceptor compartment (Rothman and Orci, 1992). The bi-directional nature of interorganelle transport is crucial for proper functioning of the pathway: anterograde transport serves to direct the forward transport of secretory cargo (Palade, 1975), while retrograde transport is essential both for retrieval of proteins which have escaped their site of residence and for recycling of transport factors required to mediate further rounds of anterograde traffic (Letourneur et al., 1994; Pelham, 1994). Because the coat complexes that direct these trafficking events participate directly or indirectly in cargo selection, vesicle coats play a critical role in maintaining the directional flow of protein transport in the secretory pathway (Aridor and Balch, 1996; Rothman and Wieland, 1996; Schekman and Orci, 1996).

Two vesicle coat complexes, COPI/coatomer and COPII, have been identified that regulate transport between the endoplasmic reticulum and Golgi complex in both yeast and mammalian cells. COPII was initially identified in yeast and is comprised of the Sec23p/Sec24p and Sec13p/Sec31p complexes, the small GTP-binding protein Sar1p (Barlowe et al., 1994), and most likely Sec16p (Esen-shade et al., 1995). The COPII complex is required for formation of cargo-containing, ER-derived transport vesicles and thus functions directly in anterograde traffic between the ER and the cis-Golgi (Bednarek et al., 1995).

In contrast to the role of COPII in ER to Golgi transport, the direct role(s) of COPI/coatomer (composed of seven subunits: α, β, β′, γ, δ, ε, ζ; Waters et al., 1991) in ER/Golgi transport has recently been the subject of signif-
Materials and Methods

Strains and Media

Yeast strains used in this study are shown in Table I. Standard genetic techniques were used throughout (Sherman et al., 1979). Strains EGY1181-1, EGY121-1, and EGY1211-2 were constructed by crossing SEY5188, SF309-1C, SEY5107, and RSY454, respectively, with either SEY6210 or SEY6211, sporulating, and isolating haploid progeny carrying desired markers and mutations. Cells were grown in either YPD (yeast extract, peptone, dextrose) or YNB (yeast nitrogen base) media supplemented as necessary (Sherman et al., 1979).

Plasmids and DNA

Plasmid p315SEC21 was provided by R. Duden and R. Schekman (University of California, Berkeley, CA) and was constructed by subcloning a NotI-HindIII fragment (containing the entire SEC21 ORF) out of a yeast YEPL3 complementing library plasmid (Hosobuchi et al., 1992) into the vector P315 (Pickart et al., 1990). Plasmid p416SEC21 contains the same NotI-HindIII fragment of SEC21 in pRS416 (Sikorski and Hieter, 1989). To generate the sec21::HIS3 disruption construct, an EcoRI-BglII fragment of SEC21 (comprising >95% of the SEC21 ORF) was replaced with the HIS3 gene in p315SEC21. This plasmid was digested with SpeI, and the SpeI fragment containing the HIS3-disrupted SEC21 gene was isolated by gel purification and used to make the strain EGY021. Plasmids pCYI-20, -50, -156, and -433 were described previously (Johnson et al., 1989). Plasmid pOH containing the hemagglutinin (HA)-tagged Ochlp was provided by S. Harris and G. Waters (Princeton University, Princeton, NJ) and was described previously (Harris and Waters, 1996).

SEC21 Mutagenesis and Double Mutant Construction

The yeast strain used in the plasmid shuffle, EGY021, was constructed by first disrupting one chromosomal copy of SEC21 in an SEY6210/SEY6211 diploid with HIS3 (using the linearized SpeI fragment described above) by homologous recombination. p416SEC21 (CEN, URA3, SEC21) was transformed into this disrupted diploid, which was then sporulated. His+, Ura+ haploids were isolated, one of which was designated EGY021. Mutations in SEC21 were generated by random PCR mutagenesis (Muhlrad et al., 1992). Primers annealing into nucleotides 5′ of the BamHI site and 150 nucleotides 3′ of the BglII site in SEC21 were used to PCR-amplify a 2,200-nucleotide fragment (~80% of the SEC21 ORF) using Taq polymerase (Perkin-Elmer Corp., Norwalk, CA) under standard conditions, except that limiting (50 μM) dATP was used. A gapped (linearized) acceptor plasmid was made by digesting p315SEC21 (CEN, LEU2, SEC21) with BamHI and BgII and isolating the vector by gel purification. Equimolar amounts of gapped plasmid and mutagenized DNA were cotransformed into EGY021. Homologous recombination in yeast resulted in mutated SEC21 DNA being incorporated into the p315SEC21 vector, and transformants were selected on ~Leu plates. Leu+ colonies were replica plated onto 5-fluoroorotic acid (5-FOA, which selects against URA3-containing plasmids) plates to shuffle out p416SEC21 (CEN, URA3, SEC21; Sikorski and Boeke, 1991). Temperature-sensitive mutants which grew on 5-FOA at 26°C but not 37°C were isolated. p315sec21 (CEN, LEU2, sec21ts) mutant plasmids were isolated and reshuffled into EGY021 for further mutant characterization.

Double mutant strains EGY231/213 (sec21::sec21-3) and EGY112/123 (sec11::sec21-3) were generated by crossing EGY1231-2 or EGY111-2, respectively, with EGY021, sporulating, and isolating temperature sensitive, His+, Ura+ haploid progeny exhibiting the appropriate mutant phenotype. p315sec21-3 was then shuffled into these strains as described.

Cell Labeling, Immunoprecipitation, Subcellular Fractionation, Immunoblotting, and Brefeldin A (BFA) Treatment

Cell labeling, immunoprecipitation, reimmunoprecipitation with secondary antiseras, endoglycosidase H treatment, and subcellular fractionation

1. Abbreviations used in this paper: ALP, alkaline phosphatase; ARF, ADP-ribosylation factor; BFA, Brefeldin A; CPS, carboxypeptidase S; CYP, carboxypeptidase Y; CV, coated vesicle; HA, hemagglutinin; ts, temperature sensitive.
were performed essentially as previously described (Gaynor et al., 1994), with the following variations. Before labeling, cells were grown to early logarithmic phase in YNB supplemented with amino acids. Cells were labeled at 5 OD/ml in YNB media containing amino acids, 100 μg/ml α2-macroglobulin, and 500 μg/ml BSA. To assay internal and external invertase, cells were converted to spheroplasts after pulse-chase by adding an equal volume of 2× spheroplast buffer (50 mM Tris, pH 7.5, 2 M sorbitol, 30 mM NaCl, 40 mM NaF, 20 mM DTT), incubating on ice for 10 min, and then centrifuged at 16,000 g for 15 min, and 0.25 OD equivalents were loaded for SDS-PAGE. For immunoprecipitation of HSP150 or invertase from a mutant, cell labeling, immunoprecipitation, and subcellular fractionation were performed as described, except that the media also contained 50 mM Na-Hepes, pH 7.0. Either 100 μg/ml BFA or an equivalent volume of 95% ethanol was added to the cells 2 min before labeling.

**Fluorescence Microscopy**

Indirect immunofluorescence was performed as previously described (Redding et al., 1991), with the following variations. Cells were fixed for 16 h in 4% formaldehyde, spheroplasted with 45 μg/ml Zymolyase 100T (Seikagaku America, Inc., Rockville, MD), and then treated with 1% SDS. Fixed spheroplasts were incubated first with either the 12CA5 monoclonal antibody at a 1:400 dilution or an affinity-purified rabbit anti-Mnn1p (Graham et al., 1994) at a 1:10 dilution for 16 h at 4°C. This was followed by incubations with either 0.5 μg/ml goat anti-mouse IgG or 0.5 μg/ml goat anti-rabbit for 2 h at 4°C, and then a 1:200 dilution of Cy3-conjugated donkey anti-goat IgG for 2 h at 4°C (antibodies from Jackson ImmunoResearch Labs., West Grove, PA).

