

Active and specific recruitment of a soluble cargo protein for endoplasmic reticulum exit in the absence of functional COPII component Sec24p

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Summary

Exit of proteins from the yeast endoplasmic reticulum (ER) is thought to occur in vesicles coated by four proteins, Sec13p, Sec31p, Sec23p and Sec24p, which assemble at ER exit sites to form the COPII coat. Sec13p may serve a structural function, whereas Sec24p has been suggested to operate in selection of cargo proteins into COPII vesicles. We showed recently that the soluble glycoprotein Hsp150 exited the ER in the absence of Sec13p function. Here we show that its ER exit did not require functional Sec24p. Hsp150 was secreted to the medium in a *sec24-1* mutant at restrictive temperature 37°C, while cell wall invertase and vacuolar carboxypeptidase Y remained in the ER. The determinant guiding Hsp150 to this transport route was mapped to the C-terminal domain of 114 amino acids by

deletion analysis, and by an HRP fusion protein-based EM technology adapted here for yeast. This domain actively mediated ER exit of Sec24p-dependent invertase in the absence of Sec24p function. However, the domain was entirely dispensable for ER exit when Sec24p was functional. The Sec24p homolog Sfb2p was shown not to compensate for nonfunctional Sec24p in ER exit of Hsp150. Our data show that a soluble cargo protein, Hsp150, is selected actively and specifically to budding sites lacking normal Sec24p by a signature residing in its C-terminal domain.

Key words: Hsp150, COPI, Yeast, Secretion, Membrane traffic

Introduction

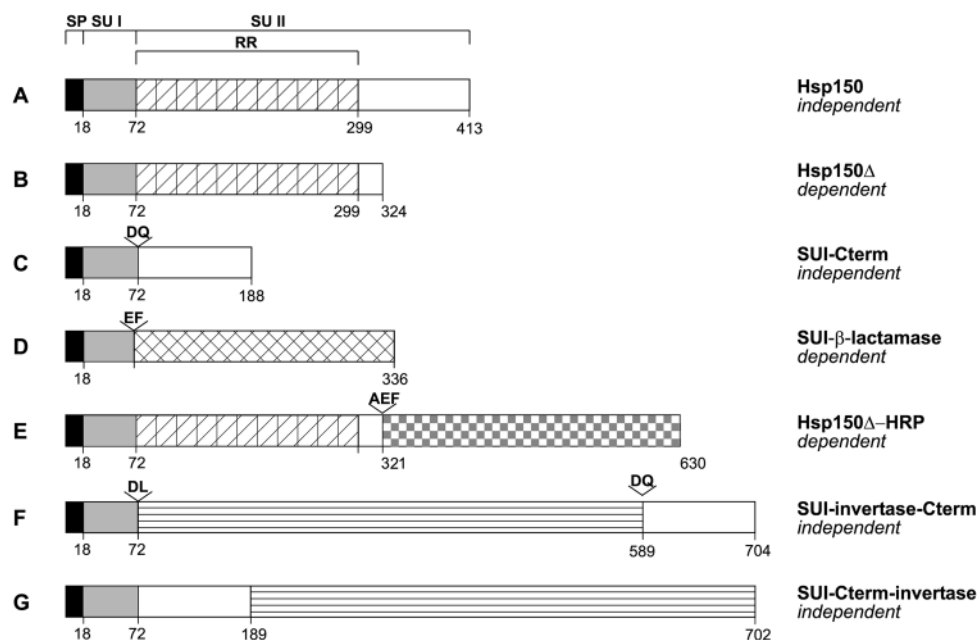
Exit of soluble and membrane proteins from the yeast endoplasmic reticulum (ER) occurs in vesicles covered with the COPII coat, which consists of the proteins Sec23p, Sec24p, Sec13p and Sec31p, and the small GTPase Sar1p (Kaiser and Schekman, 1990; Pryer et al., 1993; Salama et al., 1993; Barlowe et al., 1994; Barlowe, 2000). The assembly of the COPII coat starts by recruitment of cytosolic GDP-bound Sar1p by the ER membrane protein Sec12p to the budding site (Barlowe and Schekman, 1993). Exchange by Sec12p of GDP to GTP on Sar1p recruits the Sec23p/24p complex to the ER membrane, whereafter Sec13p/Sec31p binds to the complex, culminating in vesicle budding (Yoshihisa et al., 1993; Barlowe et al., 1994; Matsuoka et al., 1998). Three-dimensional reconstruction studies have suggested that Sec13p mediates essential interactions between Sec13p/31p and Sec23p/24p complexes, and thus appears to fulfill a structural function in the COPII coat (Lederkremer et al., 2001). ER exit of cell wall invertase and vacuolar CPY is strictly dependent on the function of all COPII components. However, both proteins were able to slowly exit the ER in the absence of Sec13p in cells with mutated *BST1*, *BST2/EMP24* or *BST3* genes. These genes were suggested to encode proteins that prevent cargo proteins from entering buds covered with incomplete COPII coats (Elrod-Erickson and Kaiser, 1996; Springer et al., 2000). We showed recently that the glycoprotein Hsp150 exited the

ER and was secreted to the medium in the absence of normal Sec13p function in cells that harboured wild-type *BST1*, *BST2/EMP24* and *BST3* genes (Fatal et al., 2002). This suggested that a subset of soluble cargo could be selected in living cells to budding sites covered with coats varying in their compositions of functional COPII proteins.

Sec24p has two homologs, Sfb2p (Iss1p/Sec24B) and Sfb3p (Lst1p/Sec24C), which have 56% and 23% identity with Sec24p, respectively (Kurihara et al., 2000; Roberg et al., 1999). All homologs have been shown to serve as COPII components and operate in ER exit. Sec24p is essential, whereas deletion of either of the homologs individually or in combination does not compromise exocytic membrane traffic or viability (Pagano et al., 1999). However, overexpression of Sfb2p rescued viability of cells lacking the *SEC24* gene, and allowed intracellular transport of several cargo proteins, showing that Sfb2p can compensate for Sec24p in COPII coats (Kurihara et al., 2000). Overexpression of Sfb3p could not complement the lack of *SEC24*, but Sfb3p could replace Sec24p in in vitro ER budding assays (Miller et al., 2002). Deletion of *SFB3* from *sec24* mutants is lethal, but that of *SFB2* is not (Roberg et al., 1999; Peng et al., 2000).

