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Sec24p and Iss1p Function Interchangeably in Transport Vesicle Formation from the Endoplasmic Reticulum in *Saccharomyces cerevisiae*

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The Sec23p/Sec24p complex functions as a component of the COPII coat in vesicle transport from the endoplasmic reticulum. Here we characterize *Saccharomyces cerevisiae SEC24*, which encodes a protein of 926 amino acids (YIL109C), and a close homologue, *ISS1* (YNL049C), which is 55% identical to *SEC24*. *SEC24* is essential for vesicular transport in vivo because depletion of Sec24p is lethal, causing exaggeration of the endoplasmic reticulum and a block in the maturation of carboxypeptidase Y. Overproduction of Sec24p suppressed the temperature sensitivity of *sec23-2*, and overproduction of both Sec24p and Sec23p suppressed the temperature sensitivity of *sec16-2*. *SEC24* gene disruption could be complemented by overexpression of *ISS1*, indicating functional redundancy between the two homologous proteins. Deletion of *ISS1* had no significant effect on growth or secretion; however, *iss1*Δ mutants were found to be synthetically lethal with mutations in the v-SNARE genes *SEC22* and *BET1*. Moreover, overexpression of *ISS1* could suppress mutations in *SEC22*. These genetic interactions suggest that Iss1p may be specialized for the packaging or the function of COPII v-SNAREs. Iss1p tagged with His_6 at its C terminus copurified with Sec23p. Pure Sec23p/Iss1p could replace Sec23p/Sec24p in the packaging of a soluble cargo molecule (α-factor) and v-SNAREs (Sec22p and Bet1p) into COPII vesicles. Abundant proteins in the purified vesicles produced with Sec23p/Iss1p were indistinguishable from those in the regular COPII vesicles produced with Sec23p/Sec24p.

INTRODUCTION

Vesicular transport from the endoplasmic reticulum (ER) to the Golgi is the first step in the intracellular trafficking of proteins destined for the Golgi apparatus, lysosomes (vacuoles), plasma membrane, and extracellular space. Most, if not all, anterograde vesicular transport from the ER to the Golgi complex is carried out by vesicles coated with a protein complex known as COPII. In vitro assays have shown that the minimal components needed for formation of the COPII coat are three cytosolic proteins: Sar1p, the Sec23p/Sec24p complex, and the Sec13p/Sec31p complex (Barlowe *et al.*, 1994). During vesicle formation, coat proteins are sequentially recruited to the ER: first Sar1p, then Sec23p/Sec24p, and finally Sec13p/Sec31p (Matsuoka *et al.*, 1998).

Sar1p is a small GTPase (21 kDa) of the Ras superfamily (Nakano and Muramatsu, 1989; Oka *et al.*, 1991). Sec12p, an integral membrane protein of the ER, facilitates exchange of GTP for GDP on Sar1p and is thought to recruit Sar1p to the ER (Barlowe and Schekman, 1993). The GTP-bound form of Sar1p is required for budding, and GTP must be hydrolyzed to GDP before vesicles can fuse with the Golgi (Barlowe *et al.*, 1994). A nonhydrolyzable GTP analogue, 5′-guanylyl imidodiphosphate (GMP-PNP), satisfies the nucleotide requirement for vesicle formation, but the vesicles produced with GMP-PNP cannot fuse with the Golgi membrane. Presumably, GTP hydrolysis by Sar1p is a prerequisite for the dissociation of coat proteins from the vesicles to produce fusion-competent vesicles. Yoshihisa *et al.* (1993) found that

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Abbreviations used: CPY, carboxypeptidase Y; ER, endoplasmic reticulum; GMP-PNP, 5′-guanylyl imidodiphosphate; gpαF, glycosylated pro-α-factor; MSS, medium-speed supernatant; Ni-NTA, nickel-nitriloacetic acid; SC, synthetic complete; TCA, trichloroacetate.

