Selective Protein Exit from Yeast Endoplasmic Reticulum in Absence of Functional COPII Coat Component Sec13p

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Sec13p has been thought to be an essential component of the COPII coat, required for exit of proteins from the yeast endoplasmic reticulum (ER). We show herein that normal function of Sec13p was not required for ER exit of the Hsp150 glycoprotein. Hsp150 was secreted to the medium under restrictive conditions in a *sec13-1* mutant. The COPII components Sec23p and Sec31p and the GTP/GDP exchange factor Sec12p were required in functional form for secretion of Hsp150. Hsp150 leaves the ER in the absence of retrograde COPI traffic, and the responsible determinant is a peptide repeated 11 times in the middle of the Hsp150 sequence. Herein, we localized the sorting determinant for Sec13p-independent ER exit to the C-terminal domain. Sec13p-dependent invertase left the ER in the absence of normal Sec13p function, when fused to the C-terminal domain of Hsp150, demonstrating that this domain contained an active mediator of Sec13p-independent secretion. Thus, Hsp150 harbors two different signatures that regulate its ER exit. Our data show that transport vesicles lacking functional Sec13p can carry out ER-to-Golgi transport, but select only specific cargo protein(s) for ER exit.

INTRODUCTION

Anterograde and retrograde membrane traffic between the endoplasmic reticulum (ER) and Golgi are mediated by coated vesicles (Barlowe, 2000). The COPII coat operates directly and exclusively in vesicle formation at the ER membrane and is composed of the structural protein complexes Sec23p/24p and Sec13p/31p, and the small GTPase Sar1p (Salama *et al.*, 1993; Barlowe *et al.*, 1994). The sequence of events in the assembly of the COPII coat, elucidated in vitro by using purified components, starts by recruitment of cytosolic GDP-bound Sar1p by the ER membrane protein Sec12p to the budding site (Barlowe and Shekman, 1993). Sec12p exchanges GDP to GTP on Sar1p, resulting in recruitment of the Sec23p/24p complex to the ER membrane. Thereafter Sec13p/31p is bound to the prebudding complex and budding of the carrier vesicle occurs (Yoshihisa *et al.*, 1993; Barlowe *et al.*, 1994; Matsuoka *et al.*, 1998). Sec13p, like the other components, is thought to be generally required for vesicle formation at the ER membrane and thus to be essential for protein transport from the ER to the Golgi (Kaiser and Schekman, 1990; Pryer *et al.*, 1993).

Another coat protein complex, the COPI coatomer, consisting of seven different protein components, operates in yeast directly in Golgi-to-ER traffic (Hosobuchi *et al.*, 1992; Gaynor and Emr, 1997). In mammalian cells it has been implicated also in ER-to-Golgi and intra-Golgi traffic (Rothman and Orci, 1992; Pepperkok *et al.*, 1993; Duden *et al.*, 1994; Letourneur *et al.*, 1994; Bednarek *et al.*, 1995; Orci *et al.*, 1997). The COPI and COPII pathways are coupled, because ongoing transport in COPI-coated vesicles from the Golgi to the ER is required also for proteins to exit the ER. Retrograde transport may return from the Golgi component(s), which are needed for forward traffic. Two yeast glycoproteins, invertase and Hsp150, were found to be secreted to the exterior of the cell under conditions, where the COPI coat is not assembled (Gaynor and Emr, 1997). Both proteins contained active sorting signals. When fused to invertase, also pro-CPY left the ER in the absence of COPI function (Gaynor and Emr, 1997), and the same was true for an Hsp150-pro-CPY fusion protein (our unpublished data). These results implied that different cargo molecules require different components for ER exit. Hsp150 (Figure 10A) is a soluble O-glycosylated protein composed of three domains, Subunit I of 54 amino acids, 11 tandem repeats of homologous peptides of mostly 19 amino acids, and a unique C-terminal domain of 114 amino acids (Russo *et al.*, 1992; Jämsä *et al.*, 1995; Paunola *et al.*, 1998). Deletion analysis showed that the