**Results**

**Rationale for Generation of New sec21 Temperature-Conditional Alleles**

Before this work, analysis of COPI function in yeast had been restricted to studying a small number of COPI mutant alleles, all of which had been isolated in screens selecting specifically for either anterograde (sec mutants) or retrograde (ret mutants) secretory transport defects. While these mutants have yielded significant insight regarding the role of COPI in secretory transport, the means by which they were identified and the fact that only one or two mutant alleles for each coatomer subunit had been isolated both limits and biases what can be learned from them. Additionally, the range of defects observed for different COPI mutants, coupled with the fact that it has been impossible to determine whether these mutations may simply alter or reduce function of the encoded proteins rather than represent true temperature-conditional loss of function alleles, has made it difficult to establish the most primary defect(s) associated with loss of COPI function.

**Table I. Saccharomyces cerevisiae Strains Used in This Study**

| Strain     | Description | Source or reference |
|------------|-------------|---------------------|
| SEY6210    | MATa ura3 leu2 his3 trp1 lys2 sec21Δ | (Robinson et al., 1988) |
| SEY6211    | MATa ura3 leu2 his3 trp1 ade2 sec24Δ | (Robinson et al., 1988) |
| EGY024     | MATa ura3 leu2 his3 trp1 sec21Δ | This study |
| EGY1213    | MATa ura3 leu2 his3 trp1 sec24Δ | This study |
| SEYS188    | MATa sec18-1 ura3 leu2 sec24Δ | (Graham and Emr, 1991) |
| EGY1181-5  | MATa sec18-1 ura3 leu2 his3 sec2Δ | This study |
| SF309-1C   | MATa sec23-1 | R. Schekman* |
| EGY1231-1  | MATa sec23-1 ura3 leu2 trp1 sec2Δ | This study |
| EGY1231-2  | MATa sec23-1 leu2 his3 sec2Δ | This study |
| EGY23123   | MATa sec23-1 leu2 his3 sec24Δ | This study |
| EGY5017    | MATa sec1-1 ura3 leu2 sec2Δ | Lab strain |
| EGY111-2   | MATa sec1-1 ura3 leu3 his3 sec2Δ | This study |
| EGY111213  | MATa sec1-1 ura3 leu3 his3 sec24Δ | This study |
| RSY454     | MATa sec22-1 ura3 leu2 his4 pep4Δ URA3 | R. Schekman |
| EGY1221-2  | MATa sec22-1 ura3 leu3 his4 trp1 sec2Δ | This study |
| RSY957     | MATa sec33-1 ura3 his4 | R. Schekman |
| PC130      | MATa ret2-1 ura3 leu2 his4 lys2 sec2Δ | P. Cosson† |
| RSY580     | MATa sec20-1 ura3 leu2 ade2 | R. Schekman |
| RSY945     | MATa bet1-1 his4 | S. Ferro-Novick§ |
| RSY317     | MATa sec16-1 leu2 | R. Schekman |
| EGY101     | MATa ret1-1 ura3 leu2 his5 trp1 sec2Δ | Letourneur et al., 1994) |
| CKB100     | MATa sec27-1 ura3 leu2 | C. Kaiser§ |
| PC159      | MATa ret1-1 ura3 leu2 his4 trp1 sec2Δ | P. Cosson |
| TGY413-6D  | MATa erg6(isl1) ura3 leu2 his3 sec2Δ | (Graham et al., 1993) |

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As an alternative genetic approach, we used random PCR mutagenesis to generate multiple new temperature-conditional mutant alleles of one COPI gene, SEC21 (γ-COP). Mutants were isolated by selecting only for temperature-conditional growth and not by screening for directional transport defects. By eliminating this bias, we hoped to reveal the primary function(s) of Sec21p/γ-COP. We chose to mutagenize SEC21 because the two existing sec21 mutant alleles exhibit different phenotypes: both are partially defective for retrieval of dilsyne-containing proteins, but while sec21-1 also exhibits a partial block in anterograde transport, sec21-2 does not (Hosobuchi et al., 1992; Letourneur et al., 1994).

SEC21, like all COPI genes, is essential. We employed the standard techniques of PCR mutagenesis (Muhlrad et al., 1992) and plasmid shuffle (Sikorski and Boeke, 1991) to generate strains in which the wild type SEC21 gene was replaced with mutagenized SEC21-containing plasmids (see Materials and Methods). Six temperature-conditional alleles were identified which were viable at 26 but not 37°C. Different growth properties at intermediate temperatures suggested that they represented at least four distinct alleles (Table II). Somewhat surprisingly, however, the sorting and transport phenotypes of these mutants were remarkably similar: all were partially defective for retrieval of a KKXX-containing Inv-Wbp1 fusion (data not shown; Gaynor et al., 1994; Letourneur et al., 1994), and all exhibited striking and nearly identical cargo-specific anterograde transport defects. Therefore, while the analyses described below were performed for each new mutant, results are only shown for one representative allele, designated sec21-3.

The sec21 ts Mutants Exhibit a Rapid and Complete Block in Export of CPY and α-factor from the ER at Nonpermissive Temperature

In yeast, anterograde secretory transport can be assayed by observing the processing and maturation of a well characterized, soluble vacuolar hydrolase, carboxypeptidase Y (CPY). CPY is synthesized initially as a proenzyme which is first modified with core oligosaccharides in the ER, generating the p1 form, and next in the Golgi complex, where the core sugars are elongated, generating the p2 form. After delivery to the vacuole, the pro region is cleaved to yield the mature (m) enzyme (Stevens et al., 1982). sec21-3 cells were incubated at either permissive (26°C) or non-permissive (37°C) temperature for 10 min and subjected to pulse–chase analysis, and CPY was recovered by immunoprecipitation. At 26°C, processing and maturation of CPY occurred normally (Fig. 1A), with kinetics similar to wild type cells (data not shown). In contrast, at 37°C, CPY was found exclusively as the p1, ER-glycosylated form (Fig. 1A). Similar results were observed when the sec21-3 mutant was preincubated at 37°C for either 1 or 30 min before labeling or when cells were chased for longer times (i.e., 60 min); in addition, maturation of two membrane-associated

![Image](322x319 to 490x595)

**Table II. Growth Phenotype of sec21 ts Mutants**

| Temperature (°C) | 26 | 30 | 33 | 37 |
|-----------------|----|----|----|----|
| Wild type       | ++ | ++ | +  | +  |
| sec21-3         | ++ | +  | +  | +  |
| sec21-4         | ++ | +  | +  | +  |
| sec21-5         | +  | +/-|    | -  |
| sec21-6         | ++ | ++ | +  | +  |

Growth of new sec21 ts mutants at various temperatures was tested on rich YPD plates. sec21-7 and sec21-8 mutant alleles exhibited similar growth phenotypes as sec21-6 and sec21-3, respectively.

++ = wild type growth.
++ = growth slower than wild type.
+ = very slow growth (minimal single colony formation).
+/- = almost no growth (no single colony formation).
- = complete lethality.