Sec23p (85 kDa) stimulates the hydrolysis of GTP by Sar1p. Sec24p (105 kDa) was discovered as a subunit of a protein complex containing Sec23p (Hicke *et al.*, 1992). Although the Sec24p subunit of this complex is required for in vitro vesicle formation, it has no significant effect on the GTPase-activation activity of Sec23p (Yoshihisa *et al.*, 1993). Sec13p is a 33-kDa protein containing six WD repeat motifs that make up most of the protein (Salama *et al.*, 1993; Saxena *et al.*, 1996). Sec31p is a 150-kDa phosphoprotein that contains seven WD repeats near the N terminus (Salama *et al.*, 1997). Phosphorylation of this protein is important for vesicle formation. Direct interactions among COPII components have been shown by two-hybrid analysis and in vitro binding assays: the N terminus of Sec24p binds to Sec23p (Gimeno *et al.*, 1996); Sec13p and the N-terminal region of Sec31p interact with each other, possibly through their WD repeat motifs (Shaywitz *et al.*, 1997); and Sec23p and Sec24p interact with a central region of Sec31p (Shaywitz *et al.*, 1997). Sec16p, a 240-kDa peripheral ER membrane protein, has also been implicated in COPII coat assembly, because Sec16p is required for vesicle formation in vivo and Sec16p binds to Sec23p, Sec24p, and Sec31p (Espenshade *et al.*, 1995; Gimeno *et al.*, 1996; Shaywitz *et al.*, 1997).

Cargo molecules are selectively packaged into COPII vesicles. Because some cargo molecules are known to be concentrated into COPII vesicles (Balch *et al.*, 1994), an active uptake mechanism must exist for at least some proteins. Sar1p and Sec23p/Sec24p probably play a central role in cargo recruitment. When Sar1p and Sec23p/Sec24p are incubated with microsomes in the presence of GTP or GMP-PNP and in the absence of Sec13p/Sec31p, a prebudding complex forms that contains various cargo molecules, such as glycosylated pro-α-factor (gpαF), amino acid permeases, Emp24p, and SNARE molecules in yeast (Kuehn *et al.*, 1998) and vesicular stomatitis virus glycoprotein in a mammalian system (Aridor *et al.*, 1998). Importantly, resident ER proteins are excluded from these prebudding complexes, indicating that at this stage at least some cargo sorting has already taken place. The v-SNARE vesicle proteins Bet1p and Bos1p bind to Sec23p/Sec24p as well as to Sar1p, indicating that sorting may be achieved by direct interaction with these coat proteins (Springer and Schekman, 1998). Recent work in mammalian cells has identified two sorting motifs within the cytoplasmic domains of transmembrane cargo molecules: a di-acidic motif (Asp-X-Glu, where X represents any amino acid) on the cytoplasmic tail of vesicular stomatitis virus glycoprotein (Nishimura and Balch, 1997), and a double phenylalanine motif (Phe-Phe) on the cytoplasmic tail of p24 proteins (Fiedler *et al.*, 1996; Dominguez *et al.*, 1998) and ERGIC-53 (Kappeler *et al.*, 1997). These motifs are important for the efficient exit of these cargo molecules out of the ER. Moreover, peptides containing the latter motif were shown to bind to the mammalian Sec23p/Sec24p complex (Kappeler *et al.*, 1997; Dominguez *et al.*, 1998).

Despite the fact that Sec24p has long been recognized to play an essential role in the budding of COPII-coated vesicles, the structure and function of this protein have not yet been described in detail. Here we report the characterization of Sec24p and a homologue, Iss1p. We demonstrate that Sec24p and Iss1p can function interchangeably in vesicle formation from the ER.

