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 bidirectional 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 (Espenshade 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-

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icant debate. In vitro work in mammalian cells initially revealed that COPI, along with the small GTP-binding protein ADP-ribosylation factor (ARF)[1], binds Golgi membranes and induces formation of functional transport vesicles (Malhotra et al., 1989; Rothman and Orci, 1992). Several lines of evidence support a role for COPI in anterograde transport. In mammalian cells, both in vitro and in vivo work has indicated that inhibition of COPI function results in a block in ER to Golgi transport (Pepperkok et al., 1993; Peter et al., 1993; Dascher and Balch, 1994; Zhang et al., 1994). COPI-coated vesicles have also been observed to bud from purified yeast nuclei (ER membranes); however, unlike COPII-coated vesicles, ER-derived COPI-coated vesicles are devoid of any known cargo (Bednarek et al., 1995). Finally, three of the COPI subunits (β, β′, γ) were initially identified as *SEC* gene products in yeast; *sec* mutants were isolated as defective for anterograde secretory transport (Hosobuchi et al., 1992; Duden et al., 1994).

More recently, COPI has been implicated as playing a critical role in Golgi to ER retrograde transport. A dilysine (KKXX) motif directing retrieval of type I membrane proteins (Jackson et al., 1993; Gaynor et al., 1994; Townsley and Pelham, 1994) was found to bind specifically to COPI proteins in vitro (Cosson and Letourneur, 1994). Furthermore, a screen for yeast mutants defective for KKXX-mediated retrieval (*ret* mutants) identified three COPI subunits (α, δ, ζ) as products of *RET* genes (Letourneur et al., 1994; Cosson et al., 1996). Interestingly, all COPI mutants, whether *sec* or *ret* mutants, are defective for KKXX-mediated retrieval, while only a small subset exhibit deficiencies in anterograde ER to Golgi transport (Letourneur et al., 1994; Cosson et al., 1996). These observations have led to the speculation that COPI may directly function only in retrograde traffic, and that anterograde transport defects in these mutants are a secondary consequence of an inability to recycle transport components required for anterograde traffic to continue (Letourneur et al., 1994; Pelham, 1994; Lewis and Pelham, 1996). However, direct verification of this hypothesis has not yet been provided.

To dissect the role of COPI in yeast more thoroughly, we used random PCR mutagenesis to generate new temperature-sensitive (*ts*) alleles of *SEC21*, which encodes the yeast γ-COP subunit (Hosobuchi et al., 1992). In these *sec21 ts* mutants, certain cargo proteins (i.e., CPY and α-factor) were blocked in the ER, while other cargo proteins (i.e., HSP150, invertase, CPY-invertase hybrid proteins) were not blocked and were secreted normally or sorted properly to the vacuole. Similar cargo-specific ER to Golgi transport was also observed in BFA-treated cells; however, no ER to Golgi transport was detected in COPII or NSF mutants. Our observations support a model whereby COPI is not directly required for anterograde transport at any step in the secretory pathway (ER to Golgi, Golgi to plasma membrane, or Golgi to vacuole); instead, ER to Golgi traffic of some cargo proteins may require COPI for retrieval of limiting transport factors essential for packaging these cargo into anterograde, ER-derived COPII-coated vesicles. This is likely to be an active process mediated by sorting determinants contained within the cargo proteins themselves.

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.

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 EGY1181-5, EGY1231-1 and -2, EGY111-2, and EGY1221-2 were constructed by crossing SEY5188, SF309-1C, SEY5017, 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 YEP13 complementing library plasmid (Hosobuchi et al., 1992) into the vector pRS315 (Sikorski and Hieter, 1989). Plasmid p416SEC21 contains this same NotI-HindIII fragment of *SEC21* in pRS416 (Sikorski and Hieter, 1989). To generate the *sec21*::*HIS3* disruption construct, an AvaI–BclI fragment of *SEC21* (comprising >95% of the *SEC21* ORF) was replaced with the *HIS3* gene in p315SEC21. This plasmid was digested with Spe1, and the Spe1 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., 1987). Plasmid pOH containing the hemagglutinin (HA)-tagged Och1p 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 160 nucleotides 5′ of the BamHI site and 150 nucleotides 3′ of the BclI 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 BclI 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 mutagenized *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-conditional mutants which grew on 5-FOA at 26° 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 (*sec23-1*/*sec21-3*) and EGY11/213 (*sec1-1*/*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 antisera, endoglycosidase H treatment, and subcellular fractionation