Here we show that Sec24p was not required for ER exit of Hsp150 in living yeast cells. Hsp150 was secreted to the medium in the absence of normal Sec24p function, albeit slowly. The signature on Hsp150 for selection to the Sec24p-

Fig. 1. Hsp150 variants and dependence of their ER exit on functional Sec24p. (A) The product of the *HSP150* gene has an N-terminal 18 amino acid signal peptide (SP, black). The ER form consists of a 54 amino acid subunit I (SU I, gray) and subunit II (SU II), which is composed of a repetitive region (RR) where homologous peptides of mostly 19 amino acids are repeated 11 times (diagonally striped boxes), followed by a unique C-terminal fragment (white area). (B) The last 89 amino acids of Hsp150 were deleted. (C) The C-terminal 114 amino acids of Hsp150 were joined to subunit I. (D) *E. coli* β -lactamase (criss-crossed) was joined to subunit I. (E) HRP (checkered) was fused to the first 321 amino acids of Hsp150. (F) Invertase (horizontally striped) was fused to subunit I, and Cterm was fused to the C-terminus of invertase. (G) Invertase was fused to the C-terminus of SUI-Cterm. The last amino acids of the various domains are numbered. Letters indicate extra amino acids resulting from cloning strategy. All other proteins had a Kex2p recognition site at the C-terminal end of subunit I, except SUI- β -lactamase (D) and Hsp150 Δ -HRP (E). The dependency of ER exit on functional Sec24p is indicated.



independent ER exit route resided in the C-terminal domain of 114 amino acids. The determinant was an active mediator of ER exit occurring independently of Sec24p function. The C-terminal domain recruited Hsp150 specifically to ER exit sites lacking functional Sec24p, because it was completely dispensable for secretion in cells with wild-type Sec24p. Nonfunctional Sec24p was not compensated by the homolog Sfb2p in ER exit of Hsp150.

Materials and Methods

Cloning and strain construction

A *sec24-1* strain was rendered auxotrophic for tryptophan by disrupting the *TRP1* gene as described previously (Fatal et al., 2002). The resulting strain (H1237) was transformed with linearized plasmid pKTH5056 (SUI-Cterm-invertase, see Fig. 1) (Fatal et al., 2002) to create strain H1544. An integrative plasmid for expression of fusion protein SUI-invertase-Cterm (plasmid pKTH5057, see Fig. 1) was constructed as follows: the invertase gene *SUC2* was amplified without its signal sequence and stop codon, using oligos 5' AAT TAA GGA GAT CTG TCA ATG ACA AAC GAA ACT AGC GAT AG 3' and 5' ATA TTA AGA TCT TTT ACT TCC CTT ACT TGG AAC T 3'. The PCR fragment was digested with *Bgl*III and ligated into *Bcl*II-digested pKTH5006 (Fatal et al., 2002), to create pKTH5057, which was transformed into yeast strains H4, H1233, H1236 and H1237 to create strains H1575, H1577, H1578 and H1579, respectively. The *HSP150Δ*-HRP cDNA was constructed by amplifying the synthetic horseradish peroxidase isoenzyme C (HRP-C) gene (Pubmed access number J05552) from HRP-pBS (Stinchcombe et al., 1995) using primers E385 (5' CCG AAT TCC AGT TAA CCC CTA CAT TCT ACG AC 3') and E386 (5' CCA AGC TTT TAT TAG TTG CTG TTG ACC ACT CTG C 3'), adding 5' terminal *Eco*RI and 3' terminal *Hind*III cloning sites, respectively, to the PCR product. The resulting HRP cDNA was cleaved with *Eco*RI, blunted with *T4* and finally cleaved with *Hind*III, giving rise to a DNA-strand with a blunt end and a sticky end, and inserted between the *T4*-blunted *Kpn*I site and

the *Hind*III site of pKTH4536. The resulting plasmid pKTH4979 was then cleaved with *Bam*HI and the fragment containing the *HSP150Δ*-HRP coding sequence under the *HSP150* promoter, and the *ADH1*-terminator sequences, was introduced into the *Bam*HI site of pRS425, a 2 μ yeast vector, resulting in plasmid pKTH5011. The yeast strains H1455, H1458 and H1459 were constructed by transforming strains H3, H1101 and H481, respectively, with pKTH5011. The *SFB2* gene was disrupted by *loxP*-*KanMX*-*loxP* from the *sec24-1* mutant H1101 using primers F0669 (5' TGA ACC TTC TTC CAT TAA TGA TCG ACA GCT GCA GTG AAT ACC AGC TGA AGC TTC GTA CGC 3') and F0671 (5' GAT CGG TTA ATA AAG ATA AAG ATT AAA GAA AGA CTG ATT GAT AGG CCA CTA GTG CAT CTG 3') yielding strain H1555. The disruption was confirmed by PCR.

Electron microscopy

Cells were fixed by adding an equal volume of 6% formaldehyde plus 1% of glutaraldehyde in 0.2 M sodium citrate, pH 4.8, to the growth medium for a 2 hour incubation at 37°C, followed by an overnight incubation at 4°C in 3% formaldehyde in 0.1 M sodium citrate. The cell wall was weakened by digestion with zymolyase (25 U/ml; Seikagaku, Japan) in TPC buffer (10 mM Tris-HCl, 10 mM CaCl₂ and 1.2 M sorbitol) for 15 minutes at room temperature. A DAB treatment was adapted from Brown and Farquhar (Brown and Farquhar, 1989) as follows. The cells were washed three times for 5 minutes in 50 mM Tris-HCl buffer, pH 7.6, and incubated then for 10 minutes in the dark in 0.15% DAB/Tris-HCl buffer, and then for 30 minutes in 0.15% DAB/Tris-HCl containing 0.063% hydrogen peroxide. After three washes with Tris-HCl buffer and another three washes with distilled water, the cells were treated with 1% OsO₄/1.5% K₄Fe(CN)₆ for 1 hour at 4°C. The cells were washed with water, and left overnight in water. The cell pellets were then dehydrated in an increasing gradient of ethanol, 3×20 minutes in 70%, 3×20 minutes in 94% and 3×20 minutes in 99.5% ethanol, followed by an overnight incubation in the last. The cells were finally embedded in Spurr resin (Agar Scientific, UK) as follows: 1 hour in 50% and then 1 hour in 67% Spurr in ethanol, and finally 14 hours in 100% Spurr. Polymerization was at