Table 1. Strains used in this study

Name	Genotype
RSY255	*MATα leu2-3,112 ura3-52*
RSY612	*MATα can1-100 leu2-3,112 his3-11,15 trp1-1 ura3-lade2-1 GAL2*
	MATα can1-100 leu2-3,112 his3-11,15 trp1-1 ura3-lade2-1 GAL2
RSY620	*MATα leu2-3,112 ura3-52 ade2-1 trp1-1 his3-11,15PEP4::TRP1*
RSY866	RSY612 derivative, one of whose two *SEC24* genes is disrupted by *LEU2*
RSY875	*sec24::LEU* haploid carrying pTYY214 derived from RSY866 [pTYY214]
CKY496	*MATα sec24-1 ura3-52 leu2-3,112*
CKY499	*MATα iss1-Δ2::TRP1 ura3-52 leu2-Δ1 his3-Δ200 lys2-801 ade2-101 trp1-Δ63*
CKY500	*MATa iss1-Δ2::TRP1 ura3-52 leu2-Δ1 his3-Δ200 lys2-801 ade2-101 trp1-Δ63*
CKY39	*MATα sec12-4 ura3-52 his4-619*
CKY40	*MATa sec12-4 ura3-52*
CKY46	*MATa sec13-1 ura3-52 his4-619*
CKY51	*MATa sec16-2 ura3-52*
CKY55	*MATa sec17-1 ura3-52 his4-619*
CKY58	*MATα sec18-1 ura3-52 his4-619*
CKY59	*MATa sec18-1 ura3-52 his4-619*
CKY70	*MATα sec22-3 ura3-52 his4-619*
CKY71	*MATa sec22-3 ura3-52*
CKY72	*MATα sec22-1 ura3*
CKY78	*MATa sec23-1 ura3-52 leu2-3,112*
CKY85	*MATa bet1-1 ura3-52*
GPY60	*MATα ura3-52 trp1-289 his4-579 leu2-3,112 prb1 pep4::URA3 gal2*
YPH501	*MATa ura3-52 lys2-801^{amber} ade2-101^{ochee} trp1-Δ63 his3-Δ200 leu2-Δ1*
	MATα ura3-52 lys2-801^{amber} ade2-101^{ochee} trp1-Δ63 his3-Δ200 leu2-Δ1
TKY5	*MATa SEC24* (pTKY6) derived from RSY866 (pTKY6)
TKY6	*MATα SEC24* (pTKY6) derived from RSY866 (pTKY6)
TKY7	*MATa sec24::LEU2* (pTKY6) derived from RSY866 (pTKY6)
TKY8	*MATα sec24::LEU2* (pTKY6) derived from RSY866 (pTKY6)
TKY10	*MATα ISS1 ura3-52 lys2-801^{amber} ade2-101^{ochee} trp1-Δ63 his3-Δ200 leu2-Δ1*
TKY12	*MATα iss1-Δ1::HIS3 ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*
TKY22	Haploid (*sec24::LEU2* [pTKY11]) derived from RSY866 (pTKY11)
TKY23	Haploid (*SEC24* [pTKY11]) derived from RSY866 (pTKY11)

MATERIALS AND METHODS

Strains and Plasmids

Strains and plasmids used in this study are listed in Table 1 and Table 2, respectively, and their construction is described below

Media

YPD is a complex medium with 1% Bacto yeast extract (Difco, Detroit, MI) and 2% Bacto peptone (Difco) supplemented with glucose (2%, unless noted otherwise). YPGal is the same as YPD except that it contains galactose (2%, unless noted otherwise) instead of glucose. MVCA medium consists of 0.67% yeast nitrogen

Table 2. Plasmids used in this study

Name	Description	Source
pTYB121	pBS II KS(+) carrying partial *SEC24* (0.45 kb) amplified by PCR	This study
pTYB131	pBS II SK(+) carrying 4.2-kb *Xho*I–*Hin*dIII fragment containing *SEC24*	This study
pTYY111–114	Positive clones from YEp24 library in *SEC24* screening	This study
pTYY211–213	Positive clones from YCp50 library in *SEC24* screening	This study
pTYY115	YEp352 (2μ *URA3*) with 4.2-kb fragment containing *SEC24*	This study
pTYY116	YEp352 (2μ *URA3*) with 4.2-kb fragment containing *SEC24* and 3.5-kb fragment containing *SEC23*	This study
pTYY122	YEp352 (2μ *URA3*) with 3.5-kb fragment containing *SEC23*	This study
pTYY214	pBM743 (*CEN URA3 GAL1* promoter) with *SEC24*	This study
pTYY303	pTYB131 derivative with *SEC24* disrupted by *LEU2*	This study
pRH200	pCT3 (*CEN URA3*) with 7.0-kb *Cla*I–*Xba*I fragment containing *ISS1*	This study
pTKB1	pGEM-4Z with 7.0-kb *Cla*I–*Xba*I fragment containing *ISS1*	This study
pTKB2	pGEM-4Z with 7.0-kb *Cla*I–*Xba*I fragment containing His_6-tagged version of *ISS1*	This study
pTKY4	YEp352 (2μ *URA3*) with 7.0-kb *Hin*dIII–*Xba*I fragment containing *ISS1*	This study
pTKY6	YEp352 (2μ *URA3*) with 7.0-kb *Hin*dIII–*Xba*I fragment containing His_6-tagged version of *ISS1*	This study
pTKY7	pGAL425GAL1 (2μ *LEU2 GAL1* promoter and *CYC1* terminator) with 3.5-kb *Pst*I fragment containing His_6-tagged version of *ISS1*	This study
pTKY9	pGAL426GAL1 (2μ *URA3 GAL1* promoter and *CYC1* terminator) with 2.9-kb *Nco*I–*Hin*dIII fragment containing *SEC23*	This study
pTKY11	pGAL426GAL1 (2μ *URA3 GAL1* promoter and *CYC1* terminator) with 3.5-kb fragment containing His_6-tagged version of *ISS1*	This study