Table I. Saccharomyces cerevisiae Strains Used in This Study

Strain	Description	Source or reference
SEY6210	*MATα ura3 ura3 leu2 his3 trp1 lys2 suc2Δ9*	(Robinson et al., 1988)
SEY6211	*MAT***a** *ura3 ura3 leu2 his3 trp1 ade2 suc2Δ9*	(Robinson et al., 1988)
EGY021	*MATα ura3 leu2 his3 trp1 suc2Δ9 sec21::HIS3* containing p416SEC21 (*URA3 CEN6 SEC21*)	This study
EGY1213	*MATα ura3 ura3 leu2 his3 trp1 suc2Δ9 sec21::HIS3* containing p315sec21-3 (*LEU2 CEN6 sec21-3*)	This study
SEY5188	*MATα sec18-1 ura3 leu2 suc2Δ9*	(Graham and Emr, 1991)
EGY1181-5	*MATα sec18-1 ura3 leu2 his3 suc2Δ9*	This study
SF309-1C	*MAT***a** *sec23-1*	R. Schekman*
EGY1231-1	*MATα sec23-1 ura3 leu2 trp1 suc2Δ9*	This study
EGY1231-2	*MAT***a** *sec23-1 leu2 his3 suc2Δ9*	This study
EGY231/213	*MAT***a** *sec23-1 leu2 his3 suc2Δ9 suc21::HIS3* containing p315sec21-3 (*LEU2 CEN6 sec21-3*)	This study
SEY5017	*MAT***a** *sec1-1 ura3 leu2 suc2Δ9*	Lab strain
EGY111-2	*MAT***a** *sec1-1 ura3 leu2 his3 suc2Δ9*	This study
EGY11/213	*MATα sec1-1 ura3 leu2 his3 suc2Δ9 sec21::HIS3* containing p315sec21-3 (*LEU2 CEN6 sec21-3*)	This study
RSY454	*MATα sec22-1 ura3 leu2 his4 pep4::URA3*	R. Schekman
EGY1221-2	*MATα sec22-1 ura3 leu2 his4 trp1 suc2Δ9*	This study
RSY957	*MATα sec33-1 ura3 his4*	R. Schekman
PC130	*MAT***a** *ret2-1 ura3 leu2 his4 lys2 suc2Δ9*	P. Cosson‡
RSY580	*MAT***a** *sec20-2 ura3 leu2 ade2*	R. Schekman
RSY945	*MATα bet1-1 his4*	S. Ferro-Novick§
RSY317	*MATα sec16-1 leu2*	R. Schekman
EGY101	*MAT***a** *ret1-1 ura3 leu2 his3 trp1 suc2Δ9*	Letourneur et al., 1994)
CKY100	*MAT***a** *sec27-1 ura3 leu2*	C. Kaiser‖
PC159	*MAT***a** *ret3-1 ura3 leu2 his4 trp1 suc2Δ9*	P. Cosson
TGY413-6D	*MATα erg6(ise1) ura3 leu2 his3 suc2Δ9*	(Graham et al., 1993)

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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, 40 mM NaN_3, 40 mM NaF, 20 mM DTT), incubating on ice for 10 min, then adding 25 μg zymolyase/ml cell suspension and incubating 30 min at 30°C. To separate cells or spheroplasts from media, cell suspensions were centrifuged at 5,000 *g* for 5 min. To assay media proteins, media fractions were precipitated by addition of TCA to a final concentration of 10%. After washing the pellets twice in acetone, proteins were solubilized by sonication in Laemmli sample buffer plus 5% β-mercaptoethanol, boiled, and 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 internal/external fractions, OD equivalents of cells or spheroplasts and media were harvested and precipitated by adding TCA to a 10% final concentration. Media fractions were prepared as described. Cells were prepared for immunoprecipitation as previously described (Gaynor et al., 1994). Spheroplasts were similarly prepared but without glass bead lysis. Antisera to CPY (Klionsky et al., 1988), α factor (Graham and Emr, 1991), HSP150 (Russo et al., 1992), invertase (Gaynor et al., 1994), and α1,6 and α1,3 mannose (Franzusoff and Schekman, 1989) were described previously. Immunoblotting to detect Och1-HA was done using the 12CA5 monoclonal antibody (provided by David Levin, Johns Hopkins University, Baltimore, MD, and made by Berkeley Antibody Co., Berkeley, CA) at a 1:5,000 dilution and visualized by ECL. BFA was from Epicentre Technol. Corp. (Madison, WI) and was solubilized at 10 mg/ml in 95% ethanol before use. For experiments involving BFA treatment of the *erg6* 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.