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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 MATa <i>sec18-1 trp1-289 leu2-3, 112 ura3-52 his⁻</i>	R. Schekman
H23	MATa <i>ura3-1 his3-11,15 leu2-3, 112 trp1-1 ade2-1 can1-100 hsp150::URA3</i>	Russo <i>et al.</i> , 1992
H229	SF226-1C MATa <i>sec12-4</i>	Novick and Schekman, 1980
H230	HMSF163 MATa <i>sec13-1</i>	Novick and Schekman, 1980
H238	HMSF190 MATa <i>sec23-1</i>	Novick and Schekman, 1980
H245	W303-1A MATa <i>ura3-1 his3-11, 15 leu2-3, 112 trp1-1 ade2-1 can1-100</i>	K. Kuchler/J. Thorner
H247	W303-1B MATa <i>ura3-1 his3-11,15 leu2-3, 112 trp1-1 ade2-1 can1-100</i>	K. Kuchler/J. Thorner
H259	RSY153 MATa <i>sec63-1 leu2-3, 112 ura3-52</i>	R. Schekman
H340	MATa <i>sec7-1 his-leu2-3, 112 trp1-289 ura3-52 URA3::HSP150Δ-β-lactamase</i>	Simonen <i>et al.</i> , 1994
H393	MATa <i>sec18-1 trp1-289 leu2-3, 112 ura3-52 his⁻ URA3::HSP150Δ-β-lactamase</i>	Simonen <i>et al.</i> , 1994
H430	MATa <i>ura3-1 his3-11, 15 leu2-3, 112 trp1-1 ade2-1 can1-100 hsp150::URA3 LEU2::HSP150Δ</i>	This study
H440	MATa <i>sec18-1 trp1-289 leu2-3, 112 ura3-52 his⁻ LEU2::HSP150Δ</i>	This study
H675	MATa <i>ade2-101 ura3-52 leu2-3, 112 suc2-Δ9 gal2 LEU2::HSP150Δ-β-lactamase</i>	Suntio <i>et al.</i> , 1999
H839	MATa <i>ade2-101 ura3-52 leu2-3, 112 suc2-Δ9 gal2 LEU2::SUI-β-lactamase</i>	Suntio <i>et al.</i> , 1999
H840	MATa <i>sec18-1 trp1-289 leu2-3, 112 ura3-52 his⁻ LEU2::SUI-β-lactamase</i>	Suntio <i>et al.</i> , 1999
H1064	MATa <i>sec13-1 leu2::loxP-KanMX-loxP</i>	This study
H1065	MATa <i>sec13-1 leu2::loxP-KanMX-loxP LEU2::HSP150Δ-β-lactamase</i>	This study
H1067	MATa <i>sec13-1 leu2::loxP-KanMX-loxP LEU2::SUI-β-lactamase</i>	This study
H1102	CKY555 MATa <i>sec31-2 ura3-52 leu2-3, 112</i>	C. Kaiser
H1107	MATa <i>sec13-1 leu2::loxP-KanMX-loxP LEU2::HSP150Δ</i>	This study
H1233	MATa <i>ura3-1 his3-11, 15 leu2-3, 112 trp1-1 ade2-1 can1-100 hsp150::loxP-KanMX-loxP</i>	This study
H1234	MATa <i>ura3-1 his3-11, 15 leu2-3, 112 trp1-1 ade2-1 can1-100 hsp150::loxP-KanMX-loxP</i>	This study
H1236	MATa <i>sec13-1 trp1::loxP-KanMX-loxP</i>	This study
H1284	MATa <i>sec13-1 ura3::loxP-KanMX-loxP</i>	This study
H1400	MATa <i>sec13-1 ura3::loxP-KanMX-loxP URA3::SUI-R3-β-lactamase</i>	This study
H1429	MATa <i>sec13-1 trp1::loxP-KanMX-loxP TRP1::SUI-Cterm</i>	This study
H1431	MATa <i>sec63-1 leu2-3,112 ura3-52 URA3::SUI-R3-β-lactamase</i>	This study
H1432	MATa <i>sec18-1 trp1-289 leu2-3, 112 ura3-52 his⁻ URA3::SUI-R3-β-lactamase</i>	This study
H1433	MATa <i>sec7-1 his4-580 ura3-52 leu2-3, 112 trp1-289 URA3::SUI-R3-β-lactamase</i>	This study
H1508	MATa <i>ura3-1 his3-11, 15 leu2-3, 112 trp1-1 ade2-1 can1-100 hsp150::loxP-KanMX-loxP TRP1::SUI-Cterm</i>	This study
H1540	MATa <i>ura3-1 his3-11, 15 leu2-3, 112 trp1-1 ade2-1 can1-100 100 hsp150::loxP-KanMX-loxP TRP1::SUI-Cterm-invertase</i>	This study
H1541	MATa <i>sec13-1 trp1::loxP-KanMX-loxP TRP1::SUI-Cterm-invertase</i>	This study
H1542	MATa <i>sec18-1 trp1-289 leu2-3,112 ura3-52 his⁻ TRP1::SUI-Cterm-invertase</i>	This study
H1545	MATa <i>sec13-1 trp1-1 leu2-3, 112 ura3-1 his3-11,15 hsp150::loxP-KanMX-loxP LEU2::HSP150Δ</i>	This study

repetitive peptide was responsible for selection of Hsp150 to the COPI-independent transport route (Suntio *et al.*, 1999).

Herein, we show that Hsp150 was able to leave the ER in the absence of functional Sec13p. Thus, carrier vesicles can bud off the ER membrane and fuse with the Golgi membranes in the absence of normal Sec13p function. However, only a subset of soluble cargo molecules, or Hsp150 alone, could be recruited to such prebudding complexes. The signature required for selection to the Sec13p-independent ER exit pathway was mapped to the C-terminal domain of Hsp150. When fused to the C-terminal domain, ER exit of invertase became independent of functional Sec13p.

MATERIALS AND METHODS

Yeast Strain Construction

Yeast cells were grown in YPD medium containing 2% glucose, or SC medium lacking appropriate amino acids or nucleotides and containing 2% glucose, unless otherwise stated. Transformations were done with the lithium acetate method (Hill *et al.*, 1991). *LEU2*,

TRP1, and *URA3* disruption cassettes were constructed by polymerase chain reaction (PCR) with pUG6 as a template and oligonucleotides that carry at their 3' termini a sequence homologous to *loxP-kanMX-loxP* on the pUG6 plasmid, and at their 5' termini homologous sequences to *LEU2*, *TRP1*, or *URA3*, respectively (Güldener *et al.*, 1996). They were transformed into yeast strain H230 to create strains H1064, H1236, and H1284, respectively (for genotypes of yeast strains, see Table 1). Transformation of pKTH4660 (*HSP150Δ-β-lactamase*, Figure 10B; Paunola *et al.*, 1998) into H1064 created strain H1065. An *HSP150* cDNA variant (pKTH4570) encoding Hsp150Δ (Figure 10D) was made like pKTH4568 (Jämsä *et al.*, 1995): pKTH4553 (Simonen *et al.*, 1994) was linearized with *KpnI* and subjected to mung bean nuclease digestion, and a portion of the 3' end was removed by *ClaI* digestion. After blunting with Klenow, ligation and transformation into *Escherichia coli*, the sequence of the 3' end of the *HSP150* fragment was found to encode Cys Lys Thr Ser Asp Leu Ile Asp Cys. The *BamHI* fragment from pKTH4570 containing *HSP150Δ* was ligated to the *BamHI* site of pFL26 (Bonneaud *et al.*, 1991), creating pKTH4606. Transforming pKTH4606 into yeast strains H23, H4, and H1064 created strains H430, H440, and H1107, respectively. Endogenous *HSP150* was disrupted from strains H245 and H247 by *loxP-KanMX-loxP*, creating strains H1233 and H1234.