Figure 1. CPY transport analysis in sec21-3. CPY is schematized at the top, showing the signal sequence (black), pro segment (hatched lines), and four N-linked glycosylation sites (Y). (A) Processing and maturation of CPY. sec21-3 (EGY1213) cells were incubated at 26 or 37°C for 10 min, labeled for 10 min with Tran35S, and then chased for the indicated times. CPY was recovered by immunoprecipitation, subjected to SDS-PAGE, and visualized by fluorography. The positions of precursor (p1/ER; p2/Golgi) and mature (m/vacuolar) forms of CPY are indicated. (B) Golgi-specific modification of CPY in sec21-3. Wild type (SEY6210), sec21-8 (EGY1181-5), and sec21-3 (EGY1213) cells were incubated at 37°C for 10 min, labeled for 10 min, and chased for 30 min. CPY was recovered by immunoprecipitation, split into two equal aliquots, subjected to secondary immunoprecipitation and visualized by SDS-PAGE and fluorography. (C) Subcellular localization of CPY in sec21-3. Wild type (SEY6210) and sec21-3 (EGY1213) cells harboring pOH (Och1-HA) were incubated at 37°C for 10 min, labeled for 15 min, and chased for 45 min. Lysed spheroplasts were fractionated by differential centrifugation, and P13, P100, and S100 fractions were harvested. CPY was recovered by quantitative immunoprecipitation and visualized by SDS-PAGE and fluorography. Fractions were also immunoblotted with the 12CA5 monoclonal antibody which recognizes the HA-tagged Och1p.
vacuolar hydrolases, alkaline phosphatase (ALP) and carboxypeptidase S (CPS), was also completely blocked in sec21-3 at 37°C (data not shown). This is in contrast to the partial anterograde transport defects observed for other COPI mutants (Duden et al., 1994; Letourneur et al., 1994). Even in sec21-1, for instance, we observed significant maturation of CPY at 37°C after backcrossing this mutation into our strain background (data not shown).

To address whether the p1CPY which accumulated in sec21-3 at 37°C had reached the yeast Golgi complex, Golgi-specific carbohydrate modifications on CPY were analyzed. It has previously been shown that the yeast Golgi complex can be functionally divided into at least four distinct compartments. From cis to trans, these compartments are defined by: initial α,1,6 mannosyltransferase (Och1p), elongating α,1,6 mannosyltransferase, α,1,3 mannosyltransferase (Mnn1p), and Kex2p activities (Franzusoff et al., 1991; Graham and Emr, 1991; Nakashishi-Shindo et al., 1993; Gaynor et al., 1994). p1CPY is generated in the ER by the addition of core oligosaccharides to appropriate asparagine residues. Upon arrival in the Golgi, the core sugars are modified first with initial α,1,6 mannosase in the cis-most compartment and then with α,1,2 and α,1,3 mannosase in a medial compartment (Graham and Emr, 1991). The presence of these mannose moieties indicates how far the protein has progressed in the secretory pathway. Cells were incubated at 37°C for 10 min, pulse-labeled, and chased for 30 min. CPY was recovered by immunoprecipitation, and then split into equal aliquots and subjected to a second immunoprecipitation with antisera specific to either CPY or α,1,6 mannosase (Fig. 1 B). 

Figure 2. α-factor processing and maturation in sec21-3. α-factor is schematized at the top, showing the signal sequence (black), pro segment (hatched lines), three N-linked glycosylation sites (Y), and late Golgi cleavage sites (white and arrows) which generate the four mature peptides. sec21-3 (EGY1213) cells were incubated for 5 min at 23 or 37°C, pulse-labeled for 10 min with Tran35S, then chased for 15 min. Equal numbers of cells were removed at indicated timepoints. α-factor was recovered by immunoprecipitation and visualized by SDS-PAGE and fluorography. The positions of core (ER)-, α,1,6 mannosase (Golgi)-, and α,1,3 mannosase (Golgi)-modified forms are indicated, as is the position of the mature, secreted peptide.

In wild type cells, CPY was efficiently α,1,6 mannosase-modified, while in a sec18-1 (NSF) mutant, which blocks ER to Golgi transport, CPY does not acquire α,1,6 mannosase (Fig. 1 B). In sec21-3, as in sec18-1, p1CPY was found exclusively in the P13 fraction (Fig. 1 C), which has previously been shown to be ER-resident (Wbp1p). ER resident proteins fractionate almost entirely in the P13, while Golgi proteins (i.e., Mnn1p and Kex2p), even cis-Golgi proteins (i.e., Och1p), are found primarily in the P100 (data not shown; Gaynor et al., 1994; Harris and Waters, 1996). Indeed, immunoblot analysis indicated that ~75% of an HA-tagged Och1p (a resident of the cis-most Golgi compartment) fractionated in the P100 in both wild type and sec21-3 cells (Fig. 1 C). Further support for ER localization of p1CPY in sec21-3 was provided by resolution of P13 material on a 2-step sucrose gradient, where p1CPY migrated in the ER-enriched fraction but Och1p did not (data not shown; Gaynor et al., 1994). Together, these data provide strong evidence that CPY transport was blocked at the level of the ER in sec21-3 at nonpermissive temperature.

To assay anterograde transport of a secreted protein in the new sec21 ts mutants, pulse–chase analysis of the mating pheromone α-factor was performed. α-factor is synthesized as a precursor which acquires up to three core N-linked oligosaccharides in the ER. Pro-α-factor then transits rapidly through the Golgi complex, where it is sequentially modified with α,1,6 and α,1,3 mannosae, then proteolytically processed into the mature peptide pheromone in the trans-Golgi before being secreted (Julius et al., 1984; Graham and Emr, 1991). In sec21-3 at permissive temperature (23°C), α-factor was very rapidly Golgi-modified and then converted to the mature, 13 amino acid peptide hormone (Fig. 2). This is identical to what was observed in wild type cells; in addition, the mature α-factor peptide was secreted to the medium in sec21-3 at 23°C (data not shown). After a 5-min preincubation at 37°C, however, only the core-glycosylated, ER form of α-factor was recovered (Fig. 2). The protein also remained entirely intracellular, and even after long chase times, appearance of mature α-factor was not observed (data not shown). Therefore, shifting the sec21 ts mutants to nonpermissive tem-
temperature yielded a rapid and complete ER block for several vacuolar hydrolases (CPY, ALP, and CPS), as well as for a secreted protein, α-factor; this rapid and absolute onset also suggests that the temperature shift resulted in immediate inactivation of Sec21p.

**Anterograde Transport of a Subset of Secreted Proteins (i.e., HSP150) Is Not Affected in the sec21 ts Mutants**

Rather than limit our analysis of transport defects in the sec21 ts mutants to specific known proteins, we took an alternative approach to assess general secretion defects in these and other sec mutants. Most proteins secreted from yeast cells remain in the periplasmic space or are associated with the cell wall; however, a few proteins are efficiently secreted into the growth medium (Robinson et al., 1988). We took advantage of this to address (a) whether some proteins might escape the strong ER block observed for CPY and α-factor in the sec21 ts mutants and (b), whether COPII mutants are competent for secretion of some cargo molecules; if so, this might suggest that COPI and COPII can both form cargo-containing anterograde ER to Golgi transport vesicles. Whole cells were incubated at 37°C for 30 min, pulse-labeled, and chased for 30 min. Cells and media were separated by centrifugation, and proteins in the media were precipitated with TCA and resolved by SDS-PAGE. At least nine protein bands were apparent in media from wild type cells (Fig. 3, lane 1). No media proteins were secreted from either sec18-1 (NSF) or sec23-1 (COPII) mutants (Fig. 3, lanes 2 and 3), nor were any media proteins secreted from a sec1-1 mutant (Fig. 3, lane 5), which blocks docking/fusion of TGN-derived secretory vesicles with the plasma membrane (Novick and Schekman, 1979; Bennett, 1995). Interestingly, in the sec21-3 mutant, some media proteins were efficiently secreted (Fig. 3, lane 4, arrows) while others were blocked (●). Identical results were obtained after either a 1 or 60 min preincubation of the sec21-3 mutant at either 37 or 38°C (data not shown). This analysis thus revealed that secretion of a distinct subset of proteins was unaffected in the COPII mutant. These proteins trafficked via the normal secretory route, because (a) COPII NSF, and Sec1p were absolutely required for their secretion (which also implied that COPII is required for all anterograde ER to Golgi transport), and (b) all media proteins secreted from wild type and sec21-3 cells were precipitable with Con A–sepharose (data not shown), demonstrating that they received glycosyl modifications typical of proteins transiting the secretory pathway.