base without amino acids (Difco), 0.5% vitamin assay casamino acid (Difco), and 5% carbon source. MV-lowS medium contains 0.67% yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.1 mM $(NH_4)_2SO_4$, and 5% carbon source. MV-noS medium lacks $(NH_4)_2SO_4$ from MV-lowS medium. Nutrients corresponding to auxotrophic markers were supplemented to the MVCA, MV-lowS, and MV-noS media. Synthetic complete (SC) medium contains 0.67% yeast nitrogen base without amino acids and 2% glucose as a carbon source (unless noted otherwise) as well as various nutrients (Sherman, 1991). SC dropout medium lacks one or two nutrients from SC medium.

Purification and Amino Acid Sequencing of Sec24p

The Sec23p/Sec24p complex was purified as described (Hicke *et al.*, 1992), precipitated with 5% trichloroacetate (TCA), denatured in SDS-PAGE sample buffer, and separated by 7.5% SDS-PAGE. After transfer to a nitrocellulose membrane, proteins were stained with Ponceau S. The protein band of Sec24p (105 kDa) was excised, destained with Tris-buffered saline, and washed thoroughly with distilled water. Sec24p bound to nitrocellulose was digested with trypsin, and the released peptides were purified with C_{18} reverse-phase HPLC. Several peaks were recovered and sequenced: P1, IWQIFQ; P2, SVQ(D/F)ILATYK; P3, VGLLATTINTLLQNL; and P4, VTAQLLSCQDSTY.

Cloning of SEC24

Three sets of sense and antisense degenerate oligonucleotides were synthesized based on the amino acid sequences of tryptic peptides P1 (1 and rev-1) and P2 (2A, rev-2A, 2B, and rev-2B): 1, 5′-AT(A/T/C)TGGCA(A/G)AT(A/T/C)TT(T/C)CT-3′; rev-1, 5′-TG(A/G)AA(A/T/G)AT(T/C)TGCCA(A/G/T)AT-3′; 2A, 5′-AT(A/T/C)TT(A/G)GCNACNTA (T/C)AA-3′; rev-2A, 5′-TT(A/G)TANGTNGC(T/C)AA(A/T/G)AT-3′; 2B, 5′-AT(A/T/C)CTNGCNACNTA(T/C)AA-3′; rev-2B, 5′-TT(A/G)TANGTNGCNAG(A/T/G)AT-3′.

PCR was conducted with the primer pairs 1/rev-2A or rev-2B and rev-1/2A or 2B with the use of genomic DNA from RSY255 as a template. Thirty reaction cycles (each cycle was 0.5 min at 93°C, 1.5 min at 50°C, and 3 min at 72°C) were carried out, followed by a 5-min incubation at 72°C. A PCR product of 0.45 kilobase (kb) was obtained with primers 1 and rev-2A. This product was also detected in a similar reaction at a higher annealing temperature (53°C). This fragment was subcloned into an *Sma*I site of pBluescript II KS(+) (Stratagene, La Jolla, CA), giving pTYB121, and sequenced. An ORF was shown to span the entire 453-base pair (bp) insert. This insert was isolated, labeled with [α-^{32}P]dCTP by means of the random primer DNA-labeling system (Amersham, Arlington Heights, IL), and used as a probe to screen yeast genomic libraries in YEp24 and YCp50. Screening was carried out according to the protocol provided by Amersham. We isolated four positive clones from YEp24 libraries (pTYY111–pTYY114) and three from YCp50 libraries (pTYY211–pTYY213). All seven clones shared a 0.5-kb *Eco*RI fragment, a 0.9-kb *Pst*I fragment, and a 3.0-kb *Nco*I fragment that hybridized with the probe DNA. The *Eco*RI fragments from pTYY113 (3.3 and 0.5 kb) were subcloned into an *Eco*RI site of pBluescript II KS(+), and the *Nco*I fragment from pTYY212 (3.0 kb), converted to blunt ends by the Klenow enzyme, was introduced into a *Sma*I site of pBluescript II KS(+). These plasmids were used for sequencing.