Table 1. Yeast strains

Strain	Genotype	Reference/Source
H1	SEy2101a MATa <i>ade2-101 ura3-52 leu2-3,112 suc2-Δ9 gal2</i>	R. Schekman*
H3	SF821-8A MATa <i>sec7-1 his4-580 ura3-52 leu2-3,112 trp1-289</i>	R. Schekman
H4	mBY12-6D MATα <i>sec18-1 trp1-289 leu2-3,112 ura3-52 his</i>	R. Schekman
H23	MATα <i>ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 hsp150::URA3</i>	Russo et al., 1992
H430	MATα <i>ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 hsp150::URA3 LEU2::HSP150Δ</i>	Fatal et al., 2002
H440	MATα <i>sec18-1 trp1-289 leu2-3,112 ura3-52 his⁻ LEU2::HSP150Δ</i>	Fatal et al., 2002
H481	RSY282 MATa <i>sec23-1 ura3-52 leu2-3,112</i>	R. Schekman
H1101	E382-6 MATα <i>sec24-1 ura3-52 leu2-3,112</i>	C. Kaiser†
H1141	JPY206 <i>sec24B::HIS ura3 trp1 (SEC24B=SFB2)</i>	J. P. Paccaud‡
H1142	JPY207 <i>sec24C::HIS (SEC24C=SFB3)</i>	J. P. Paccaud
H1143	JPY205 <i>sec24B::HIS sec24::HIS lys2</i>	J. P. Paccaud
H1233	MATa <i>ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 hsp150Δ::loxP-KanMX-loxP</i>	Fatal et al., 2002
H1236	MATa <i>sec13-1 trp1::loxP-KanMX-loxP</i>	Fatal et al., 2002
H1237	MATα <i>sec24-1 ura3-52 leu2-3,112 trp1::loxP-KanMX-loxP</i>	This study
H1429	MATa <i>sec13-1 trp1::loxP-KanMX-loxP TRP1::SUI-Cterm</i>	Fatal et al., 2002
H1455	MATa <i>sec7-1 his4-580 ura3-52 leu2-3,112 trp1-289 LEU2::HSP150Δ-HRP</i>	This study
H1458	MATα <i>sec24-1 ura3-52 leu2-3,112 LEU2::HSP150Δ-HRP</i>	This study
H1459	MATa <i>sec23-1 ura3-52 leu2-3,112 LEU2::HSP150Δ-HRP</i>	This study
H1499	MATα <i>sec24-1 ura3-52 leu2-3,112 hsp150::loxP-KanMX-loxP LEU2::HSP150Δ</i>	This study
H1500	MATα <i>sec24-1 ura3-52 leu2-3,112 trp1::loxP-KanMX-loxP TRP1::SUI-Cterm</i>	This study
H1508	MATa <i>ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 hsp150Δ::loxP-KanMX-loxP TRP1::SUI-Cterm</i>	Fatal et al., 2002
H1540	MATa <i>ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 hsp150Δ::loxP-KanMX-loxP TRP1::SUI-Cterm-invertase</i>	Fatal et al., 2002
H1542	MATα <i>sec18-1 trp1-289 leu2-3,112 ura3-52 his⁻ TRP1::SUI-Cterm-invertase</i>	Fatal et al., 2002
H1544	MATα <i>sec24-1 ura3-52 leu2-3,112 trp1::loxP-KanMX-loxP TRP1::SUI-Cterm-invertase</i>	This study
H1555	MATα <i>sec24-1 ura3-52 leu2-3,112 sfb2Δ::loxP-KanMX-loxP</i>	This study
H1575	MATα <i>sec18-1 trp1-289 leu2-3,112 ura3-52 his⁻ TRP1::SUI-invertase-Cterm</i>	This study
H1577	MATa <i>ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 hsp150Δ::loxP-KanMX-loxP TRP1::SUI-invertase-Cterm</i>	This study
H1578	MATa <i>sec13-1 trp1::loxP-KanMX-loxP TRP1::SUI-invertase-Cterm</i>	This study
H1579	MATα <i>sec24-1 ura3-52 leu2-3,112 trp1::loxP-KanMX-loxP TRP1::SUI-invertase-Cterm</i>	This study

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60°C. Sections were cut and picked on single-slot copper grids, post-stained with uranyl acetate and lead citrate, using the Leica EMSTAIN device. Sections were examined with an FEI Tecnai12 transmission electron microscope at 80 kV. Formaldehyde, glutaraldehyde and osmium tetroxide (OsO₄) were obtained from Electronic Microscopic Sciences (Washington, PA); diaminobenzidine (DAB) was from TAAB Laboratories Equipment, UK. All reagents were EM grade.

Other methods

Metabolic labeling with ³⁵S-methionine/cysteine (1000 Ci/mmol; Amersham International, Buckinghamshire, UK) and immunoprecipitation with antisera against Hsp150 (1:400), β-lactamase (1:100), CPY (1:100) and HRP (1:200, RDI), and SDS-PAGE (8% gels) were as described previously (Paunola et al., 1998). Internal and cell wall activities of invertase were determined as described in Simonen et al. (Simonen et al., 1994). Activity staining of invertase in nondenaturing gels was according to Novick et al. (Novick et al., 1980). Endoglycosidase H (Boehringer, Mannheim, Germany) digestion (20 mU/50 μl of 80 mM sodium acetate buffer, pH 5.2) was performed for 4 hours at 37°C on immunoprecipitated substrate protein adhering to protein A-Sepharose beads. Trichloroacetic acid (TCA) precipitation was performed with 10% acid, followed by washing of the precipitate with ice-cold acetone.