One particularly abundant protein secreted from sec21-3 cells (Fig. 3, lane 4, top arrow) migrated at a molecular mass similar to that observed for a previously characterized media protein, HSP150 (Russo et al., 1992); subsequent analysis with HSP150 antiserum confirmed that HSP150 was the identity of this protein (see below). HSP150 is extensively O-glycosylated, and its synthesis increases ~6-fold upon shift to high temperature (Russo et al., 1992). Glycosylation in the ER causes this 414-amino acid protein to migrate at ~80–100 kD. After carbohydrate modification in the Golgi, HSP150 migrates at ~150 kD (Russo et al., 1992; Jamsa et al., 1994). HSP150 secretion was assayed by immunoprecipitating HSP150 from intra-
Invertase Is Underglycosylated but Efficiently Secreted from the sec21 ts Mutants

Given the striking result that several media proteins were efficiently secreted from sec21 ts and other COPI mutant cells, we next decided to analyze secretion of the well characterized periplasmic enzyme invertase (encoded by the SUC2 gene). Previous analysis of the original sec21-1 mutant indicated that invertase secretion was partially blocked in this mutant (Novick et al., 1980). Subsequent analysis of sec21-1 and sec33-1 also yielded partial invertase secretion phenotypes (Wuestehube et al., 1996). However, these experiments were done using invertase enzyme assays following derepression of the SUC2 promoter, which necessitated long incubations in low glucose media and long incubations at nonpermissive temperature, conditions which could yield secondary effects that might mask the true behavior of invertase in COPI mutants. To avoid these difficulties, we performed pulse-chase analysis using a constitutively expressed form of invertase, which eliminated the need for SUC2 derepression. Invertase contains 9–12 N-linked glycosylation sites which are heterogeneously and extensively modified in the Golgi complex. By the time it is secreted, invertase migrates as a high molecular mass smear on SDS gels. To assay invertase secretion, cells were incubated at 37°C, pulse-labeled, chased briefly, converted to spheroplasts, and separated into intracellular (I) and extracellular (E) fractions from which invertase was immunoprecipitated. Unexpectedly, we found that invertase, like HSP150, was efficiently and rapidly secreted from both sec21-3 and from all aforementioned COPI mutants; this was observed at all restrictive conditions described for CPY and HSP150, even 60 min preincubations at 38°C (Fig. 5 A and data not shown). Under similar conditions, in sec18-1, sec23-1, sec22-1, and sec1-1 cells, invertase remained entirely intracellular (Fig. 5 A and data not shown). Surprisingly, invertase secreted from both sec21-3 and ret2-1 migrated neither as the high molecular mass smear observed in wild type cells nor as the ER-retained, core-glycosylated ladder of bands observed for sec18-1, but instead as a distinct intermediate form (Fig. 5 A, *).

To observe postER modifications on invertase in sec21-3 and to determine if the shift in molecular mass occurred as a result of incubation at nonpermissive temperature, cells were incubated at 26 or 37°C, pulse-labeled, and chased. Invertase was immunoprecipitated and split into equal aliquots for reimmunoprecipitation with antisera against ei-
munoprecipitation with antisera to either invertase, 10 min. Invertase was recovered by immunoprecipitation and shown for the 37°C experiment. Invertase was visualized by SDS-PAGE and fluorography. Migration of molecular mass standards is shown to the right. At the left, the positions of core (ER) and hyperglycosylated (Golgi) modified invertase are noted. The asterisk (*) denotes the form of invertase is schematized at the top, showing the signal sequence (black) and N-linked glycosylation sites (Y). (A) Wild type (SEY6210), sec18-1 (EGY1181-5), sec21-3 (EGY1213), and ret2-1 (PC130) cells harboring pCYI-20 (encoding constitutively expressed, full-length, secreted invertase) were incubated for 30 min at 37°C, labeled with Tran35S for 10 min, and then chased for 10 min. Transport was stopped by adding NaN3 and NaF to a final concentration of 20 mM each. Cells were converted to spheroplasts and then separated by centrifugation into internal (I) and external (E) fractions. Invertase was recovered by immunoprecipitation and visualized by SDS-PAGE and fluorography. Migration of molecular mass standards is shown to the right. At the left, the positions of core (ER) and hyperglycosylated (Golgi) modified invertase are noted. The asterisk (*) denotes the form of invertase secreted from sec21-3 and ret2-1. (B) Wild type (SEY6210) and sec21-3 (EGY1213) cells harboring pCYI-20 were incubated at 26°C or 37°C for 10 min, labeled for 10 min, and then chased for 10 min. Invertase was recovered by immunoprecipitation and then split into three equal aliquots and subjected to a second immunoprecipitation with antisera to either invertase, α1,6, or α1,3 mannose linkages. Mannose-specific antisera results are only shown for the 37°C experiment. Invertase was visualized by SDS-PAGE and fluorography. Molecular mass standards are shown to the right.

Figure 5. Invertase secretion and Golgi-specific modification. Invertase is schematized at the top, showing the signal sequence (black) and N-linked glycosylation sites (Y). (A) Wild type (SEY6210), sec18-1 (EGY1181-5), sec21-3 (EGY1213), and ret2-1 (PC130) cells harboring pCYI-20 (encoding constitutively expressed, full-length, secreted invertase) were incubated for 30 min at 37°C, labeled with Tran35S for 10 min, and then chased for 10 min. Transport was stopped by adding NaN3 and NaF to a final concentration of 20 mM each. Cells were converted to spheroplasts and then separated by centrifugation into internal (I) and external (E) fractions. Invertase was recovered by immunoprecipitation and then subjected to SDS-PAGE and fluorography. Migration of molecular mass standards is shown to the right. At the left, the positions of core (ER) and hyperglycosylated (Golgi) modified invertase are noted. The asterisk (*) denotes the form of invertase secreted from sec21-3 and ret2-1. (B) Wild type (SEY6210) and sec21-3 (EGY1213) cells harboring pCYI-20 were incubated at 26°C or 37°C for 10 min, labeled for 10 min, and then chased for 10 min. Invertase was recovered by immunoprecipitation and then split into three equal aliquots and subjected to a second immunoprecipitation with antisera to either invertase, α1,6, or α1,3 mannose linkages. Mannose-specific antisera results are only shown for the 37°C experiment. Invertase was visualized by SDS-PAGE and fluorography. Molecular mass standards are shown to the right.

other invertase, α1,6, or α1,3 mannose linkages. At 26°C, invertase from sec21-3 cells migrated as a high molecular mass smear similar to wild type cells (Fig. 5 B), indicating that the aberrant migration in sec21-3 was due to the temperature conditional defect. However, unlike in sec18-1 cells at 37°C (data not shown), in sec21-3 at 37°C, invertase was efficiently modified with both α1,6 and α1,3 mannose (Fig. 5 B). This analysis revealed that (a) invertase secreted from sec21-3 did not bypass Golgi glycosyltransferase activities, and (b) the severe underglycosylation was most likely due to defects in elongating α1,6 mannosyltransferase activity.

BFA Induces Cargo-selective ER to Golgi Transport Defects

As an independent test of the requirement for COPI in ER to Golgi transport, we employed the fungal metabolite BFA and the BFA-sensitive yeast mutant erg6 (ise1). Treatment of mammalian cells with BFA severely perturbs secretory pathway function (Pelham, 1991; Klausner et al., 1992), blocking ER export of several secretory proteins (Misumi et al., 1986) and inducing redistribution of Golgi enzymes to the ER (Lippincott-Schwartz et al., 1989). These events are thought to occur because BFA inhibits a guanine nucleotide exchange factor which catalyzes the exchange of GTP for GDP on ARF (Donaldson et al., 1992; Helms and Rothman, 1992). This inhibition causes ARF and thus coatamer to dissociate from Golgi membranes (Donaldson et al., 1990, 1991). Not surprisingly, BFA also prevents formation of COPI-coated vesicles in vitro (Orci et al., 1991); however, BFA does not affect the membrane association of COPII proteins (Shaw et al., 1995).