Strains H1234 and H1107 were crossed and sporulated. A spore that did not grow at 37°C and expressed only Hsp150Δ and not Hsp150, verified by Western blotting, was designated H1545. *SUI-R3-β-lactamase* (pKTH5005; Figure 10C) was constructed by replacing the *XhoI*- and *KpnI*-cut fragment derived from pKTH4544 (Simonen *et al.*, 1994) with a *XhoI*-/*KpnI*-digested PCR fragment made using oligos 5'-ATGGTAGGAATCCTCGAGATATAAAAAGG-3' and 5'-CTTAAGGTACCAGTCTTAGCAGAGGTAGTCTT-3', and using pKTH4544 (Simonen *et al.*, 1994) as a template. Transforming pKTH5005 into yeast strains H259, H3, H4, and H1284 created strains H1431, H1433, H1432, and H1400, respectively. The *SUI-Cterm* fragment (Figure 10E) was constructed by annealing two PCR fragments, made with oligonucleotides 5'-ATGGTAGGAATCCTCGAGATATAAAAAGG-3' and 5'-GGCTGCAGAAGCCTGATCAGCAGCTCTCTTGGCCTTAGATGAG-3' or 5'-GCCAAGAGAGCTGCTGATCAGGCTTCTGCAGCCGCTACCTCCAC-3' and 5'-AAATTTAAGCTTAACAGTCTATCAAATCGATAG-3' using pKTH4529 (Simonen *et al.*, 1994) as a template, and filling the ends with DNA polymerase I. After *HindIII* and *XhoI* digestion, the fragment was inserted to *HindIII*-/*XhoI*-digested pKTH4696 (Sievi *et al.*, 2001), creating pKTH5006. Transforming pKTH5006 to yeast strains H1236 and H1233 created strains H1429 and H1508, respectively. Plasmid pKTH4592 (*SUI-β-lactamase*, Figure 10F; Suntio *et al.*, 1999) was transformed into strain H1064, creating strain H1067. To construct *SUI-Cterm-SUC2* (encoding *SUI-Cterm*-invertase; Figure 10G), the invertase gene *SUC2*, without the signal sequence, was amplified using oligonucleotides 5'-AAGCTATCGATTGATAGACTGTCAATGACAAACGAACTAGCGATAG-3' and 5'-ATTAATAAGCTTATTTACTTCCCTTACTTGGAACTTG-3'. The fragment was digested with *ClaI*/*HindIII* and ligated to similarly digested pKTH5006 to create pKTH5056, which was transformed to control, *sec13-1*, and *sec18-1* cells to create strains H1540, H1541, and H1542, respectively (Table 1). All *HSP150* variants were expressed under the *HSP150* promoter (Russo *et al.*, 1993).

Other Methods

Metabolic labeling with [³⁵S]methionine/cysteine (1000 Ci/mmol; Amersham Biosciences UK, Little Chalfont, Buckinghamshire, United Kingdom) and immunoprecipitation with antisera against Hsp150 (1:400), β-lactamase (1:100), and CPY (1:100), and SDS-PAGE (8% gels unless otherwise stated) were as described previously (Paunola *et al.*, 1998). β-Lactamase and invertase activities were determined as described in Simonen *et al.* (1994) and Makarow (1988), activity staining of invertase in nondenaturing gels was according to Novick *et al.* (1980), and indirect immunofluorescence staining was according to Makarow (1988). Release and separation of cell wall components from spheroplasts was as described previously (Kapteyn *et al.*, 1999).

RESULTS

Secretion of Hsp150 in Absence of Normal Sec13p Function

Herein, we studied whether the COPII components were required for Hsp150 (Figure 10A) to exit the yeast ER in vivo. A temperature-sensitive *sec13-1* mutant (yeast strain numbers are indicated in figure legends, and their genotypes and references are given in Table 1) was preincubated for 15 min at 37°C to inactivate Sec13p, labeled with [³⁵S]methionine/cysteine for 5 min, and chased in the presence of cycloheximide (CHX) (Figure 1A). Immunoprecipitation with Hsp150 antiserum and SDS-PAGE analysis detected after the pulse in the lysate the ER form of Hsp150 (100 kDa) (lane 2) together with some mature form (150 kDa), and very little was found in medium (lane 1). The biosynthetic intermediates of Hsp150 have been

Figure 1. Secretion of Hsp150 in *sec13-1* mutant. (A) *Sec13-1* cells (H230) were preincubated for 15 min and pulse labeled for 5 min at 37°C with [³⁵S]methionine/cysteine (lanes 1 and 2). Parallel cell samples were chased in the presence of CHX at 37°C. Medium (m) and cell lysate (c) samples were immunoprecipitated with Hsp150 antiserum followed by SDS-PAGE analysis. ER (100 kDa) and mature forms (150 kDa) of Hsp150 are indicated. (B) Relative signal intensities of A were quantitated by PhosphorImager, and the fraction of the mature 150-kDa form (cell wall-associated plus secreted protein) is plotted against chase time (circles). The experiment of A was repeated except that preincubation at 37°C was 1 h, and the fraction of the 150-kDa form was quantitated (squares).