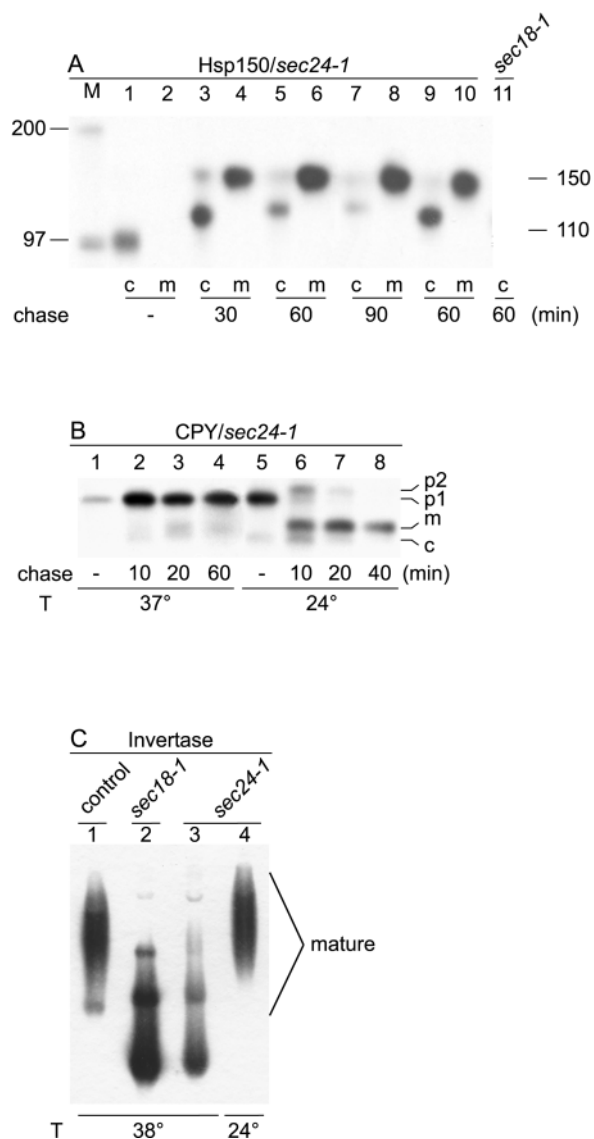
Results

Secretion of Hsp150 in the absence of normal Sec24p function

A temperature-sensitive *sec24-1* mutant (for strain numbers and genotypes, see figure legends and Table 1) was preincubated for 15 minutes at restrictive temperature 37°C, labeled with ³⁵S-methionine/cysteine for 5 minutes and chased

in the presence of cycloheximide (CHX). Immunoprecipitation with Hsp150 antiserum and SDS-PAGE analysis revealed after the pulse an Hsp150 variant of about 97 kDa (Fig. 2A, lane 1). It comigrated with the ER form of Hsp150, accumulated in the *sec18-1* mutant, where at 37°C ER-derived vesicles cannot fuse with the Golgi membrane (lane 11). No Hsp150 was found in the respective medium sample of the *sec24-1* mutant (lane 2). After a 30-minute chase, roughly half of Hsp150 was in mature form (150 kDa) in the medium (lane 4). The respective cell-associated form had grown in apparent molecular weight, probably because of increased O-glycosylation. Subunits I and II are extensively O-glycosylated, whereas N-glycosylation sites are absent from Hsp150 (Russo et al., 1992; Jämsä et al., 1995) (see Fig. 1A). The lysate also contained little of the mature form (lane 3). After 1 hour of chase most of Hsp150 was in the medium (lane 6), some of the immature and mature forms remaining cell-associated (lane 5). When the preincubation of *sec24-1* cells at restrictive temperature before labeling was extended to 30 minutes, again most of Hsp150 was found in the medium after 1 hour of chase (lanes 9 and 10). In normal cells Hsp150 is secreted at 37°C with a half time of about 5 minutes (Jämsä et al., 1995).

We used pro-CPY and invertase as internal controls for nonfunctional Sec24p in the above experiment; both are shown to require all structural components of the COPII coat for ER exit (Novick et al., 1980; Stevens et al., 1982). In the same *sec24-1* cells where Hsp150 continued to be slowly secreted at restrictive temperature, newly synthesized vacuolar CPY remained in the ER-specific p1 form (Fig. 2B, lanes 1-4), indicating that it could not leave the ER (Stevens et al., 1982), whereas at permissive temperature 24°C pro-CPY was rapidly



converted via the Golgi form p2, to the mature vacuolar form (Fig. 2B, lanes 5-8). When the *sec24-1* mutant was preincubated for 15 minutes at 38°C and then derepressed for invertase synthesis in low glucose medium at the same temperature, electrophoresis under nondenaturing conditions and activity staining revealed molecules (Fig. 2C, lane 3) that comigrated with the ER form accumulating in the ER in the *sec18-1* mutant (lane 2). When invertase was synthesized at 24°C in the *sec24-1* mutant (lane 4), it comigrated with mature invertase with extended N-glycans expressed in control cells (lane 1). In this experiment 38°C was used as restrictive temperature, but similar results were obtained at 37°C. Our data show that Hsp150 was secreted to the culture medium under conditions in which the COPII component Sec24p was nonfunctional to an extent that it prevented pro-CPY and invertase from leaving the ER.

Mapping of the domain responsible for ER exit of Hsp150 in the absence of normal Sec24p function

We showed previously that the C-terminal domain of Hsp150

Fig. 2. Intracellular transport of proteins in *sec24-1* mutant. (A) Hsp150 secretion. *sec24-1* (H1101; lanes 1-10) and *sec18-1* (H4, lane 11) cells were pulse-labeled for 5 minutes with ³⁵S-methionine/cysteine and chased in the presence of CHX at 37°C as indicated. Preincubation before labeling at 37°C was for 15 minutes, except in the case of lanes 9 and 10, where it was 30 minutes. Immunoprecipitation of cell lysate (c) and medium (m) samples before SDS-PAGE analysis was with Hsp150 antiserum. Migration of ER (97 kDa) and mature (150 kDa) forms of Hsp150 are indicated on the right. (B) Transport to the vacuole of pro-CPY. *sec24-1* cells (H1101) were preincubated for 15 minutes and ³⁵S-labeled for 5 minutes and chased with CHX as indicated, at 37°C (lanes 1-4) or at 24°C (lanes 5-8). Cell lysates were immunoprecipitated with CPY antiserum. Migration of cytosolic (c, 59 kDa), ER (p1, 67 kDa), Golgi (p2, 69 kDa) and vacuolar (m, 62 kDa) forms of CPY are indicated. (C) Invertase secretion. Control (H23, lane 1), *sec18-1* (H4, lane 2) and *sec24-1* (H1101, lanes 3 and 4) cells were preincubated at 38°C (lanes 1-3) or 24°C (lane 4) for 15 minutes, shifted to low glucose (0.1%) medium to derepress invertase synthesis and incubated for 1 hour at the same temperatures. After nondenaturing gel electrophoresis the gel was stained for invertase activity. Migration of mature invertase is indicated. T, temperature.