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 dilysine-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 nonpermissive (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. 1 *A*), 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. 1 *A*). 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

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.

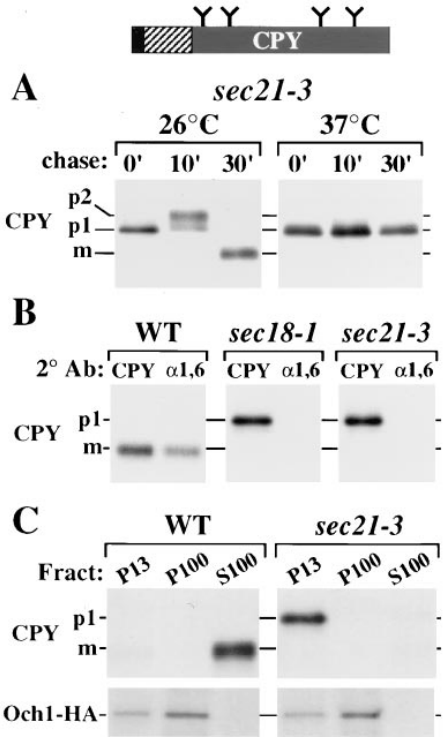

Figure 1. CPY transport analysis in *sec21-3.* CPY is schematized at the top, showing the signal sequence (*black*), pro segment (*hatched lines*), and four N-linked glycosylation sites (**Y**). (*A*) Processing and maturation of CPY. *sec21-3* (EGY1213) cells were incubated at 26 or 37°C for 10 min, labeled for 10 min with Tran^{35}S, and then chased for the indicated times. CPY was recovered by immunoprecipitation, subjected to SDS-PAGE, and visualized by fluorography. The positions of precursor (*p*1/ER; *p*2/Golgi) and mature (*m*/vacuolar) forms of CPY are indicated. (*B*) Golgi-specific modification of CPY in *sec21-3.* Wild type (SEY6210), *sec18-1* (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 with antiserum to either CPY or α1,6 mannose linkages, 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 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 α1,6 mannosyltransferase (Och1p), elongating α1,6 mannosyltransferase, α1,3 mannosyltransferase (Mnn1p), and Kex2p activities (Franzusoff et al., 1991; Graham and Emr, 1991; Nakanishi-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 α1,6 mannose in the *cis*-most compartment and then with α1,2 and α1,3 mannose 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 α1,6 mannose linkages. In wild type cells, CPY was efficiently α1,6 mannose-modified, while in a *sec18-1* (NSF) mutant, which blocks ER to Golgi transport, CPY does not acquire α1,6 mannose (Fig. 1 *B*). In *sec21-3*, as in *sec18-1*, no CPY was recovered after immunoprecipitation with α1,6 mannose antiserum (Fig. 1 *B*), suggesting that ER to Golgi transport of CPYwas blocked in *sec21-3.*

Finally, the subcellular location of p1CPY in *sec21-3* at nonpermissive temperature was examined by differential centrifugation. Spheroplasts were incubated at 37°C, pulse-labeled, chased for 45 min, and then osmotically lysed. After a clearing spin at 300 *g* to remove unbroken cells and cell wall debris, the lysate was centrifuged sequentially to generate 13,000 *g* pellet (P13), 100,000 *g* pellet (P100), and 100,000 *g* supernatant (S100) fractions. In wild type cells, mCPY fractionated in the S100 (Fig. 1 *C*), consistent with its localization to the lumen of the osmotically sensitive vacuole. In contrast, in *sec21-3* at 37°C, p1CPY was found exclusively in the P13 fraction (Fig. 1 *C*). It has previously been shown that both soluble (i.e., PDI) and membrane-associated (i.e., 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 α1,6 and α1,3 mannose, 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-