characterized before (Russo *et al.*, 1992; Jämsä *et al.*, 1995; Suntio *et al.*, 1999). Subunits I and II have many serine and threonine residues, which in the ER obtain single mannose residues, resulting in a form with an electrophoretic migration position of ~100 kDa. In the Golgi, the O-glycans are extended up to pentamannosides, and Subunit I is detached by Kex2p, resulting in mature Subunit II that is secreted to the medium and migrates in SDS-PAGE like a 150-kDa protein. During the chase in *sec13-1* cells, more and more Hsp150 appeared in the medium (lanes 3 and 5) at the expense of cell-associated forms (lanes 4 and 6), until most of it was secreted after 1 h (lane 7). We have shown that a small fraction of externalized Hsp150 remains covalently bound to the cell wall (Kapteyn *et al.*, 1999). When a parallel set of metabolically labeled cell samples was subjected to isolation of the cell walls, the mature cell-associated fraction of Hsp150 (150 kDa) was mostly found in the cell wall preparation, whereas the

immature forms (<150 kDa) were detected in the spheroplast lysate (our unpublished data). Thus, the cell-associated mature form (Figure 1A, even-numbered lanes) had in fact been externalized, but remained bound to the cell wall. According to PhosphorImager quantitation, 38% of Hsp150 was in mature form, and thus externalized, after the 5-min pulse. After 15-min chase, >50% and after 1 h of chase 82% was secreted (Figure 1B, circles). With increasing chase time, the cell-associated immature form (Figure 1A, lane 2) migrated more and more slowly (lanes 4, 6, and 8), apparently due to elongation of *O*-glycans. At permissive temperature 24°C most of Hsp150 was in the medium after 15 min of chase (our unpublished data), like in wild-type cells at 37 and 24°C (Jämsä *et al.*, 1994). When the preincubation of *sec13-1* cells at 37°C before the pulse was extended to as long as 1 h, Hsp150 was still secreted from the *sec13-1* mutant (Figure 1B, squares).

Next, we confirmed that Sec13p was nonfunctional while Hsp150 continued to be secreted, by studying the fate of also pro-CPY and invertase in the very same *sec13-1* cells. The ER form of CPY is primary *N*-glycosylated pro-CPY (p1), and extension of the glycans in the Golgi yields the p2 form. Once the protein arrives in the vacuole, the propeptide is removed, yielding mature CPY (m) (Stevens *et al.*, 1982). After preincubation of *sec13-1* cells for 15 min at 37°C and a 5-min ³⁵S pulse, p1 was detected (Figure 2Aa, lane 1). It persisted during chase (lanes 2–5), indicating that it could not leave the ER. At permissive temperature 24°C, p1 could be detected after the pulse (Figure 2Ab, lane 1), p1 and p2 after 10 min of chase (lane 2), and mostly mature CPY after 20–40 min of chase (lanes 3–5), demonstrating arrival in the vacuole. When the *sec13-1* cells were shifted to low-glucose (0.1%) medium to derepress synthesis of cell wall invertase, and incubated at 37°C, most of the activity remained intracellular (Figure 2B), whereas at 24°C most was externalized to the cell wall (squares). Novick *et al.* (1980) have shown that invertase molecules remaining inside of *sec13-1* cells at 37°C reside in the ER, because their *N*-glycans are not Golgi modified, and we confirmed this by activity staining after nondenaturing gel electrophoresis. We conclude that in cells where pro-CPY and invertase remained in the ER due to nonfunctional Sec13p, Hsp150 was secreted to the culture medium. Whether ER exit of Hsp150 was completely independent of Sec13p function, or whether some residual activity of Sec13p in the *sec13-1* mutant at the restrictive temperature was sufficient to support ER exit of Hsp150, remained unknown.

ER Exit of Hsp150 in Other COPII-defective Mutants

Next, we examined the fate of Hsp150 in mutants where the structural COPII components Sec31p or Sec23p are defective at 37°C. In a *sec31-2* mutant newly synthesized Hsp150 molecules remained cell associated in immature form (Figure 3A, uneven-numbered lanes), and very little was secreted to the medium (even-numbered lanes). The electrophoretic migration of the cell-associated form decreased during chase. This must have been due to addition of second mannose residues to the primary *O*-linked mannose residues during extended residence in the ER, as demonstrated for bulk glycoproteins (Haselbeck and Tanner, 1986). In a

Figure 2. Fate of pro-CPY and invertase in *sec13-1* mutant. (A, a) *Sec13-1* cells (H230) were preincubated for 15 min at 37°C, ³⁵S labeled for 5 min (lane 1), and chased with CHX at 37°C (lanes 2–5). The cell lysates were immunoprecipitated with anti-CPY antiserum followed by SDS-PAGE analysis. (A, b) The above-mentioned experiment was repeated at 24°C. Migration of ER (p1), Golgi (p2), and vacuolar (m) forms of CPY are indicated. (B) *Sec13-1* cells (H230) were incubated in low-glucose medium (0.1%) at 24°C (squares) or 37°C (circles). Extracellular (EX, full symbols) and intracellular (IN, open symbols) invertase activity was determined and plotted against incubation time.

sec23-1 mutant, similar results were obtained (Figure 3B, only cell lysates are shown). For *sec12-4* cells (H229), where GTP/GDP exchange on Sar1p is defective at 37°C preventing the assembly of the COPII coat (Barlowe and Shekman, 1993), the results were similar to those of the *sec31-2* and *sec23-1* mutants. In all of these strains ER-specific pro-CPY persisted during the entire chase time. Thus, the structural COPII components Sec23p and Sec31p, as well as Sec12p were required for ER exit of Hsp150.