(see Fig. 1A) was slowly secreted to the medium in the absence of normal Sec13p function (Fatal et al., 2002). This data provided us with a starting point to map the fragment guiding Hsp150 to the ER exit pathway that does not require normal Sec24p function. Subunit I of Hsp150, flanked by an authentic C-terminal recognition site for Kex2p located in the late Golgi (Wilcox et al., 1992), was joined directly to the N-terminus of the C-terminal domain of Hsp150, creating SUI-Cterm (see Fig. 1C). Cells expressing SUI-Cterm were preincubated for 15 minutes, pulse-labeled for 5 minutes and chased up to 1 hour at 37°C, followed by immunoprecipitation of cell lysates and respective media samples, and SDS-PAGE analysis. In the case of control cells, very little – if any – SUI-Cterm could be detected in the lysates (Fig. 3C, lanes 1-4). After the 5-minute pulse, very little protein was detected in the medium (Fig. 3C, lane 5), but after 15-60 minutes of chase, proteins of 16.5 and 14 kDa could be immunoprecipitated from the medium (lanes 6-8). Apparently, SUI-Cterm was difficult to detect from the lysate after the pulse because of heterogeneity in glycosylation, which resulted in heterogeneity in electrophoretic migration. The 14 and 16.5 kDa bands represent the C-terminal fragment, released from subunit I by Kex2p. The two forms apparently arose from different degrees of processing of the C-terminus by other peptidases after the Kex2p cleavage. In the control cells, secretion of the C-terminal fragment to the medium was completed within 15 minutes of chase. In the *sec24-1* mutant, uncleaved SUI-Cterm could be detected after the pulse in the lysate (Fig. 3A, lane 1). Thereafter, the fusion protein disappeared from the cells (lanes 2-4), and the released C-terminal fragment appeared in the medium (lanes 5-8). An apparently maximal amount of Cterm was in the medium in 30 minutes. For comparison, we show a similar experiment performed on *sec13-1* cells. In this case, more of the intracellular uncleaved form was detected (Fig. 3B, lanes 1-4), and some uncleaved form appeared also in the medium (lanes 5-8). The secretion of released Cterm in *sec24-1* cells (H1500) was also compared at permissive and restrictive temperature. Similar relative amounts of Cterm could be detected in the

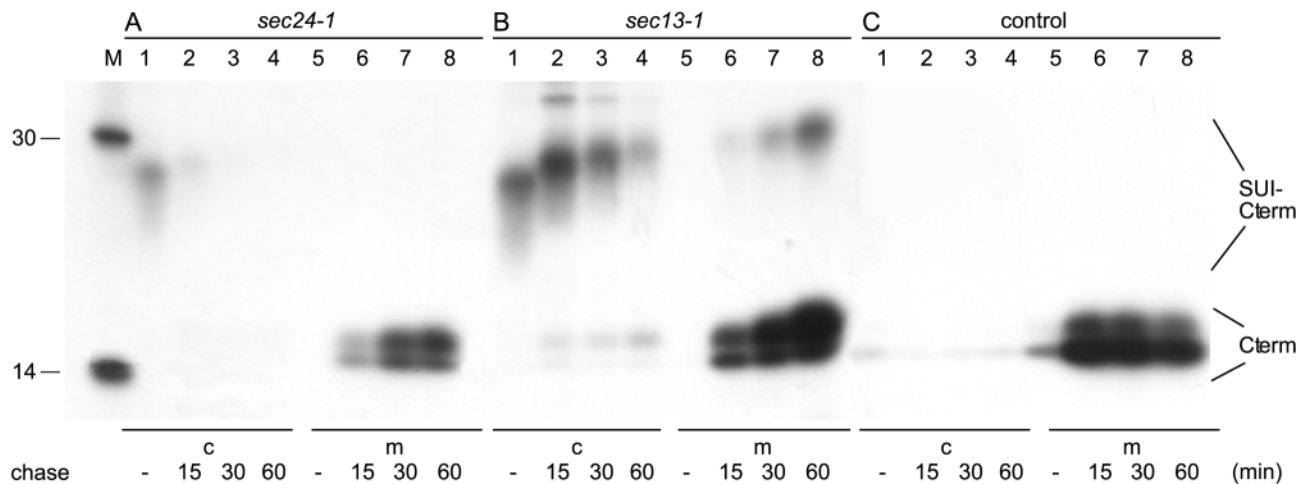


Fig. 3. Secretion of the C-terminal domain of Hsp150 in a *sec24-1* mutant. *sec24-1* (H1500, A), *sec13-1* (H1429, B) and control cells (H1508, C) were preincubated for 15 minutes, ^{35}S -labeled for 5 minutes and chased as indicated, at 37°C. Cell lysate samples (c) and the respective medium samples (m) were subjected to immunoprecipitation with Hsp150 antiserum and SDS-PAGE (15% gels) analysis. SUI-Cterm and released Cterm are indicated.

medium after a 30-minute chase both at 37°C and 24°C (data not shown).

A construction in which the C-terminal fragment was replaced by β -lactamase (SUI- β -lactamase, Fig. 1D) served as a control to confirm that it was the C-terminal fragment rather than subunit I that was responsible for ER exit in the absence of normal Sec24p function. A similar pulse-chase experiment as above showed that SUI- β -lactamase remained intracellular at 37°C in a *sec24-1* background, and comigrated in SDS-PAGE with the form retained in the ER in a *sec18-1* mutant (not shown). We conclude that the C-terminal fragment harbored a signature recruiting Hsp150 to the Sec24p-independent ER exit pathway.