The yeast erg6 (ise1) mutant was previously demonstrated to exhibit dramatic growth and anterograde secretory transport defects upon treatment with BFA (Graham et al., 1993; Vogel et al., 1993). To observe the immediate effects of BFA treatment on transport of distinct cargo proteins from the ER to the Golgi, erg6 cells were incubated in media buffered to pH 7.0 either in the presence or absence of 100 µg/ml BFA for 2 min, pulse-labeled for 10 min, and then chased for 30 min. Invertase and CPY were recovered by immunoprecipitation and then subjected to immunoblotting with antisera against either the protein itself or α1,6 mannose linkages. Interestingly, invertase from BFA-treated cells was quantitatively α1,6 mannose modified and migrated at a mobility remarkably similar to that observed for sec21-3 cells at nonpermissive temperature (Fig. 6 A). HSP150 was also modified with Golgi-specific carbohydrates in BFA-treated cells (data not shown). In contrast, BFA treatment resulted in <10% α1,6 mannose modification of CPY (Fig. 6 B). Retrograde flux of Golgi enzymes to the ER was not previously detected in BFA-treated erg6 cells (Graham et al., 1993). Therefore, this analysis suggested that in the presence of BFA, nearly all of the newly synthesized invertase and HSP150 reached the Golgi complex, while >90% of CPY did not.

To confirm that cargo-selective ER to Golgi transport occurred in the presence of BFA, the subcellular distribution of invertase and CPY was determined. Invertase was quantitatively modified with Golgi-specific carbohydrates (Fig. 6 A); however, its secretion, like that of media proteins such as HSP150, was completely blocked after BFA treatment (data not shown). We thus analyzed the intracellular location of invertase and CPY by differential centrifugation. erg6 cells were converted to spheroplasts, incubated in media buffered to pH 7.0 in the presence of 100 µg/ml BFA for 2 min, and then pulse-labeled and chased. The lysed spheroplasts were then subjected to differential centrifugation as described for Fig. 1 C, after which CPY and invertase were recovered by immunoprecipitation.
Because secretion was also blocked in BFA treated cells, this analysis also suggests that BFA acts at multiple sites in the yeast secretory pathway, consistent with previous observations in mammalian cells (Pelham, 1991; Klausner et al., 1992; Strous et al., 1993; Torii et al., 1995).

**CPY-Invertase Hybrids Exit the ER and Are Sorted to the Vacuole in sec21 ts Mutants**

Thus far, our analysis has shown that the sec21 ts mutations (and BFA treatment) prevented ER export of some proteins while others were entirely unaffected. We next decided to address which of these two events is dominant; for instance, does a protein like CPY contain a signal for ER retention in this mutant, or might invertase contain a signal for its active ER export even under conditions where other proteins are blocked?

We therefore examined the behavior of a series of CPY-invertase fusion proteins. These fusion proteins use the CPY promoter and contain either 50, 156, or 433 amino acids of CPY fused to full-length invertase (Fig. 7A). Each fusion protein has previously been shown to be efficiently targeted to the vacuole and proteolytically cleaved in a vacuolar hydrolase-dependent manner at the CPY–invertase junction (Johnson et al., 1987). Cells harboring these fusion proteins were incubated at 37°C, pulse-labeled, and chased. Fusion proteins were recovered by immunoprecipitation with invertase antiserum and treated with endoglycosidase H, which removes NH2-linked sugars and reduces invertase to a single band, to reveal the vacuolar processing event. In sec18-1, the fusion proteins migrated at the predicted molecular mass for the intact CPY-invertase hybrids (Fig. 7B, ●), indicating that they were retained in the ER and not transported to the vacuole. In both wild type and sec21-3 cells at nonpermissive temperature, however, only the cleaved, vacuolar form of the hybrid proteins was recovered (Fig. 7B, arrow). This demonstrated that each of these fusion proteins, even CYI-433 (which contains ~80% of CPY), escaped the sec21-3 ER block and was subsequently targeted to the vacuole and proteolytically processed. Interestingly, this analysis also revealed that, as with Golgi to plasma membrane transport, transport and sorting from the Golgi to the vacuole was unaffected in sec21-3.

**The cis-Golgi Is Disrupted in the sec21 ts Mutants**

In mammalian cells, disruption of COPI function leads to significant morphological changes in Golgi structure (Donaldson et al., 1990; Guo et al., 1994; Kreis et al., 1995). Our observation that invertase was significantly underglycosylated in the sec21 ts mutants suggested that Golgi structure might also be physically disrupted upon shift of these yeast COPI mutants to nonpermissive temperature. We performed indirect immunofluorescence experiments to look at the distribution of two Golgi resident proteins, Och1p (earliest cis-Golgi: Nakamichi-Shindo et al., 1993; Gaynor et al., 1994) and Mnn1p (medial/trans-Golgi: Graham and Emr, 1991; Graham et al., 1994). For Och1p, we used an HA epitope-tagged form of the protein which has previously been shown to substitute functionally for and exhibit near-identical fractionation characteristics with native Och1p (Harris and Waters, 1996). Cells were incu-
bated for 30 min at 26 or 37°C, and then fixed and prepared for indirect immunofluorescence with either the 12CA5 monoclonal antibody directed against the HA epitope or an affinity-purified anti-Mnn1p antibody. In wild type cells at 26 and 37°C (Fig. 8 A and data not shown) and in sec21-3 cells at 26°C (Fig. 8 B), Och1p localized to punctate structures (∼5–10/cell in a focal plane) characteristic of the yeast Golgi. However, in sec21-3 at 37°C (Fig. 8 C), Och1p exhibited a much more diffuse staining pattern, with some faint punctate spots visible but with most of the signal dispersed through the cell, perhaps staining either small vesicular or tubulo-vesicular structures. This staining was specific to Och1p, since cells not harboring the HA-Och1p plasmid did not stain with the 12CA5 antibody (data not shown). The diffuse staining in sec21-3 at 37°C did not result from decreased protein expression, transport to and/or degradation in the vacuole, or significant accumulation of the protein in the ER, since immunoblot analysis yielded a similar signal and subcellular fractionation pattern for HA-Och1p in both wild type and sec21-3 cells at 26 and 37°C (Fig. 1 C and data not shown). After longer preincubations at 37°C (>1 h), ∼10% of sec21-3 cells also exhibited modest ER staining (data not shown), suggesting that appearance of Och1p in the ER may represent a secondary, more terminal phenotype in this mutant. Interestingly, in contrast to Och1p, Mnn1p exhibited a punctate Golgi staining pattern in both wild type cells at 26 or 37°C (Fig. 8 D and data not shown) and in sec21-3 cells at either 26 (Fig. 8 E) or 37°C (Fig. 8 F), with ∼8–15 distinct punctate structures observed per cell in a focal plane. Therefore, while the sec21-3 mutation caused a dramatic change in distribution of a cis-Golgi protein, a medial/trans-Golgi protein appeared to be unaffected. This suggests that COPI plays a role in maintenance of early but not late Golgi structure.
Discussion