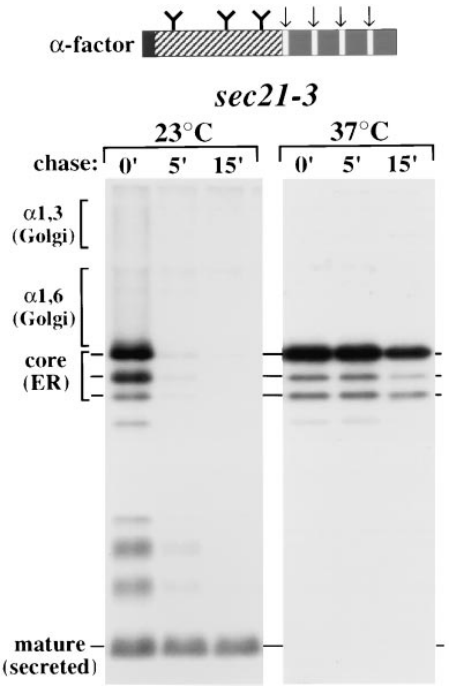

Figure 2. α-factor processing and maturation in *sec21-3.* α-factor is schematized at the top, showing the signal sequence (*black*), pro segment (*hatched lines*), three N-linked glycosylation sites (**Y**), and late Golgi cleavage sites (*white* and arrows) which generate the four mature peptides. *sec21-3* (EGY1213) cells were incubated for 5 min at 23 or 37°C, pulse-labeled for 10 min with Tran^{35}S, 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*)-, α1,6 manose (*Golgi*)-, and α1,3 mannose (*Golgi*)-modified forms are indicated, as is the position of the mature, secreted peptide.

perature 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 COPI 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 intracellular (*I*) and extracellular (*E*) fractions of cells which had been pulse-labeled and chased at 37°C as described. CPY was also immunoprecipitated from the intracellular fraction to ensure that each mutant assayed exhibited an appropriate secretion block (data not shown). This analysis revealed that >95% of HSP150 was secreted from wild type cells and from *sec21-3* cells (Fig. 4, *top panel*) with nearly identical kinetics (data not shown). Even under severely restrictive conditions (i.e., preincubation at 38°C for 60 min before labeling), >95% of HSP150 was secreted from all new *sec21 ts* mutants (data not shown), including *sec21-5* which exhibits growth defects at <30°C (Table II) and likely represents a nearly null allele. >95% of HSP150 was also secreted from two other COPI mutants, *ret2-1* (δ-COP) and *sec33-1* (α-COP) (Fig. 4 *top panel*), each of which exhibits defects in anterograde transport of CPY (data not shown; Cosson et al., 1996; Wuestehube et al., 1996). All other COPI mutants tested (*ret1-1*, α-COP; *sec27-1*, β′-COP; and *ret3-1*, ζ-COP) also secreted >95% of HSP150. Thus the lack of an anterograde transport de-

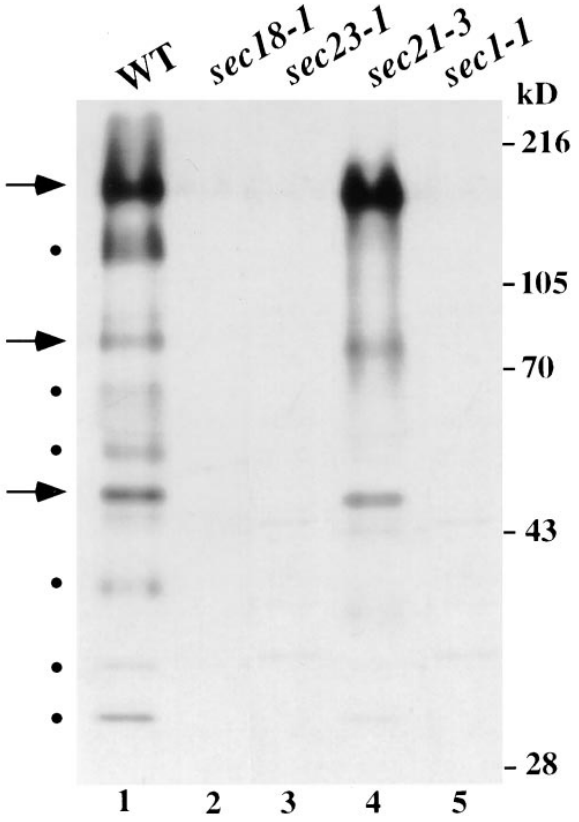

Figure 3. Assay for general secretion competence in *sec* mutants. Wild type (SEY6210), *sec18-1* (EGY1181-5), *sec23-1* (EGY1231-1), *sec21-3* (EGY1213), and *sec1-1* (EGY111-2) cells were incubated at 37°C for 30 min, labeled with Tran^{35}S for 10 min, and then chased for 30 min. Transport was stopped by addition of NaN_3 and NaF to a final concentration of 20 mM each. Cells and media were separated by centrifugation, and proteins secreted into the media during the pulse–chase were visualized by SDS-PAGE and fluorography. Migration of molecular mass standards is shown to the right. At the left, arrows (→) indicate proteins secreted from both wild type and *sec21-3* cells, while the dot (●) indicates proteins secreted from wild type but not *sec21-3* cells.