Figure 3. Fate of Hsp150 in other COPII mutants. (A) *Sec31-2* (H1102) and (B) *Sec23-1* (H238) cells were preincubated for 15 min, ^{35}S labeled for 5 min, and chased with CHX at 37°C. Cell lysate (c) and medium (m) samples were immunoprecipitated with Hsp150 antiserum before SDS-PAGE analysis. Mature and ER forms of Hsp150 are indicated on the right, and molecular weight markers (kilodaltons) on the left.

ER Exit of Hsp150 Variants Lacking C-Terminal Domain Is Sec13p Dependent

The unique C-terminal fragment of Hsp150 (Figure 10A, white area) was replaced by *E. coli* β -lactamase, creating Hsp150 Δ - β -lactamase (Figure 10B), and it was expressed in the *sec13-1* mutant. In normal yeast cells, the β -lactamase portion folds in the ER into a catalytically active conformation and the fusion protein is secreted to the culture medium within <20 min (Simonen *et al.*, 1994; Paunola *et al.*, 1998; Suntio *et al.*, 1999). To determine whether the reporter was secreted, in *sec13-1*, the transformants were incubated at 37°C for 60 min, CHX was added, and the cells shifted to 24°C. Intracellular and cell wall-bound β -lactamase activity, and activity in the medium were determined. During the 37°C incubation, intracellular activity increased (Figure 4A, open circles), but no activity accumulated in the cell wall (squares) or medium (closed circles). After chase at 24°C, the intracellular activity started to decline after 1 h, with concomitant increase of activity in the medium. Secretion of β -lactamase activity in control cells is shown for reference in Figure 4B. Thus, Hsp150 Δ - β -lactamase remained intracellular in the absence of Sec13p function. Slow reversion of secretion (Figure 4A) was not due to characteristics of Hsp150 Δ - β -lactamase, but to slow reversion of the function of the mutant Sec13 protein. This was shown by examining the reversion of transport of pro-CPY in the same *sec13-1* mutant cells. After preincubation and pulse labeling at 37°C and chase at 24°C, pro-CPY was converted to the vacuolar

Figure 4. Secretion of β -lactamase activity. *Sec13-1* cells (H1065) (A) and control cells (H675) (B) grown at 24°C were shifted to fresh medium and incubated at 37°C. In A, CHX was added and the cells shifted to 24°C. Catalytic activity inside of the cells (open circles), in the cell wall (squares), and in the medium (closed circles) was determined and plotted against incubation time.

form as slowly (our unpublished data) as Hsp150 Δ - β -lactamase was secreted (Figure 4A).

To determine the location of intracellular Hsp150 Δ - β -lactamase, the *sec13-1* cells were incubated at 37°C and subjected to indirect immunofluorescence staining by using β -lactamase antiserum. The nuclear membrane and the plasma membrane were stained (Figure 5c). This is a typical staining pattern for ER proteins. In yeast cells, the ER usually lies underneath the plasma membrane, as shown in Figure 5a for BiP/Kar2p, a resident ER protein. Similar staining was observed when Hsp150 Δ - β -lactamase was blocked in the ER in a *sec18-1* mutant (Figure 5b). When blocked in the Golgi in a *sec7-1* mutant, immunostaining of Hsp150 Δ - β -lactamase revealed a punctate pattern (Figure 5d), like in the case of Kex2p, a late Golgi marker (Sievi *et al.*, 2001). We suggest that in the absence of the C-terminal fragment our reporter protein was not able to leave the ER in the absence of normal Sec13p.

Next, we wanted to confirm by a biochemical assay that Hsp150 Δ - β -lactamase did not reach the Golgi in the absence of functional Sec13p. Kex2 cleavage between Subunits I and II could be used as an indication of arrival to the late Golgi, however, the modification is too small a change to be de-

Figure 5. Indirect immunofluorescence staining of Hsp150Δ-β-lactamase. *Sec13-1* (strain H1065, a and c), *sec18-1* (H393, b), and *sec7-1* mutants (H340, d) were incubated at 37°C for 1 h (c) or 2 h (a, b, and d), followed by a 1-h (b and d) or 2-h (c) chase with CHX before fixation. Immunostaining was with antiserum against BiP/Kar2p (a) or β-lactamase (b–d).

tected in SDS-PAGE. Thus, we used a variant, where the number of the repetitive peptides was reduced to three (SUI-R3-β-lactamase; Figure 10C). When *sec13-1* cells expressing SUI-R3-β-lactamase were preincubated and ³⁵S-labeled at 37°C, proteins with electrophoretic migration positions of 43.5 and 57 kDa could be immunoprecipitated with β-lactamase antiserum from the cell lysate (Figure 6, lane 1). The 43.5-kDa form was an untranslocated form, as verified by pulse-labeling of SUI-R3-β-lactamase blocked in the cytosol in a translocation-deficient *sec63-1* mutant (lane 13). The 57-kDa form must have been a primary glycosylated ER intermediate. After chase for 15 min of the *sec13-1*

mutant, both forms disappeared with concomitant appearance of a 63-kDa species (lane 2), which comigrated with SUI-R3-β-lactamase arrested in the ER in a *sec18-1* mutant (lane 14). With increasing chase time the latter protein's migration was retarded to correspond to 66 kDa (lanes 3 and 4), apparently due to elongation of O-glycans upon prolonged residence in the ER, like suggested above for authentic Hsp150. No protein could be found in the respective medium samples (lanes 7–10). When the cells chased for 60 min at 37°C were shifted to permissive temperature 24°C, a species migrating like a 53-kDa protein was detected in the cell lysates (lanes 5 and 6), and a comigrating species in the medium (lanes 11 and 12). We suggest that R3-β-lactamase was cleaved from Subunit I by Kex2p only when Sec13p was functional, at 24°C, indicating that in the absence of Sec13p function, SUI-R3-β-lactamase was not transported to the late Golgi.