Recent controversy over the precise and primary role of the COPI/coatomer complex has yielded several speculative models to explain how COPI may function both in retrograde and anterograde ER/Golgi transport (Pelham, 1995; Aridor and Balch, 1996; Lewis and Pelham, 1996; Schekman and Orci, 1996). These models have remained speculative in part because of uncertainty over whether existing yeast COPI mutants were simply weak alleles which may partially allow both retrograde and anterograde transport to continue. Thus it has been difficult to establish a primary phenotype associated with loss of COPI function in yeast. We have isolated and analyzed multiple new temperature-conditioned sec21 (γ-COP) mutants (represented by sec21-3 in this study). Surprisingly, even though these mutants were not identified by screening for directional transport defects, each new mutant rapidly and completely blocked ER export of some cargo proteins. However, further analysis of these mutants revealed that (a) other cargo proteins were unaffected in these and other COPI mutants, (b) these cargo proteins may contain active recognition determinants directing their packaging into COPII vesicles, (c) anterograde transport to the cell surface and to the vacuole is not affected in the sec21 ts mutants, and (d) these COPI mutations cause significant perturbation of early but not late Golgi structure and function. We also found that BFA induced nearly identical cargo-specific ER to Golgi transport defects as observed in the sec21 ts mutants. Together, our results are consistent with a model whereby COPI does not act directly in anterograde transport but instead plays a critical role in regulated retrograde transport between the Golgi and the ER, and that this role indirectly influences ER to Golgi transport of selected cargo proteins.

Model for an Indirect Requirement for COPI in ER to Golgi Transport of Some but Not All Cargo

In the transport model we propose (Fig. 9), cargo destined for export from the ER would first be concentrated and recruited into budding COPII-coated vesicles (COPII-CVs) by cargo-specific “receptors” or transport factors. All cargo would then travel to the earliest cis-Golgi (Och1p) compartment in COPII-CVs. Although our model implies that this step is mediated by a single type of COPII-CV, our data do not rule out the possibility that multiple types of COPII-CVs may carry distinct cargo to the cis-Golgi. After COPII vesicles fuse with the cis-Golgi, transport factors (i.e., receptors) for cargo such as CPY and α-factor would cycle back to the ER in COPII-CVs to be reutilized for further rounds of traffic. The absence of Sec21p/COPI function would result in rapid depletion of receptor pools in the ER, causing anterograde transport of cargo dependent on these receptors to cease. Without COPI, for instance, these receptors may not be recognized in the cis-Golgi and, like other proteins dependent on COPI for retrieval to the ER (i.e., invertase-Wbp1 fusion proteins; Letourneur et al., 1994), be directed to the vacuole and degraded. Anterograde transport of cargo insensitive to the COPII mutant block (i.e., HSP150 and invertase) could be maintained by one of three putative mechanisms. One possibility is that these cargo do not require sorting receptors and thus are not sensitive to a block in retrieval of these proteins. Second, HSP150 and invertase may use receptor(s) with sufficiently high rates of expression such that their de novo synthesis allows efficient packaging of these cargo into COPII vesicles. Third, HSP150 and invertase may interact with receptor(s) which do recycle but which do not absolutely require Sec21p/COPI for recognition in the cis-Golgi and retrieval to the ER. The latter two possibilities seem most plausible (Fig. 9), since evidence for cargo-selective transport in other yeast mutants already exists (Schimmoller et al., 1995), and proteins like invertase and HSP150 are likely to be actively packaged into COPII-CVs. If these receptors do recycle, how might this con-

Figure 9. Model for an indirect requirement for COPI in anterograde ER to Golgi transport. In the ER, cargo molecules first associate with specific transport factors (i.e., “sorting receptors”) which mediate their concentration and packaging into COPII-coated vesicles. Cargo proteins like CPY () and α-factor () utilize one type of receptor (T), while cargo like invertase () and HSP150 () utilize a different type of receptor (i.e., U-shaped receptor). COPII vesicles dock and fuse with the cis-Golgi via a v-SNARE/t-SNARE interaction. Some cargo receptors (i.e., T) are then packaged into and require COPII vesicles for retrieval back to the ER. COPII vesicles dock and fuse with the ER (via a distinct v-SNARE/t-SNARE interaction), allowing these receptors (T) to direct additional cargo molecules into anterograde COPII vesicles. Other type(s) of receptors (i.e., U-shaped receptor) do not absolutely require COPI for retrieval (see Discussion), and thus, anterograde transport of their cargo does not cease in the absence of COPI function. Not shown is the role of COPI in intra-Golgi and distal Golgi to ER retrieval. This may contribute to the altered Golgi morphology and glycosylation seen in COPI mutants (see Discussion).
continue in the sec21 ts mutants and BFA treated cells? Disruption of COPI function in mammalian cells has been shown to induce formation of Golgi–ER tubules that lead to unregulated transport of Golgi proteins to the ER (Lippincott-Schwartz et al., 1990; Kreis et al., 1995). One speculative possibility is that normally the invertase/HSP150 “receptors” may or may not recycle in COPI vesicles; however, under mutant/COPI-deficient conditions, tubule formation could allow return of these but not other “receptor” proteins.

Despite the cargo-selective anterograde transport defects observed in the sec21 ts mutants, models supporting a direct role for COPI in anterograde ER to Golgi transport seem unlikely given the following contradictory observations. First, there is no evidence in favor of COPII-independent ER to Golgi transport (Fig. 3; Barlowe et al., 1994; Aridor et al., 1995; Bednarek et al., 1995; Doering and Schekman, 1996), and COPI and COPII are unlikely to coat the same transport vesicle (Barlowe et al., 1994; Aridor et al., 1995; Bednarek et al., 1995). Second, while our results are most consistent with an ER block for both CPY and α-factor in the sec21 ts mutants (Figs. 1 and 2), α-factor is packaged exclusively into COPII-CVs in vitro (Bednarek et al., 1995), and these vesicles continue to bud even when COPI is washed off the membrane (Barlowe et al., 1994). Finally, no soluble cargo proteins have thus far been found within ER-budded COPI vesicles (Bednarek et al., 1995).

Finally, if our model is correct, then other mutants defective for the retrograde transport process might also be expected to exhibit cargo-specific anterograde transport defects. Recently, Lewis and Pelham showed that Sec20p, an ER membrane protein, and Ufe1p, a candidate retrograde ER t-SNARE, are likely to participate in retrieval of dilysine-containing proteins (Lewis and Pelham, 1996). In the SNARE hypothesis (Rothman, 1994), targeting of transport vesicles to an appropriate acceptor compartment is mediated by the interaction of a v-SNARE protein on the vesicle with its cognate t-SNARE protein on the target membrane. Interestingly, the sec20-2 mutant as well as the bet1-1 v-SNARE mutant also exhibited cargo-specific ER to Golgi transport defects (Fig. 4). These results suggest that Bet1p might act as a retrograde v-SNARE involved in targeting of recycling Golgi to ER vesicles with Ufe1p on the ER. Recently, rat homologs of Sec22p and Bet1p (rsec22 and rbet1) were identified (Hay et al., 1996). Surprisingly, these two proteins localized to distinct subcellular compartments: rsec22 localized to the ER, consistent with its role as an anterograde COPII-CV v-SNARE, while rbet1 localized to the Golgi. Together, these data support a role for Bet1p as a candidate v-SNARE for retrograde COPI-CVs.