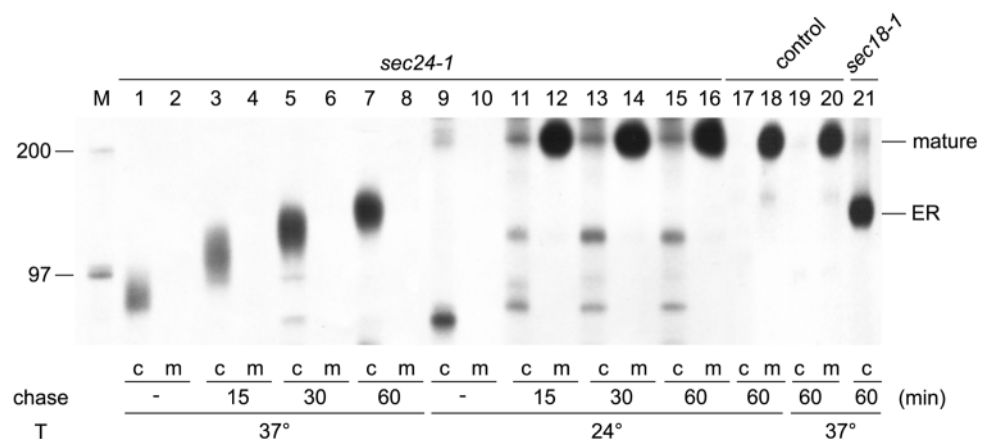
If the C-terminal domain in full-length Hsp150 is alone responsible for ER exit in the absence of normal Sec24p function, the remainder of the protein, Hsp150 Δ (see Fig. 1B), should remain in the ER in the absence of Sec24p function. We showed that this indeed is the case. Pulse-chase experiments on *sec24-1* cells lacking the endogenous *HSP150* gene detected an intracellular form of about 90 kDa after pulse at 37°C (Fig. 4, lane 1). Its electrophoretic migration slowed down to correspond to 170 kDa during the chase (lanes 3, 5 and 7), whereas no protein could be detected in the media samples

(lanes 2, 4, 6 and 8). The intracellular form detected after 60 minutes of chase (lane 7) comigrated with the ER form detected at 37°C in a *sec18-1* mutant (lane 21). At 24°C, the mature form of Hsp150 Δ migrating like a protein of more than 200 kDa appeared in the medium by 15 minutes of chase (lanes 9-16). Mature Hsp150 Δ , as well as its biosynthetic intermediates, migrate anomalously slowly in SDS-PAGE, probably because of extensive O-glycosylation, as described previously (Jämsä et al., 1995). In control cells, similar amounts of the mature form were in the media samples after 60 minutes of chase both at 24°C (lane 18) and 37°C (lane 20), whereas no intracellular forms could be detected at either temperature (lanes 17 and 19). This data shows that the C-terminal domain is dispensable for ER exit in the presence of normal Sec24p. Thus, the C-terminal domain appears to recruit authentic Hsp150 specifically to ER exit sites that lack functional Sec24p.

Electron microscopic localization of Hsp150 Δ -HRP

Next we sought for morphological evidence that an Hsp150 variant lacking the C-terminal domain is retained in the ER in the absence of Sec24p function. To this end, the C-terminal

Fig. 4. Fate of Hsp150 Δ in *sec24-1* mutant. *sec24-1* (H1499), control (H430) and *sec18-1* (H440) cells were preincubated for 15 minutes, pulse-labeled for 5 minutes and chased as indicated. The ER and mature forms of Hsp150 Δ are indicated on the right and molecular weight markers (kDa) on the left.



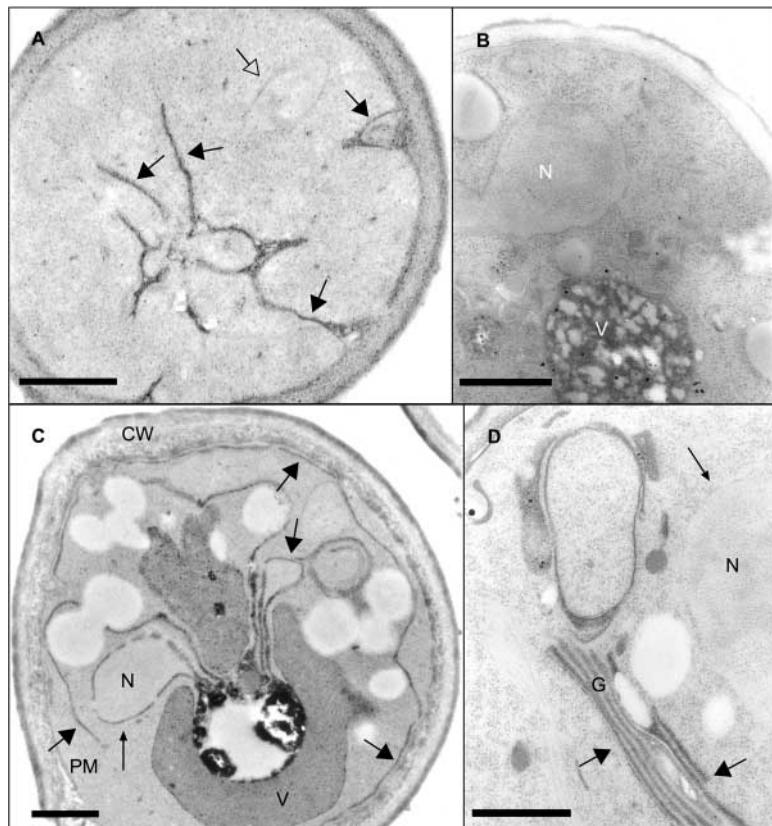


Fig. 5. Electron microscopy of Hsp150 Δ -HRP. *sec24-1* (A; H1458), *sec23-1* (C; H1459) and *sec7-1* (D; H1455) mutants expressing Hsp150 Δ -HRP, and the parental *sec7-1* strain (B; H3) were incubated for 1 hour at 37°C, fixed and processed for HRP EM. Large black arrows indicate putative stained ER (A and C), or Golgi cisternae (D). The large open arrow points to unidentified unstained membrane (A). Small arrows indicate stained (C) and unstained (D) nuclear membrane. CW, cell wall; G, Golgi; N, nucleus; PM, plasma membrane; V, vacuole. Bars, 0.5 μ m.

portion was exchanged to horseradish peroxidase (HRP; see Fig. 1E). The chimera was expressed in *sec* mutants under the *HSP150* promoter, which drives a basal level of expression at 24°C, and is upregulated at 37°C (Russo et al., 1993). The fusion protein migrating in SDS-PAGE like a 170 kDa protein was recognized by antisera against Hsp150 and HRP in western blotting and immunoprecipitation experiments (not shown). For EM, the cells were incubated for 1 hour at 37°C to impose the secretion block, fixed and processed as described in Materials and Methods. Fig. 5A shows in *sec24-1* cells membranous structures, reminiscent of ER membrane, containing HRP reaction product (large black arrows), as well as unstained membrane (large open arrow). A *sec23-1* mutant served as a control for ER. Stained ER membrane close to the plasma membrane and in the cytosol (Fig. 5C, large black arrows), as well as stained nuclear envelope (small arrows), were observed. The plasma membrane appeared not to be stained, at least not uniformly. The cell wall contained some reaction product, which was apparently due to the basal expression of secretory Hsp150 Δ -HRP at 24°C before shift to 37°C. Endogenous peroxidases were responsible for reaction product in the vacuoles. For reference we show the Golgi-specific *sec7-1* mutant (Fig. 5D), where multiple stained

cisternae were detected (large black large), whereas the nuclear envelope was unstained (small arrow). In the absence of the *HSP150* Δ -HRP gene, no deposit was observed, except for the vacuole harboring endogenous peroxidases (Fig. 5B).