Construction of Plasmids for Purification of YNL049C-encoded Protein (Iss1p)

A sequence coding for a stretch of six histidine residues was introduced in front of the termination codon of YNL049C (*ISS1*) as follows. The following four PCR primers were synthesized: TKPr1, 5′-CAGTAACCTCACTTAACCTATG-3′; TKPr2, 5′-TGTTAGTGATGGTGATGGTGATGTCTGTTGATACTAGTCTTCATAC-3′; TKPr3,5′-ACAGACATCACCATCACCATCACTAACAATCAGTCTTTCTTTAATCTT-3′; and TKPr4, 5′-ATATGGCCATTTATCACGAATAC-3′.

His_6 residues are encoded by the underlined sequence of TKPr3 and the sequence complementary to the underlined sequence of TKPr2. TKPr1 anneals to the antisense strand of the *ISS1* ORF, ~650 nucleotides upstream from the termination codon. A part of TKPr2, TCTGTTGATACTAGTCTTCATAC, anneals to the sense strand of the 3′ end of *ISS1* ORF. A part of TKPr3, TAACAATCAGTCTTTCTTTAATCTT, anneals to the 3′-flanking region and termination codon of *ISS1*. TKPr4 anneals to the 3′-flanking region of *ISS1*, ~900 nucleotides downstream from the termination codon. TKPr2 and TKPr3 anneal to each other. The plasmid pRH200 carries the *ISS1*

gene on a *Cla*I–*Xba*I genomic DNA fragment. The first-stage PCR was carried out with TKPr1 and TKPr2 and with TKPr3 and TKPr4 with the use of pRH200 as a template. We obtained an ~650-bp fragment with TKPr1 and TKPr2 and an ~900-bp fragment with TKPr3 and TKPr4. We used these two fragments as templates in the second-stage PCR, in which TKPr1 and TKPr4 were used as primers. This second-stage PCR yielded an ~1.6-kb DNA encoding the C terminus of Iss1p that included His_6 residues as well as a termination codon and the 3′-flanking region of *ISS1*.

The *Cla*I–*Xba*I fragment of pRH200 containing *ISS1* was ligated with pGEM4Z (Promega, Madison, WI) digested with *Acc*I and *Xba*I to generate pTKB1. The 1.4-kb *Acc*I–*Sna*I region of pTKB1 corresponding to the 3′ end and 3′-flanking region of *ISS1* was replaced by the 1.4-kb *Acc*I–*Sna*I fragment of the above PCR product encoding the His_6-tagged C terminus of Iss1p. This plasmid was named pTKB2. We sequenced the region of pTKB2 derived from the PCR product to ensure that no mutation had occurred as a result of PCR. *Xba*I–*Hin*dIII fragments of pTKB1 and pTKB2 were introduced into the *Xba*I–*Hin*dIII site of YEp352 (2μ *URA3*) to obtain pTKY4 and pTKY6, respectively. The *Pst*I fragment (3.5 kb) of pTKY6 encoding His_6-tagged Iss1p was introduced into the *Pst*I site of p425GAL1 (2μ *LEU2*) to express the *ISS1* coding sequence from the *GAL1* promoter (Mumberg *et al.*, 1994). This plasmid, pTKY7, was used for Iss1p purification.