**Active Cargo Concentration into COPII Vesicles via cis-acting Sorting Determinants**

Essential to our model is the existence of cargo “receptors” and whether such receptors might actively recruit and concentrate cargo into budding COPII-CVs. If this is the case, then the ER would provide the first point of discrimination between distinct cargo proteins. Two candidate cargo receptor families have already been identified. One, the p24 family of membrane proteins, has been detected in both COPI- and COPII-CVs (Schimmoller et al., 1995; Stamnes et al., 1995), and the cytoplasmic domains of these proteins have recently been shown to bind COPI proteins in vitro (Fiedler et al., 1996). Interestingly, yeast strains deleted for the first member of this family to be cloned, EMP24, exhibit a delay in anterograde transport of some but not all cargo proteins (Schimmoller et al., 1995). However, unlike the sec21-1 mutant, Δemp24 strains affect transport of invertase but not CPY; this also supports the idea that packaging of proteins like invertase into COPII vesicles requires “receptors” or transport factors (Fig. 9). It has also been suggested that lectin-like membrane proteins may comprise another candidate cargo “receptor” family (Fiedler and Simons, 1995; Schekman and Orci, 1996). In yeast, a KXXX-containing protein exhibiting lectin homology (Emp47p) has been identified and shown to cycle between the Golgi and ER in a COPII-dependent manner (Schroder et al., 1995; Lewis and Pelham, 1996). Δemp47 strains do not exhibit transport defects for any proteins tested (Schroder et al., 1995). However, BLAST (Basic Local Alignment Search Tool) analysis of the recently completed yeast genome sequence revealed a protein with significant homology to EMP47, suggesting that the two proteins may function similarly or even substitute for each other. Given that HSP150 and invertase (two of the proteins observed to be unaffected by the sec21-3 transport block) are both extensively glycosylated, it seems plausible that a lectin-like protein may play a role in their selective transport out of the ER.

Concentration of cargo into COPII-CVs has also been observed in both yeast and mammalian cells; however, the mechanism for this event is unknown, and whether this is an active process has been postulated but not yet demonstrated (Aridor and Balch, 1996; Schekman and Orci, 1996). In support of this being an active process, it is interesting to note that in the sec21 ts mutants, newly synthesized CPY and α-factor did not escape the ER, even after long incubations at nonpermissive temperature. Thus these proteins were not simply swept along (in a “bulk flow” manner) into anterograde vesicles along with invertase and HSP150, both of which were efficiently transported out of the ER in a COPII-dependent manner (Figs. 4 and 5 and our unpublished observations). In contrast, CPY-invertase hybrid proteins, like invertase, were efficiently transported out of the ER under conditions where CPY transport was blocked (Fig. 7). Thus CPY is not likely to be “actively retained” in the ER in the COPI mutant. Previous work demonstrated that HSP150 can act as a “carrier,” which, when fused to nonyeast proteins, directs their transport through the media (Simonen et al., 1994). Together, these observations suggest that both invertase and HSP150 may contain active sorting determinants mediating their packaging into anterograde COPII-CVs. Interestingly, a candidate motif for such a determinant may already exist. Previous work demonstrated that a single point mutation in the NH₂-terminal region of invertase resulted in ER accumulation of this “S2” mutant invertase in wild type cells (Schauer et al., 1985). We are currently investigating whether this region of invertase is also responsible for its COPI-independent ER to Golgi transport.

**COPI Is Required for Early but Not Late Golgi Structure and Function**

Our data also indicate that COPI plays a critical role in
maintenance of early Golgi function and structure in yeast. In the sec21-3 mutant at nonpermissive temperature, immunofluorescence revealed that the cis-Golgi was noticeably perturbed, while the medial/trans Golgi appeared normal (Fig. 8). We also observed striking underglycosylation of invertase in all COPI mutants, most notably sec21-3 and ret2-1 (Fig. 5). Because initial α1,6 and α1,3 mannosyltransferases were still functional, this was likely due to a defect in elongating α1,6 mannosyltransferase activity. One interpretation of these data is that early Golgi compartments no longer retain functional integrity because they are either “mixed,” dissociated, or vesiculated in this mutant, thereby causing inactivation of enzymes whose activities are dependent on a specific environment. “Mixing” might occur if COPI-mediated recycling of mannosyltransferases is required for maintenance of functionally distinct early Golgi compartments. For instance, loss of COPI function might cause a more random distribution of the mannosyltransferases, yielding the diffuse Och1p staining pattern and perhaps accounting for inactivation of the elongating α1,6 mannosyltransferase. Alternatively, COPI may act structurally to stabilize the cis-Golgi; in this case, loss of COPI function could result in either mixing or rapid dissociation/vesiculation of early Golgi membranes. The yeast Golgi is difficult to detect at the ultrastructural level, and EM analysis of sec21-3 shifted for 1 h to nonpermissive temperature did not reveal significant accumulation of vesiculated or tubulated membranes (our unpublished observations). However, consistent with this interpretation of our data, rapid and dramatic vesiculation of Golgi membranes was observed by EM in a mammalian e-COP mutant after incubation at nonpermissive temperature (Guo et al., 1994).

Despite early Golgi aberrancies in the sec21-3 mutant, this organelle remained remarkably competent for trafficking. Neither HSP150 nor invertase bypassed this organelle en route to the plasma membrane: both proteins ficking. Neither HSP150 nor invertase bypassed this or-ganelle en route to the plasma membrane: both proteins traversing. Both proteins, as well as membrane proteins, were retained by ER (Fig. 4). Later Golgi compartments also seemed to be unaffected in the sec21-3 mutant, both structurally and functionally; indeed, both TGN to plasma membrane and TGN to vacuole transport steps occurred normally and involved factors that suggested that the trans-Golgi retained wild type characteristics (i.e., secretion required Sec1p, and transport to the vacuole required a vacuolar sorting determinant). If COPI is required for regulated antero- grade vesicular transport through the Golgi, it is possible that fusion of Golgi cisternae in the sec21 ts mutants could mask this requirement by creating a syncytium allowing (unregulated) anterograde transport to continue. However, given the distinct localization patterns of Och1p and Mnn1p and the fact that, with the exception of the elongating α1,6 mannosyltransferase, Golgi function appears nearly normal in the sec21 ts mutants, this seems unlikely.

In summary, we have provided evidence that COPI is not likely to act directly in anterograde secretory transport but instead, along with other proteins (i.e., Sec20p and Bet1p), is more likely to be required for recycling of transport factors required to package selected cargo into COPII vesicles in the ER. Future identification and characterization of both these transport factors as well as sort-

ing determinants in cargo proteins directing their packaging into COPII vesicles will provide additional mechanistic insight into this essential sorting process.

We thank members of the Emr lab for many helpful discussions during the course of this work, especially Beverly Wendland, Chris Burd, and Matthew Seaman for also critically reading the manuscript, and Eden Estepa for excellent technical assistance. We thank Randy Schekman, Rainer Duden, Pierre Cosson, Francois Letourneur, Marja Makarow, Sandy Harris, Gerry Waters, Todd Graham, David Levin, Chris Kaiser, and Susan Ferro-Novick for providing strains, plasmids, and/or antisera.

This work was supported by grants from the National Institutes of Health (GM32703 and CA58889) to S.D. Emr. S.D. Emr is supported as an Investigator of the Howard Hughes Medical Institute.

References

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.

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. COPII- and COPII-coated vesicles bud directly from the endoplasmic reticulum in yeast. Cell. 83:1183–1196.

Bennett, M.K. 1995. SNAREs and the specificity of transport vesicle targeting. Curr. Opin. Cell Biol. 7:581–586.

Cosson, P., and F. Letourneur. 1994. Coatamer interaction with di-lysine endo-plasmic reticulum retention motifs. Science (Wash. DC). 263:1629–1631.

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

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

Doerger, T.L., and R. Schekman. 1996. GPI anchor attachment is required for Gaslp transport from the endoplasmic reticulum in COPI II vesicles. EMBO (Eur. Mol. Biol. Organ.) J. 15:182–191.

Donaldson, J.G., J. Lippincott-Schwartz, G.S. Bloom, T.E. Kreis, and R.D. Klausner. 1990. Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in brefeldin A action. J. Cell Biol. 111:2295–2306.

Donaldson, J.G., R.A. Kahn, J. Lippincott-Schwartz, and R.D. Klausner. 1991. Binding of ARF and beta-COP to Golgi membranes: possible regulation by a transmembrane G protein. Science (Wash. DC). 254:1197–1199.