To examine whether Sec13p-dependent Hsp150 fusion protein variants remained in the ER, or whether they recycled between the ER and the Golgi, we expressed SUI-R3-β-lactamase in a *sec7-1* mutant, where at 37°C membrane traffic is blocked in the Golgi. After pulse and chase, three species could be immunoprecipitated with β-lactamase antiserum from the cell lysates (Figure 6, lane 15). The 63-kDa form apparently was SUI-R3-β-lactamase decorated with ER-specific O-glycans, and the 72-kDa form the variant with Golgi-specific extended O-glycans. The 53-kDa form must be the R3-β-lactamase fragment, released from Subunit I at the Kex2p site. Because no 72-kDa form nor released R3-β-lactamase was detected in the *sec13-1* mutant at 37°C, SUI-R3-β-lactamase apparently did not recycle between ER and Golgi, but was retained in the ER.

Then, a Hsp150 variant lacking the C-terminal domain, Hsp150Δ (Figure 10D) was expressed in a *sec13-1* mutant. In normal cells, Hsp150Δ is secreted to the medium, and its electrophoretic migration is anomalously slow, probably due to poor binding of SDS to the extensively O-glycosy-

Figure 6. Kex2p cleavage of SUI-R3-β-lactamase. H1400 cells (SUI-R3-β-lactamase/*sec13-1*) were preincubated for 15 min and pulse labeled with [³⁵S]methionine/cysteine for 3 min at 37°C, followed by chase with CHX for 15–60 min at 37°C. The cells were then shifted to 24°C and chased for an additional 30 and 60 min, as indicated. The lysed cells (lanes 1–6) and media (lanes 7–12) were immunoprecipitated with β-lactamase antiserum and analyzed by SDS-PAGE. Lane 13, SUI-R3-β-lactamase/*sec63-1* (H1431). Lane 14, SUI-R3-β-lactamase/*sec18-1* (H1432). Lane 15, SUI-R3-β-lactamase/*sec7-1* (H1433). The last three strains were treated as described above and chased as indicated. On the left, marker proteins (kilodaltons), and on the right, the cytosolic form (CS; 43.5 kDa), ER forms (57–63 kDa), uncleaved Golgi form (72 kDa), and Kex2p-cleaved Golgi form (53 kDa) of SUI-R3-β-lactamase are indicated.

pressing only authentic Hsp150. After chase at both temperatures, Hsp150 was detected mostly in the medium (f, lanes 1–4). We conclude that in the very same cells where authentic Hsp150 was secreted to the medium in the absence of Sec13p function, most of the variant lacking the C-terminal domain remained in the cells in an immature form.

C-Terminal Domain Harbors a Determinant for Sec13p-independent ER Exit

To study directly whether the C-terminal portion harbored a determinant responsible for Sec13p-independent ER exit of Hsp150, the last 114 amino acids of Hsp150 were fused to Subunit I (SUI-Cterm; Figure 10E) for expression in a *sec13-1* mutant. At restrictive temperature 37°C, pulse labeling and

immunoprecipitation with Hsp150 antiserum revealed no protein in the medium (Figure 8A, lane 1), but in the cell lysate a diffuse band of ~23–29 kDa (lane 2), which probably was the heterogeneously O-glycosylated ER form of SUI-Cterm. After a 1-h chase, most of the cell-associated form had disappeared (lane 4). Concomitantly, a protein of 16.5-kDa plus a smaller species had appeared in the medium (lane 3). These must be the C-terminal fragment, released at the Kex2p site from Subunit I in the late Golgi. A very small amount of both remained cell associated (lane 4). The C-terminal fragment contains one methionine and four cysteines, whereas Subunit I has none and cannot be detected after release. Similar results were obtained at permissive temperature 24°C (lanes 5–8). When SUI-Cterm was expressed in control cells (*SEC13*⁺), similar amounts of the released C-terminal fragment were detected in the medium at 37°C (lane 9) and 24°C (lane 11), as in the case of the *sec13-1* mutant. No cell-associated smear could be detected after the pulse, which may indicate faster secretion in the control cells compared with the *sec13-1* mutant. All of these proteins were absent from the *sec13-1* mutant lacking the recombinant gene (our unpublished data). Thus, we conclude that SUI-Cterm was able to leave the ER in the absence of Sec13p function.