The *Nco*I (blunted)–*Hin*dIII fragment (2.9 kb) of pTYY121 (YEp351 containing *SEC23*) was introduced into p426GAL1 (2μ *URA3*) digested with *Spe*I (blunted) and *Hin*dIII so that *SEC23* could be expressed under control of the *GAL1* promoter (Mumberg *et al.*, 1994). This plasmid was named pTKY9.

Purification of His_6-tagged Iss1p

RSY620 was transformed with pTKY7 and pTKY9. The cells were grown in SC-Ura-Leu (2% glucose) to early stationary phase and used to inoculate 6 l of SC-Ura-Leu (2% raffinose) at an initial OD_{600} of 0.005. The cells were grown at 30°C for 1 d until an OD_{600} of ~1.2 was reached. At this time, 1/100 volume of 20% galactose was added (final concentration of 0.2%) and incubation continued for 5 h, to an OD_{600} of ~2.7, for overproduction of Iss1p and Sec23p. The cells were harvested and washed twice with distilled water. About 25 g of cells (wet weight) were obtained from a 6-l culture. The cells were stored at −80°C until use.

The frozen cells were suspended with HSLB (0.75 M potassium acetate, 50 mM HEPES, 0.1 mM EGTA, 20% [wt/vol] glycerol; final pH was adjusted to 7.0 with 5 M KOH) to a final volume of 70 ml. Immediately before cell disruption, protease inhibitors and reducing agent were added to the following final concentrations: 1.4 mM 2-mercaptoethanol, 1 μM leupeptin, 1 μM pepstatin A, 1 mM ϵ-aminocaproic acid, and 0.5 mM PMSF. Cells were disrupted in a beadbeater chamber filled with a half-volume of glass beads (0.5 mm diam, Biospec Products, Bartlesville, OK) by five 1-min periods of agitation with 2-min intervals for chilling. The lysate was recovered, and the glass beads were washed once with 20 ml of HSLB supplemented with the protease inhibitors and 2-mercaptoethanol. The total lysate (80 ml) was centrifuged at 13,000 × *g* for 10 min, and the resulting supernatant was centrifuged at 40,000 rpm (~186,000 × *g*) for 75 min (45Ti rotor, Beckman, Palo Alto, CA) to obtain a high-speed supernatant fraction.

The supernatant (46 ml) was loaded onto a 10-ml nickel-nitriloacetic acid (Ni-NTA) agarose column (Qiagen, Valencia, CA) equilibrated with HSLB with the protease inhibitors and 2-mercaptoethanol. The column was washed successively with 90 ml of B-II [0.75 M potassium acetate, 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 0.1 mM EGTA, 20% (wt/vol) glycerol, 40 mM imidazole (pH 6.3 adjusted with 5 M KOH)], 20 ml of B-III [0.75 M potassium acetate, 50 mM HEPES, 0.1 mM EGTA, 0.25 M sorbitol, 20% (wt/vol) glycerol, 40 mM imidazole (pH 7.0)], 35 ml of B-IV100 (same as B-III except 100 mM imidazole), and finally 35 ml of B-IV200 (same as B-III except 200 mM imidazole). B-II, B-III, and B-IV100 contained the protease inhibitors. Sec23p/Iss1p was eluted from the column with B-IV500 (same as B-III except 500 mM imidazole). In a typical preparation, 1.5 mg of Sec23p/Iss1p was obtained from 25 g of cells (wet weight). Fractions that contained Sec23p/Iss1p were frozen in liquid nitrogen and stored at −80°C.

Disruption of SEC24 and ISS1

pTYB131 is a pBluescript II SK(+) derivative harboring the 4.2-kb *Xho*I–*Hin*dIII fragment from pTYY113 with the entire *SEC24* gene. The *Bgl*II–*Sal*I fragment (1.6 kb) of pTYB131 was replaced by the 2.5-kb *Bgl*II–*Sal*I fragment of YEp13 containing the *LEU2* gene to yield pTYY303. A 3.7-kb *Bam*HI–*Hin*dIII fragment from pTYY303 containing a partial *SEC24* disrupted by *LEU2* was introduced into the diploid strain RSY612 to disrupt one of the chromosomal copies of *SEC24*. The resulting heterozygous disruption was named RSY866. We confirmed the disruption by Southern blot analysis. Tetrad analysis was performed as described (Sherman *et al.*, 1983).