The C-terminal domain of Hsp150 harbors an active signal recruiting invertase for Sec24p-independent ER exit

Next we studied whether the C-terminal fragment of Hsp150 was able to recruit invertase to the Sec24p-independent ER exit pathway. Invertase was used as cargo, as in authentic form it requires functional Sec24p for ER exit. Invertase was fused to the C-terminus of subunit I, and the C-terminal Hsp150 domain was joined to the C-terminus of invertase, creating SUI-invertase-Cterm (see Fig. 1F). A Kex2p recognition site joined subunit I to invertase. Cleavage at the Kex2p site would indicate that the fusion protein had exited the ER and reached at least the late Golgi, where the Kex2 protease resides. The chimera was placed under the control of the *HSP150* promoter. Thus, in full glucose (2%) medium, at either temperature, invertase activity would be due only to the recombinant gene, and not to endogenous invertase expressed only in low (0.1%) glucose medium.

The invertase portion acquired a catalytically active conformation and the fusion protein acquired a transport-competent form, because high levels of invertase activity were detected in control (H1577), *sec24-1* (H1579), *sec13-1* (H1578) and *sec18-1* (H1575) cells after growth at 24°C in full glucose medium. At least 75% of the total activity was in the cell wall and the rest was in spheroplast lysates in each strain, with no activity in the medium (data not shown). Immunoprecipitation of ³⁵S-labeled control cell lysates, followed by endoglycosidase H digestion

to remove the heterogenous N-glycans of the invertase portion, revealed in SDS-PAGE analysis a protein migrating at 73 kDa (Fig. 6A, lane 4). In a *sec18-1* mutant, labeled at 37°C, a protein migrating at 81 kDa was detected (lane 6). The difference in electrophoretic migration, 9 kDa, corresponds to the 54 amino acid O-glycosylated subunit I. Thus, we expect that the 81 kDa form was SUI-invertase-Cterm, retained in the pre-Golgi in the *sec18-1* mutant, whereas in control cells subunit I had been removed by Kex2p in the late Golgi, resulting in invertase-Cterm. In *sec24-1* cells both forms were detected (lane 5). When the same experiment was performed for reference in *sec13-1* cells, similar results were obtained (lane 7). We suggest that in the *sec24-1* and *sec13-1* mutants about half of SUI-invertase-Cterm had left the ER at 37°C during a 40-minute chase, and reached at least the late Golgi. Similar results were obtained when the C-terminal fragment was placed to the N-terminus of invertase (SUI-Cterm-invertase, Fig. 1G), as shown in Fig. 6A, lanes 1-3. Parallel immunoprecipitations with CPY antiserum showed that CPY remained in the ER form in the three mutant strains (Fig. 6B, lanes 1, 3 and 4), but was converted to the mature form in the control cells (lane 2). We conclude that the C-terminal domain of Hsp150 served as an active mediator of ER exit for proteins,

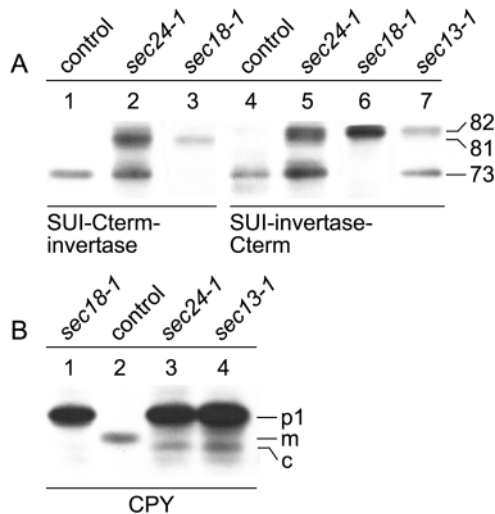


Fig. 6. The C-terminal domain of Hsp150 as mediator of ER exit of invertase. (A) Control (H1540 in lane 1, and H1577 in lane 4), *sec24-1* (H1544 in lane 2, and H1579 in lane 5), *sec18-1* (H1542 in lane 3, and H1575 in lane 6), and *sec13-1* (H1578 in lane 7) cells were ^{35}S -labeled for 5 minutes after a 15-minute preincubation, and chased with CHX for 40 minutes at 37°C. After immunoprecipitation with Hsp150 antiserum and endo H digestion, the samples were subjected to SDS-PAGE analysis. The recombinant strains expressed SUI-Cterm-invertase or SUI-invertase-Cterm, as indicated. The apparent molecular weights of the immunoprecipitates (kDa) are indicated on the right. (B) *sec18-1* (H1542), control (H1540), *sec24-1* (H1544) and *sec13-1* (H1578) cells were labeled as above, but immunoprecipitated with CPY antiserum. The ER (p1, 67 kDa), mature (m, 62 kDa) and cytosolic (c, 59 kDa) forms are indicated.

which in authentic form were dependent on functional Sec24p for recruitment to ER-derived COPII-coated vesicles.

Role of Sec24p homologs in ER exit of Hsp150

Deletion of either of the *SEC24* homologs, *SFB2* or *SFB3* alone or in combination from a normal cell (strains H1141-H1143, see Table 1), did not affect Hsp150 secretion (not shown). Hsp150 appeared in the culture medium with a half time of 5 minutes as described previously for normal cells (Jämsä et al., 1995; Fatal et al., 2002). Disruption of the *SFB3* gene from thermosensitive *sec24* mutants is lethal (Roberg et al., 1999; Peng et al., 2000). Thus, we disrupted the *SFB2* gene from the *sec24-1* mutant. Pulse-chase experiments showed that Hsp150 was secreted to the medium at 37°C in this double mutant (Fig. 7B), with similar kinetics as in the

sec24-1 mutant carrying a normal *SFB2* gene (Fig. 7A). Thus, Sfb2p did not compensate for nonfunctional Sec24p in ER exit of Hsp150.