To confirm that the C-terminal domain alone, and not Subunit I, was responsible for Sec13p-independent ER exit, SUI- β -lactamase (Figure 10F) was expressed in a *sec13-1* mutant. A pulse-chase experiment showed that even after a 90-min chase, SUI- β -lactamase remained cell associated (Figure 8B, lanes 1, 3, 5, 7, and 9), and no protein appeared in the medium (respective even-numbered lanes). The electrophoretic migration was similar to that of SUI- β -lactamase retained in the pre-Golgi compartment due to the *sec18-1* mutation (lane 11). Mature SUI- β -lactamase found in the medium of normal cells migrates more slowly due to extended O-glycans (lane 12). In normal cells SUI- β -lactamase leaves the ER rapidly (Holkeri and Makarow, 1998). We conclude that the C-terminal domain of Hsp150 harbored a determinant, which conferred Hsp150 the capability of exiting the ER in the absence of functional Sec13p.

C-Terminal Domain of Hsp150 Harbors an Active Mediator of Sec13p-independent Secretion

Finally, we studied whether the C-terminal Hsp150 domain was an active determinant, capable of guiding Sec13p-dependent protein out of the ER in the absence of Sec13p function. Cell wall invertase was used as a reporter, because its ER exit is dependent of Sec13p, and independent of COPI traffic. Our experimental setup was designed according to the strategy used by Gaynor and Emr (1997) to show that invertase is a direct mediator for COPI-independent secretion. The authors demonstrated that the profragment of a pro-CPY-invertase fusion was cleaved even in the absence of COPI function. Because this cleavage normally occurs in the vacuole, it was concluded that the chimeric protein had exited the ER and reached the vacuole. We fused invertase to the C terminus of an Hsp150 variant, which contained Subunit I followed by a Kex2p cleavage site, and the C-terminal fragment (SUI-Cterm-invertase; Figure 10G). Control, *sec13-1* and *sec18-1* cells were found to express the chimeric protein, which was catalytically active and secreted to the cell wall under permissive conditions (our unpublished

Figure 9. ER exit of SUI-Cterm-invertase in the *sec13-1* mutant. Strains expressing SUI-Cterm-invertase (SUI-Cterm-Inv) in control cells (lane 1, strain H1540), *sec13-1* mutant (lane 2, H1541), and *sec18-1* mutant (lane 3, H1542), as well as in a *sec18-1* mutant lacking the recombinant gene (lane 4, H4) were preincubated for 15 min, ³⁵S labeled for 5 min, and chased for 1 h at 37°C. In the case of lanes 1–3, expression of authentic invertase (Inv) was repressed by 2% glucose in the medium, whereas in lane 4, invertase was derepressed in 0.1% glucose medium. The cell lysates were subjected to immunoprecipitation with invertase antiserum, followed by endoglycosidase H digestion and SDS-PAGE analysis. Migration of invertase variants is indicated on the right.

data). We expected that in control cells Subunit I would be released by Kex2p while the fusion protein passed the Golgi on its way to the cell wall, and this seemed to be the case. Immunoprecipitation of ³⁵S-labeled control cell lysates with invertase antiserum, and endoglycosidase H digestion to remove the heterogenous N-glycans of the invertase portion, revealed a protein migrating at 73 kDa (Figure 9, lane 1), whereas in a *sec18-1* mutant, at 37°C, a protein migrating at 81 kDa was detected (lane 3). The difference in electrophoretic migration, 9 kDa, is compatible with the 54 amino acid long O-glycosylated Subunit I, removed by Kex2p in the control cells. When *sec13-1* cells were ³⁵S labeled at 37°C, some of the immunoprecipitated protein migrated at 82 kDa, but the majority at 73 kDa. We suggest that in the *sec13-1* cells, most of SUI-Cterm-invertase left the ER, resulting in cleavage of Subunit I in the late Golgi. The somewhat slower migration of the noncleaved protein (82 kDa) in *sec13-1*, compared with the *sec18-1*-arrested protein (81 kDa), may have been due to slight elongation of the O-glycans upon prolonged ER residence, as demonstrated above for Hsp150 (Figure 1). In low glucose medium in *sec18-1* cells at 37°C, authentic invertase (58 kDa, lane 4) could be immunoprecipitated. The other proteins except endogenous invertase were recognized with Hsp150 antiserum (our unpublished data). We conclude that the C-terminal Hsp150 fragment was able to recruit invertase out of the ER in the absence of normal Sec13p, indicating that it actively mediated ER exit in structurally or functionally incomplete COPII vesicles.

DISCUSSION

We show herein that budding of carrier vesicles from the yeast ER membrane and their fusion with the Golgi mem-

area) was fused to SUI-Cterm. The last amino acids of Hsp150 domains are numbered, and the C-terminal number indicates the total number of amino acids. 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 (F). Sec13p dependency of ER exit of the Hsp150 variants is indicated.

brane can take place in the absence of normal Sec13p function in living cells. The yeast glycoprotein Hsp150 required COPII coat assembly for ER exit, because functional Sec31p, Sec23p, and Sec12p were indispensable. However, Hsp150 was secreted to the medium under restrictive conditions in a *sec13-1* mutant. After a preincubation of 15 min at 37°C to impose the *sec13-1* phenotype, almost 40% of newly synthesized Hsp150 molecules were externalized within a 5-min pulse, and >50% after a chase of 15 min. The rest of the molecules were secreted much more slowly, within an hour of chase. At permissive temperature 24°C, almost all Hsp150 was in the medium after 15 min of chase. In normal cells Hsp150 is secreted with similar kinetics at 24 and 37°C (Jämsä *et al.*, 1994). The initial rapid secretion was not due to Sec13p still functioning normally, because simultaneously in the very same cells, invertase and pro-CYP were retained in the ER. Moreover, Hsp150 was secreted similarly even after a 1-h preincubation at restrictive temperature. The slow secretion phase of Hsp150 could have been due to retarded assembly of the COPII coat, to slow recruitment of Hsp150 to the bud, or to slow disassembly of the COPII coat preceding fusion with Golgi membranes. Sec23p serves as a GTPase-activating protein, turning GTP-Sar1p to GDP-Sar1p to trigger coat disassembly, and the Sec13p/31p complex accelerates this activity (Yoshihisa *et al.*, 1993). Thus, Sec13p seems to have a role in facilitating disassembly. Three-dimensional reconstruction of the Sec23p/24p and Sec13p/31p complexes has allowed development of a model for the architectural organization of the COPII coat, where Sec23p/24p and Sec13p/31p complexes are cross-linked and Sec13p/31p complexes associated head to head (Lederkremer *et al.*, 2001). Because Sec13p mediates these essential interactions, its presence would seem indispensable for coat