We deleted one of the chromosomal copies of *ISS1* in the diploid strain YPH501 (Sikorski and Hieter, 1989) as follows. Two PCR primers (TKPr12, 5′-CCTTCTTCCATTAATGATCGACAGCTGCAGTGAATAGCAGATTGTACTGAGAGTGCACC-3′; and TKPr13, 5′-GGTTAATAAAGATAAAGATTAAAGAAAGACTGATTGGCATATGATCCGTCGAGTTCAA-3′) were used to amplify the *HIS3* gene on pRS313 (Sikorski and Hieter, 1989). The underlined sequences of TKPr12 and TKPr13 anneal to the 5′ and 3′ regions of *HIS3*, respectively. YPH501 was transformed with the amplified DNA fragment, and His^+ transformants were selected. The transformants were sporulated and dissected to obtain a haploid cell with a disruption of the *ISS1* gene (*iss1-Δ1::HIS3*).

A second disruption (*iss1-Δ2::TRP1*) that replaced amino acids 116–622 of *ISS1* with the *TRP1* marker was made by one-step disruption of the chromosomal *ISS1* gene. The disruption plasmid (pRH247) was constructed as follows. A *Spe*I fragment of pRH200 was cloned into pRS306, creating pRH217. After deletion of the *Eco*RI site from the polylinker, the 1.5-kb *Bgl*II–*Eco*RI fragment of pRH217 was replaced with a 1-kb fragment containing the *TRP1* marker, creating pRH247.

A *trp1* diploid, CKY19, was transformed with the 2.3-kb *Spe*I fragment of pRH247, yielding CKY498. Tetrad analysis of CKY498 gave 2:2 segregation of *TRP1*. Integration of *TRP1* at the *ISS1* locus was confirmed by Southern blotting

Construction of Yeast Strains for the Galactose Shut-Off Experiment

The *SEC24* ORF was fused to the *GAL1* promotor as follows. A 5′-terminal region of *SEC24* was amplified and mutated by PCR to introduce an *Xba*I site in front of the initiation codon. No misincorporation was found by sequencing. The amplified fragment was subcloned into the *Hin*cII site of pBluescript II SK(+) to obtain pTYB133. We replaced the *Bam*HI–*Sac*I region of pTYB133 with a 3.0-kb *Bam*HI–*Sac*I fragment from pTYB131 to obtain the complete ORF (pTYB134). A 3.1-kb *Xba*I–*Hin*dIII fragment of pTYB134 was isolated and introduced downstream of the *GAL1* promoter on pBM743 followed by a multicloning site. The resulting plasmid, pTYY214, was introduced into RSY866. After sporulation of the transformant, a haploid segregant was obtained in which *SEC24* was expressed under the control of the *GAL1* promoter. RSY875 is *leu2-3,-112 his3-12,15 trp1-1 ura3-1 ade2-1 GAL2 sec24::LEU2* (pTYY214 [*URA3 Gal1p–SEC24*]).

Multicopy Suppression Analysis

The *Xho*I–*Hin*dIII fragment (4.2 kb) from pTYB131containing *SEC24* was ligated into YEp352 (2μ *URA3*) digested with *Sal*I and *Hin*dIII to obtain pTYY115. The *Hin*dIII fragment containing *SEC23* was introduced into the *Hin*dIII site of YEp352 and pTYY115, giving

pTYY122 and pTYY116, respectively. Various temperature-sensitive *sec* mutant strains were transformed with these plasmids.

Construction of ISS1-overexpressing Yeast Strains with SEC24 Disrupted

RSY866 (*MATa*/α *SEC24*/sec24::LEU2) was transformed with pTKY4 (2μ *URA3 ISS1*). The resultant strain was sporulated, and the asci were dissected. Four strains derived from the same tetrad were named TKY1 (*MATa SEC24* [pTKY4 (*URA3 ISS1*)]), TKY2 (*MATα SEC24* [pTKY4 (*URA3 ISS1*)]), TKY3 (*MATa sec24::LEU2* [pTKY4 (*URA3 ISS1*)]), and TKY4 (*MATα sec24::LEU2* [pTKY4 (*URA3 ISS1*)]).