Discussion

We used temperature-sensitive *sec24-1* mutants to show that a soluble secretory glycoprotein, Hsp150, was secreted to the culture medium in the absence of normal Sec24p function. ER exit was efficient, but occurred with a half time close to 30 minutes, whereas in normal cells the half time of secretion is about 5 minutes (Jämsä et al., 1995; Fatal et al., 2002). A signature needed for the selection of Hsp150 to the Sec24p-independent ER exit route was mapped to the C-terminal domain of 114 amino acids (see Fig. 1A). This determinant was an active ER export signal, because it drove invertase, a Sec24p-dependent exocytic cell wall protein, out of the ER in the absence of normal Sec24p function. Consequently, an Hsp150 variant lacking the C-terminal domain remained in the ER under these conditions. However, in normal cells this variant was readily secreted to the medium, suggesting that the C-terminal domain was responsible for specific recruitment to ER exit sites lacking functional Sec24p.

We showed recently that carrier vesicles can bud off the ER membrane and fuse with the Golgi membranes in the absence of normal Sec13p function, and that Hsp150 is able to leave the ER under these conditions. The signature required for selection to the Sec13p-independent ER exit pathway resided in the same C-terminal domain shown here to recruit Hsp150 for Sec24p-independent exit (Fatal et al., 2002). Whether these are in fact two different exit signatures within the C-terminal domain, each one specific for nonfunctional Sec13p and Sec24p, or a shared single signal, is not yet known.

Not only Sec24p, but also its homologs, Sfb2p and Sfb3p (Roberg et al., 1999; Pagano et al., 1999; Kurihara et al., 2000), function in ER exit. In cells lacking the *SEC24* gene but overproducing Sfb2p, the soluble cargo molecules α -factor precursor and pro-CPY, and the membrane protein Gas1p, were shown to leave the ER. Purified Sfb2p/Sec23p complexes could replace Sec24p/Sec23p in vesicle budding in vitro, and the v-SNAREs (vesicular SNAP receptors) Bet1p and Sec22p could be identified in these vesicles (Kurihara et al., 2000). Sfb3p could also compensate for Sec24p as COPII subunit in in vitro budding assays. However, such vesicles were deficient in SNAREs, suggesting that the Sec24 proteins function in cargo discrimination (Miller et al., 2002). Sfb3p was already shown earlier to select a subset of cargo in vivo, and was suggested to function as a cargo-specific adaptor protein (Roberg et al., 1999; Shimoni et al., 2000). As shown here, the C-terminal

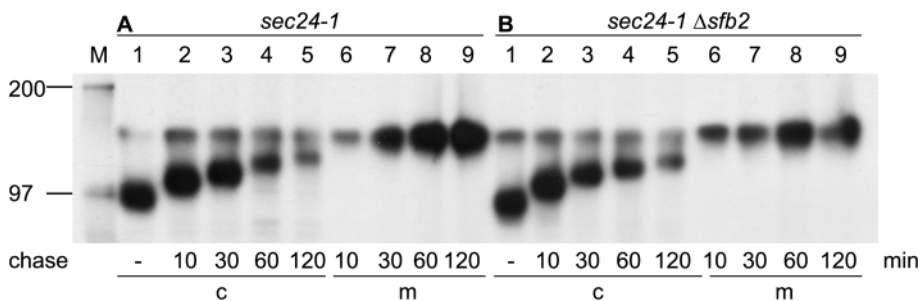


Fig. 7. Hsp150 secretion in the absence of Sfb2p. Mutants (A) *sec24-1* (H1101) and (B) *sec24-1 Δsfb2* (H1555) were preincubated at 37°C for 15 minutes and pulse-labeled for 5 minutes with ^{35}S -methionine/cysteine, followed by chase in the presence of CHX at 37°C as indicated. Immunoprecipitation of cell lysate (c) and medium (m) samples before SDS-PAGE analysis was with Hsp150 antiserum. Migration of marker proteins (M) is indicated on the left.

domain of Hsp150 caused proteins to be selected specifically to ER exit sites lacking functional Sec24p under conditions where Sfb2p and Sfb3p were normal. Deletion of one or both Sec24p homologs did not inhibit the ER exit of Hsp150 when Sec24p was fully functional. Moreover, deletion studies showed that Sfb2p did not compensate for nonfunctional Sec24p in ER exit of Hsp150. Deletion of *SFB3* is lethal in combination with mutations in any of the COPII genes *SEC13/16/23/24/31* (Roberg et al., 1999).

The exit of proteins from the yeast ER requires not only COPII traffic, but also ongoing retrograde traffic from the Golgi to the ER. The Golgi-derived vesicles are covered by the COPI coatomer consisting of seven different structural proteins (Hosobuchi et al., 1992). Retrograde transport has been suggested to return from the Golgi to the ER component(s), which are needed for forward traffic. Hsp150, as well as invertase and the C-tail anchored v-SNARE Sec22p, behave exceptionally, as they leave the ER in the absence of COPI traffic (Gaynor and Emr, 1997; Ballensiefen et al., 1998). The determinant selecting Hsp150 to the COPI-independent pathway was found to be in a region consisting mostly of a 19 amino acid peptide repeated 11 times in Hsp150 (see Fig. 1A) (Suntio et al., 1999).

In summary, Hsp150 appears to have different determinants that drive ER exit. The repetitive region promotes ER exit in the absence of components that are normally returned from the Golgi to the ER by COPI traffic and required for the ER exit of many proteins. The determinant(s) in the C-terminal domain regulate ER exit in the absence of functional Sec24p or Sec13p. None of these determinants is required for ER exit of Hsp150 in cells, where all COPII components are available in functional form and COPI traffic operates. Thus, the different determinants guide soluble cargo proteins specifically and actively to ER exit sites covered with structurally and/or functionally different COPII coats. This suggests that in normal cells the ER gives rise to heterogeneous populations of vesicles, which are covered by different coat protein compositions, resulting in the recruitment of different sets of receptors, and consequently soluble cargo proteins, for intracellular transport.

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