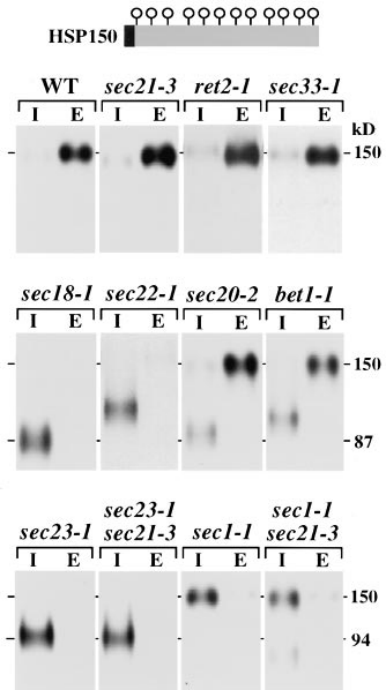

Figure 4. HSP150 secretion. HSP150 is schematized at the top, showing the signal sequence (*black*) and representative O-linked glycosylation sites (ϙ). Wild type (SEY6210), *sec21-3* (EGY1213), *ret2-1* (PC130), *sec33-1* (RSY957), *sec18-1* (EGY1181-5), *sec22-1* (EGY1221-2), *sec20-2* (RSY580), *bet1-1* (RSY945), *sec23-1* (EGY1231-2), *sec23-1*/*sec21-3* (EGY231/213), *sec1-1* (EGY111-2), and *sec1-1*/*sec21-3* (EGY11/213) cells were incubated at 37°C for 30 min, labeled with Tran^{35}S for 10 min, and then chased for 30 min. Transport was stopped by adding NaN_3 and NaF to a final concentration of 20 mM each. Cells (internal fraction, *I*) and media (external fraction, *E*) were separated by centrifugation. HSP150 was recovered by immunoprecipitation and visualized by SDS-PAGE and fluorography. Migration of molecular mass standards is shown to the right. Variation in molecular mass for HSP150 from ER-blocked mutants has been previously observed (Jamsa et al., 1994) and presumably reflects different numbers of O-linked mannoses added in the ER.

fect for HSP150 was not restricted to the new *sec21 ts* mutants but was observed for other COPI mutants as well. 70–80% of HSP150 was also secreted from *sec20-2* and *bet1-1* mutants (Fig. 4, *middle panel*) under conditions where anterograde transport of other proteins (i.e., CPY) was blocked (data not shown; Novick et al., 1980; Newman and Ferro-Novick, 1987); Sec20p has recently been implicated in retrograde Golgi–ER transport (Lewis and Pelham, 1996), while the precise function of Bet1p, a v-SNARE–like protein (Newman et al., 1992), is unknown.

In *sec18-1*, *sec23-1*, and *sec1-1* mutants, HSP150 export was completely blocked (Fig. 4, *middle*/*lower panels*). HSP150 was also retained intracellularly in *sec22-1* and *sec16-1* mutants (Fig. 4, *middle panel* and data not shown); Sec22p functions as an ER to Golgi v-SNARE (Lian and Ferro-Novick, 1993; Sogaard et al., 1994), and Sec16p has been proposed to act as a scaffold for COPII vesicle proteins (Espenshade et al., 1995). Importantly, in *sec23-1*/*sec21-3* and *sec1-1*/*sec21-3* double mutants, HSP150 remained inside the cell and migrated at the same molecular mass seen for the *sec23-1* and *sec1-1* single mutants, respectively (Fig. 4, *lower panel*). Based on mobility (Fig. 4, *top panel*) and reimmunoprecipitation with antisera to α1,3 mannose (data not shown), HSP150 also appeared to be appropriately Golgi-modified in the new *sec21 ts* and other COPI mutants. These observations demonstrate that HSP150 secretion from COPI mutants followed the normal secretory route and did not result from an aberrant bypass of secretory compartments like the Golgi. Therefore, while HSP150 secretion was abolished in COPII, Sec22p (ER–Golgi v-SNARE), Sec18p (NSF), and Sec1p mutants, it was efficiently secreted from all COPI mutants as well as from the putative retrograde transport mutants *sec20-2* and *bet1-1*.