assembly. However, intracellular transport of invertase and CYP have been found to operate, although more slowly than in normal cells, in complete absence of Sec13p, but only in cells carrying mutations in *BST1*, *BST2/EMP24*, or *BST3* genes (Elrod-Erickson and Kaiser, 1996). It was proposed that the *BST* gene products function by blocking the budding of vesicles with incomplete or incorrectly assembled COPII coats, and in the absence of Bst protein function abnormally coated vesicles could carry out ER-to-Golgi traffic. The Bst proteins apparently did not prevent ER exit of Hsp150 in the absence of functional Sec13p, but whether the gradual retardation of Hsp150 secretion was due to them, remains to be studied.

Could nonfunctional Sec13p have been replaced by a homologue in the COPII coat of vesicles carrying Hsp150? The *Saccharomyces cerevisiae* genome harbors three open reading frames encoding proteins containing 24–28% of identical amino acids with Sec13p. Two of them, Seh1p and Tup1p, have been found to function in nuclear pore complexes and as a general repressor of RNA polymerase II transcription, respectively (Keleher *et al.*, 1992; Siniosoglou *et al.*, 1996). The function of the YDR267c product, which shares 42% similar and 27% identical amino acids with Sec13p, is not known (Lucau-Danila *et al.*, 2000). We are currently studying whether it could compensate for nonfunctional Sec13p in ER exit of Hsp150. Sec24p has two homologues, Sfb2p (also called Iss1p and Sec24Bp; 55% identity with Sec24p; Kurihara *et al.*, 2000) and Sfb3p (also called Lst1p and Sec24Cp; 23% identity with Sec24p; Roberg *et al.*, 1999), and both seem to operate in ER exit. Overexpression of Sfb2p complemented disruption of the vital *SEC24* gene, suggesting that Sec24p and Sfb2p are interchangeable in COPII coats. Moreover, purified Sec23p/Sfb2p could replace Sec23p/24p in

driving ER vesicle formation in vitro. Examination of the content of these two types of vesicles failed to detect specificity in recruitment of at least major cargo proteins (Kurihara *et al.*, 2000). Sfb3p has been suggested to have a role in selection of soluble cargo for ER exit, because a subset of secretory proteins failed to appear in the medium from yeast cells lacking it (Pagano *et al.*, 1999). It was found to cooperate with Sec24p in recruitment of plasma membrane ATPase into COPII vesicles in yeast, leading to the suggestion that combinatorial subunit compositions might expand the range of cargo molecules in ER-derived carrier vesicles (Roberg *et al.*, 1999; Shimoni *et al.*, 2000).

Although Hsp150 was concentrated into ER-derived vesicles in the absence of Sec13p function, invertase and pro-CYP were excluded. This suggests that Hsp150 was recognized by a protein(s) that was selected to the prebudding complex assembling in the absence of Sec13p function. Soluble cargo is thought to interact with COPII components via transmembrane receptor or adapter proteins (Campbell and Schekman, 1997; Kuehn *et al.*, 1998). Members of the transmembrane p24 protein family of COPII-coated vesicles of *S. cerevisiae* were suggested to serve as specific cargo receptors (Schimmöller *et al.*, 1995). One of the p24 proteins, Emp24, was suggested to specifically facilitate recruitment into COPII-coated vesicles of Gas1p, a GPI-anchored plasma membrane protein (Muniz *et al.*, 2000). However, deletion of all eight p24 genes showed that the p24 proteins are not essential for vesicular transport, and that ER exit of Gas1p does not depend exclusively on p24 proteins (Springer *et al.*, 2000). A multispanning ER membrane protein, Shr3p, serves in packaging the amino acid permease family members into COPII-coated vesicles. This exemplifies another concept of recruitment, because Shr3p itself is not packaged into vesicles, but remains in the ER membrane (Gilstring *et al.*, 1999).

For selective recruitment, soluble cargo proteins need to have sorting signals. The sorting signal for COPI-independent ER exit resides in the repetitive peptide of Hsp150 (Suntio *et al.*, 1999). This signal seems to depend on the amino acid sequence rather than a three-dimensional signature, because the repetitive peptide does not adopt any regular secondary structure but occurs as a random coil (Jämsä *et al.*, 1995). Herein, we found that the C-terminal domain (Figure 10A, white area) was required for Sec13p-independent transport of Hsp150. This fragment apparently harbored an active determinant for recruitment of Sec13p-dependent passenger proteins to ER exit sites where functional Sec13p was not required for vesicle budding. Authentic invertase, which is Sec13p dependent, left the ER at restrictive temperature in a *sec13-1* mutant, when fused to the C terminus of the Hsp150 C-terminal fragment (Figure 10G). Thus, the determinants guiding proteins to the COPI-independent and Sec13p-independent ER exit pathways are different, and intracellular transport of Hsp150 is regulated by both of them.

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