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

Duden, P., M. Hosobuchi, S. Hamamoto, M. Winey, B. Byers, and R. Schekman. 1994. Yeast beta- and beta'-coat proteins (COP). Two coatamer subunits essential for endoplasmic reticulum-to-Golgi protein traffic. J. Biol. Chem. 269:24486–24495.

Epseneshade, P., R.E. Gimeno, E. Holzmacher, P. Teung, and C.A. Kaiser. 1995. Yeast SEC16 gene encodes a multidomain vesicle coat protein that interacts with Sec23p. J. Cell Biol. 131:311–324.

Fiedler, K., and K. Simons. 1995. The role of N-glycans in the secretory pathway. Cell. 131:309–312.

Fiedler, K., M. Veit, M.A. Stamnes, and J.E. Rothman. 1996. Role of brefeldin A in activating COPII vesicles. J. Cell Biol. 131:27–37.

Gaynor, E.C., S. Heesen, T.R. Graham, M. Aebl, and S.D. Emr. 1994. Signal-mediated retrieval of a membrane protein from the Golgi to the ER in yeast. J. Cell Biol. 127:653–665.

Gaynor, E.C., and S.D. Emr. 1991. Compartmental organization of Golgi-speciﬁc protein modification and vacuolar protein sorting events deﬁned in a yeast sec18 (NSF) mutant. J. Cell Biol. 114:207–218.

Graham, T.R., P.A. Scott, and S.D. Emr. 1993. Brefeldin A reversibly blocks early but not late protein transport steps in the yeast secretory pathway. EMBO (Eur. Mol. Biol. Organ.) J. 12:869–877.

Graham, T.R., M. Seeger, G.S. Payne, V.L. MacKay, and S.D. Emr. 1994.
Clathrin-dependent localization of alpha 1,3 mannosyltransferase to the Golgi complex of Saccharomyces cerevisiae. J. Cell Biol. 135:2753–2763.

Pelham, H.R. 1991. Multiple targets for brefeldin A. Cell. 67:449–451.

Pelham, H.R. 1994. About the COPVs. J. Biol. 127:667–678.

Pelham, H.R. 1995. Sorting and retrieval between the endoplasmic reticulum and Golgi apparatus. Curr. Opin. Cell. Biol. 7:530–535.

Pepperkok, R., J. Scheel, H. Horstmann, H.P. Hauri, G. Griffiths, and T.E. Kreis. 1993. Beta-COP, a non-clathrin-coated Golgi transport protein from the endoplasmic reticulum to the Golgi complex in vivo. Cell. 74:71–82.

Peter, F., H. Plutner, H. Zhi, T.E. Kreis, and W.E. Balch. 1993. Beta-COP is essential for transport of protein from the endoplasmic reticulum to the Golgi complex. J. Cell Biol. 122:1125–1137.

Redding, K., C. Holcomb, and R.S. Fuller. 1991. Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in the yeast Saccharomyces cerevisiae. J. Cell Biol. 113:527–538.

Robinson, J.S., D.J. Klionsky, J. Hauri, and S.D. Emr. 1988. Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol. Cell. Biol. 8:4936–4948.

Rothman, J.E. 1994. Mechanisms of intracellular protein transport. Nature (Lond.). 372:55–63.

Rothman, J.E., and L. Orci. 1992. Molecular dissection of the secretory pathway. Nature (Lond.). 355:409–415.

Rothman, J.E., and F.T. Wieland. 1996. Protein sorting by transport vesicles. Science (Wash. DC). 272:227–234.

Russo, P., N. Kalkkinen, H. Sareneva, J. Paakkola, and M. Makarow. 1992. A heat shock gene from Saccharomyces cerevisiae encoding a secretory glycoprotein [published erratum appears in Proc. Natl. Acad. Sci. USA. 1992 Sep 13;89(18):8857]. Proc. Natl. Acad. Sci. USA. 89:3671–3675.

Schauer, I., S. Emr, C. Gross, and R. Schekman. 1985. Inversion signal and maternal sequence substitutions that delay intercompartmental transport of active enzyme. J. Cell Biol. 100:1664–1675.

Schekman, R., and L. Orci. 1996. Coat proteins and vesicle budding. Science (Wash. DC). 271:1526–1533.

Schimmler, F., B. Singer-Kruger, S. Schroder, U. Kruger, C. Barlowe, and H. Riezman. 1995. The absence of Emp24p, a component of ER-derived COPI-coated vesicles, causes a defect in transport of selected proteins to the Golgi. EMBO (Eur. Mol. Biol. Organ.) J. 14:1329–1339.

Schroder, S., F. Schimmler, B. Singer-Kruger, and H. Riezman. 1995. The Golgi-localization of yeast Emp47p depends on its disulfyl motif but is not affected by the ret1-1 mutation in COP. J. Cell Biol. 131:885–912.

Shawitzy, D.A., L. Ori, M. Ravazolla, A. Sraapow, and C.A. Kaiser. 1995. Human SEC13Rp functions in yeast and is located on transport vesicles budding from the endoplasmic reticulum. J. Cell Biol. 128:769–777.

Sherman, F., R.G. Fink, and M. Lawrence, editors. 1979. Methods in Yeast Genetics: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics. 122:19–27.

Sikorski, R.S., and J.D. Boeke. 1991. In vitro mutagenesis and plasmid shuffling: from cloned gene to mutant yeast. Methods Enzymol. 194:302–318.

Simonen, M., E. Jamsa, and M. Makarow. 1994. The role of the caroten and disulfide formation in the folding of beta-lactamase fusion proteins in the endoplasmic reticulum of yeast. J. Biol. Chem. 269:13887–13892.

Sogasaki, M., K. Tani, R.R. Ye, S. Geromanos, P. Tempst, T. Kirchhausen, J.E. Rothman, and L. Orci. 1994. A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. Cell. 78:937–948.

Stanmases, M.A., M.W. Craighead, M.H. Hoe, N. Lampen, S. Geromanos, P. Tempst, and J.E. Rothman. 1995. An integral membrane component of coated-vesicle transport defines a family of proteins involved in budding. Proc. Natl. Acad. Sci. USA. 92:8011–8015.

Stevens, T., B. Esmon, and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. Cell. 30:439–448.

Strous, G.J., P. van Kerkhov, G. van Meier, S. Rijnboutt, and W. Stoorvogel. 1993. Differential effects of brefeldin A on transport of secretory and lysosomal proteins. J. Biol. Chem. 268:2341–2347.

Tori, S., T. Banno, T. Watanabe, Y. Ikehara, K. Murakami, and K. Nakayama. 1995. Cytotoxicity of brefeldin A correlates with its inhibitory effect on membrane binding of COP coat proteins. J. Biol. Chem. 270:11574–11580.

Vogel, J.P., J.N. Lee, D.R. Kirsch, M.D. Rose, and E.S. Sztul. 1993. Brefeldin A causes a defect in secretion in Saccharomyces cerevisiae. J. Biol. Chem. 268:3040–3043.

Waters, M.G., T. Serafini, and J.E. Rothman. 1991. ‘Coatamer’: a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. Nature (Lond.). 349:443–450.

Wiestehove, I., R. Duden, A. Eun, S. Hamamoto, P. Korn, R. Ram, and R. Schekman. 1996. New mutants of Saccharomyces cerevisiae affected in the transport of proteins from the endoplasmic reticulum to the Golgi complex. Genetics. 142:393–406.

Zhang, C.J., A.G. Rosenwald, M.C. Willingham, S. Skuntz, J. Clark, and R.A. Kahn. 1994. Expression of a dominant allele of human ARF1 inhibits membrane binding of COP coat proteins. J. Biol. Chem. 269:20305–20309.