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 utilized 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-

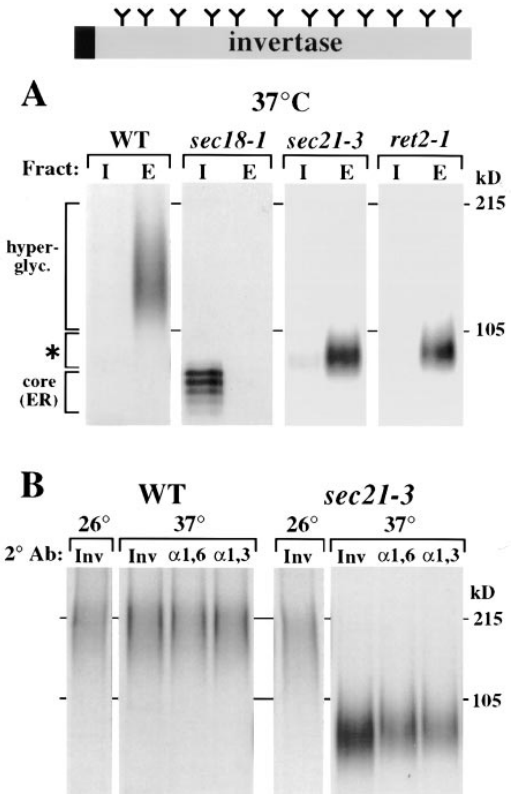

Figure 5. Invertase secretion and Golgi-specific modification. Invertase is schematized at the top, showing the signal sequence (*black*) and N-linked glycosylation sites (**Y**). (*A*) Wild type (SEY6210), *sec18-1* (EGY1181-5), *sec21-3* (EGY1213), and *ret2-1* (PC130) cells harboring pCYI-20 (encoding constitutively expressed, full-length, secreted invertase) were incubated for 30 min at 37°C, labeled with Tran^{35}S for 10 min, and then chased for 10 min. Transport was stopped by adding NaN_3 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 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.

ther 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 coatomer 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 (Shaywitz 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 reimmunoprecipitation 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.

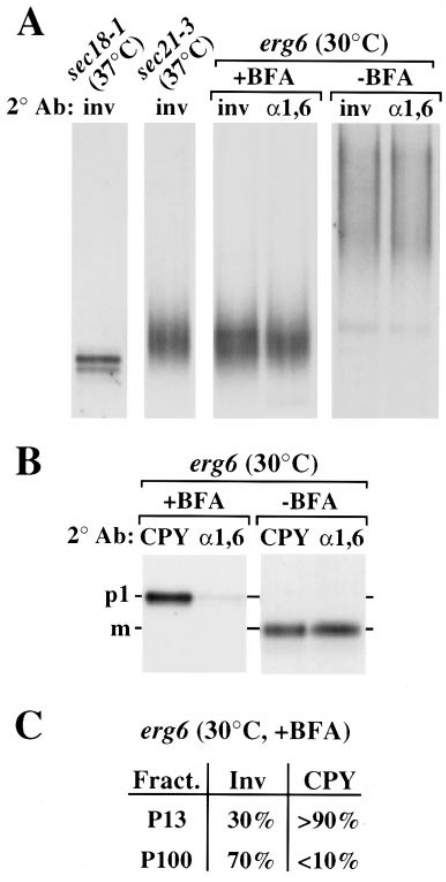

Figure 6. Cargo-specific ER to Golgi transport in BFA-treated cells. (*A*) *sec18-1* (EGY1181-5), *sec21-3* (EGY1213), and *erg6* (TGY-413-6D) cells harboring pCYI-20 were incubated at the indicated temperature in YNB media containing 50 mM Na-Hepes, pH 7.0, for 10 min. *erg6* cultures were then incubated with either 100 μg/ml BFA (+*BFA*) or an equivalent volume of 95% ethanol (−*BFA*) for 2 min. All cultures were pulse-labeled with Tran^{35}S for 10 min and then chased for 30 min. Invertase was recovered by immunoprecipitation, the *erg6* samples were split into two equal aliquots, then all samples were reimmunoprecipitated (*2°Ab*) with antisera to either invertase (*inv*) or α1,6 mannose linkages (*α1,6*). (*B*) CPY was recovered from *erg6* lysates from *A* by immunoprecipitation and then split into equal aliquots for reimmunoprecipitation with antisera to either CPY or α1,6 mannose linkages. Invertase (*A*) and CPY (*B*) were visualized by SDS-PAGE and fluorography. (*C*) *erg6* (TGY413-6D) cells harboring pCYI-20 were incubated in 100 μg/ml BFA, pulse-labeled, and chased as described in *A*. Lysed spheroplasts were fractionated by differential centrifugation, CPY and invertase were recovered by immunoprecipitation and visualized by SDS-PAGE and fluorography, and the percent of each protein in the fractions was quantitated by densitometry.

>90% of CPY was recovered in the 13,000 *g* pellet (Fig. 6 *C*), consistent with ER localization (Gaynor et al., 1994). In contrast, 70% of invertase was found in the 100,000 *g* pellet (Fig. 6 *C*), consistent with localization of invertase to the Golgi (Gaynor et al., 1994; Harris and Waters, 1996; Fig. 1 *C*). Together, these observations indicate that treatment of *erg6* cells with BFA yielded nearly identical cargo-specific ER to Golgi transport defects as those observed for the new *sec21 ts* mutants: CPY was blocked in the ER, while invertase was efficiently transported to the Golgi. 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. 7 *A*). 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 NH_2-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. 7 *B*, •), 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. 7 *B*, *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; Nakanishi-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-

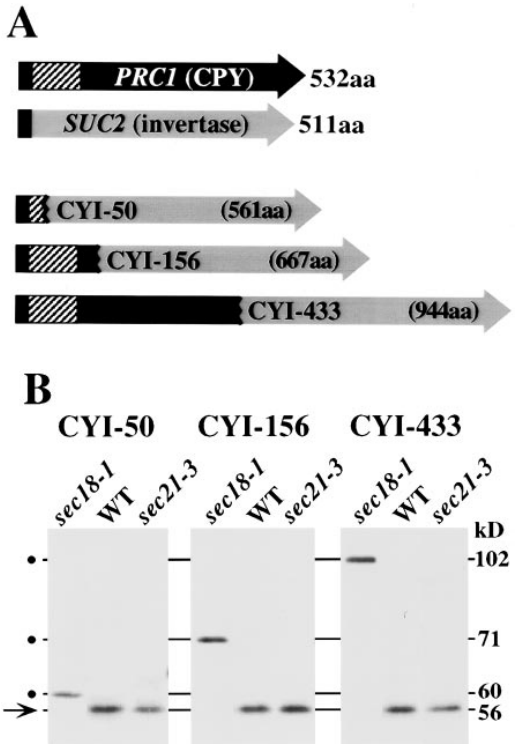

Figure 7. CPY-invertase fusion protein processing in *sec21-3*. (*A*) CPY-invertase fusion proteins are expressed from the *PRC1* (CPY) promoter and contain either 50 (*CYI-50*), 156 (*CYI-156*), or 433 (*CYI-433*) amino acids of CPY fused to the NH_2 terminus of full-length invertase, encoded by the *SUC2* gene. Each fusion contains the CPY signal sequence and the sorting determinant in the pro segment of CPY required for targeting to the vacuole. The CPY/invertase junction is denoted by the black, zig-zag line and is the site where the fusions are proteolytically cleaved in the vacuole. (*B*) *sec18-1* (EGY1181-5), wild type (SEY6210), and *sec21-3* (EGY1213) cells harboring pCYI-50, pCYI-156, or pCYI-433 were incubated at 37°C for 30 min, pulse-labeled with Tran^{35}S label for 10 min, and then chased for 20 min. Fusion proteins were recovered by immunoprecipitation with invertase antiserum, treated with endoglycosidase H, and visualized by SDS-PAGE and fluorography. Approximate molecular masses of the deglycosylated fusion proteins are shown to the right. To the left, the dots (●) indicate positions of the uncleaved fusion proteins, while the arrow (→) indicates the position of the vacuolar protease-processed fusions.

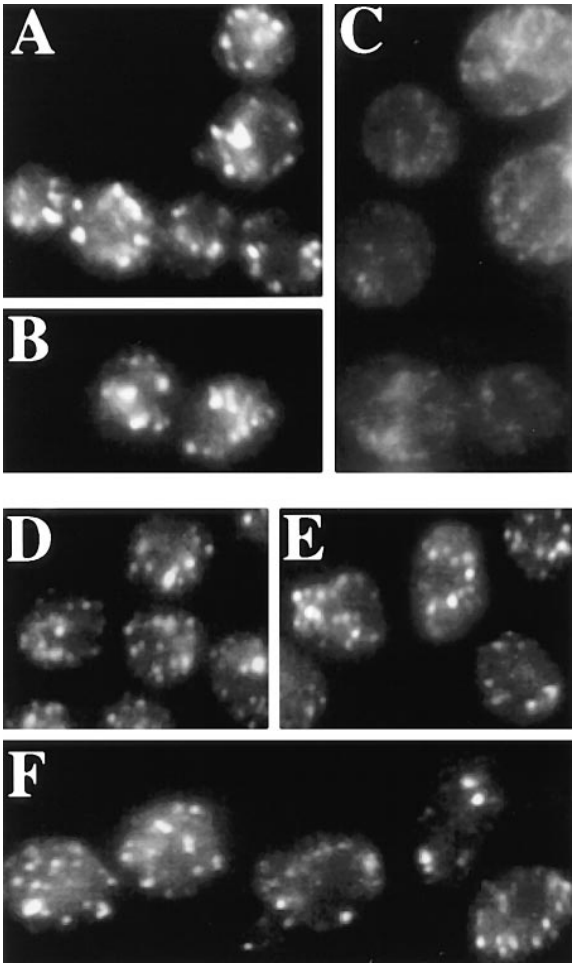

Figure 8. Localization of Och1p and Mnn1p in *sec21-3*. Wild type (SEY6210) and *sec21-3* (EGY1213) cells harboring pOH (Och1-HA) were incubated at 26 or 37°C for 30 min and then fixed for indirect immunofluorescence to visualize Och1p (*A–C*) or Mnn1p (*D–F*). Wild type cells at 37°C are shown in *A* and *D*, *sec21-3* cells at 26°C are shown in *B* and *E*, and *sec21-3* cells at 37°C are shown in *C* and *F*.

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 anlyzed multiple new temperature-conditional *sec21* (γ-COP) mutants (represented by *sec21-3* in this study). Surprisingly, even though these mutants were not identifed 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 COPI-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 COPI 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-

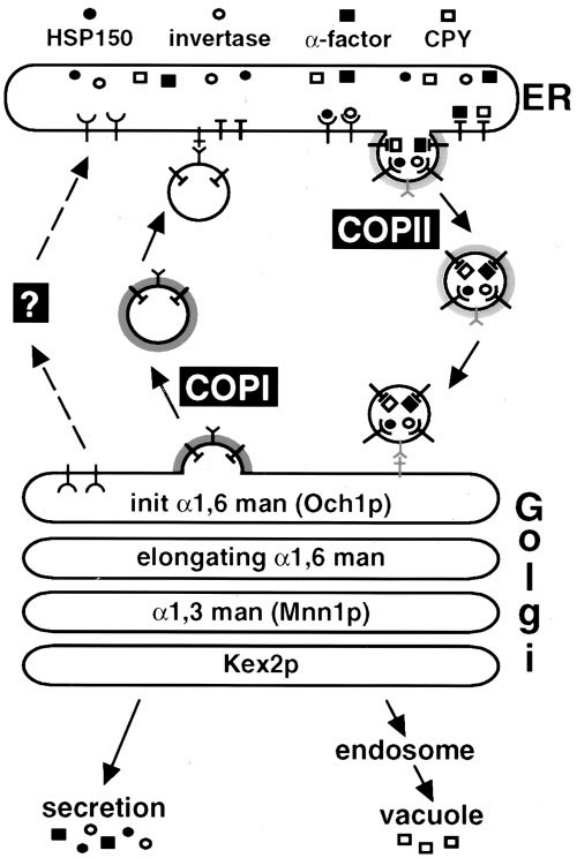

Figure 9. Model for an indirect requirement for COPI in anterograde ER to Golgi transport. In the ER, cargo molecules first associate with specific transport factors (i.e., "sorting receptors") which mediate their concentration and packaging into COPII-coated vesicles. Cargo proteins like CPY (□) and 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 COPI vesicles for retrieval back to the ER. COPI 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).

tinue 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 in vitro ER budding assays (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 *sec21-3* 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 KKXX-containing protein exhibiting lectin homology (Emp47p) has been identified and shown to cycle between the Golgi and ER in a COPI-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 NH_2-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 ε-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 were Golgi modified in the *sec21-3* mutant, and in a *sec21-3*/*sec23-1* double mutant, HSP150 transport was blocked (Figs. 4 and 5). 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 anterograde 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 syncitium 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 sorting determinants in cargo proteins directing their packaging into COPII vesicles will provide additional mechanistic insight into this essential sorting process.

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