Similar strains were constructed with pTKY6 (2μ *URA3* His6-tagged version of *ISS1*) instead of pTKY4 and named TKY5 (*MATa SEC24* [pTKY6]), TKY6 (*MATα SEC24* [pTKY6]), TKY7 (*MATa sec24::LEU2* [pTKY6]), and TKY8 (*MATα sec24::LEU2* [pTKY6]).

We constructed the following plasmid and yeast strain to regulate the *ISS1* expression level in the *sec24*-disrupted background. The *Bam*HI–*Hin*dIII fragment (3.5 kb) of pTKY7 containing *ISS1* was introduced into p426GAL1 (2μ *URA3*) digested with *Bam*HI and *Hin*dIII. The resultant plasmid (pTKY11) was introduced into the diploid strain RSY866, in which one of the *SEC24* genes had been disrupted by *LEU2*. The transformant was sporulated and dissected on a YPGal plate to allow the expression of *ISS1*. We obtained a haploid Leu^+ and Ura^+ strain, TKY22 (*sec24::LEU2* [pTKY11 (*URA3 Gal1p–ISS1*)]), and a Ura^+ strain, TKY23 (*SEC24* [pTKY11 (*URA3 Gal1p–ISS1*)]).

Pulse-Chase Experiment

For pulse-chase analysis of carboxypeptidase Y (CPY) during Sec24p depletion, RSY875 was grown in MV-lowS (galactose) with appropriate nutrients and then transferred to fresh MV-lowS (galactose) or MV-lowS (glucose) with the supplements. After 9, 12, and 15 h of incubation, 3.0-OD_{600}-unit cells were collected, washed, and transferred to 5 ml of MV-noS (galactose) or MV-noS (glucose). Cells were labeled with 9.3 MBq Trans^{35}S-label (ICN, Costa Mesa, CA) for 10 min and then chased for 60 min. Aliquots (1.2 OD_{600} units of cells) were withdrawn before and after the chase, and lysates were prepared with glass beads as described (Rothblatt and Schekman, 1989). Radioactive proteins immunoprecipitated with anti-CPY antibody were separated on SDS-PAGE and detected with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

We conducted a pulse-chase experiment for the *iss1* null strain and the *ISS1*-overexpressing, *sec24*-disrupted strain as follows. The cells were grown in SC dropout medium to late log phase and then transferred to fresh SC dropout medium (initial OD_{600} = 0.15). After a 2.5-h incubation at 30°C, the cells were collected, washed three times with SC-Met dropout medium, and suspended in SC-Met dropout medium (OD_{600} = 0.3). After a 15-min incubation at 30°C, ^{35}S-Promix (Amersham) was added (1.5 MBq for 0.3-OD_{600}-unit cells). After a 7-min incubation at 30°C, methionine and cysteine were added (final concentration of each amino acid was 0.6 mg/ml) and incubation was continued at 30°C. Cells (0.3 OD_{600} unit) were taken from the solution 0, 5, 15, 30, and 60 min after the addition of methionine and cysteine. The cell suspension was mixed with an equal volume of 10 mM NaN_3/10 mM NaF on ice, collected by centrifugation, and washed once with 10 mM NaN_3/10 mM NaF. The cells were resuspended in lysis buffer (1% SDS, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF) (100 μl for 0.3-OD_{600}-unit cells) and disrupted with glass beads. Radioactive proteins immunoprecipitated with anti-CPY antibody or anti-Gas1p antibody were separated by SDS-PAGE and detected with a PhosphorImager (Molecular Dynamics).

Electron Microscopy

RSY875 grown in YPGal (5%) to OD_{600} = 0.15–0.6 was collected and suspended in sterile distilled water to OD_{600} = 6. Fifty milliliters of YPD (5%) and YPGal (5%) were inoculated with 0.1 ml of the RSY875 suspension. After a 9-h incubation at 30°C, the cells were fixed with glutaraldehyde followed by potassium permanganate, as described by Kaiser and Schekman (1990). Briefly, 50% glutaraldehyde was added to cultures (final concentration, 1%) for 10 min. Then the cells were centrifuged, washed, and resuspended in 4% $KMnO_4$ for 2–4 h at 4°C. Fixed cells were collected, washed several times in water, and incubated in 2% uranyl acetate for 12–16 h at 4°C. After several rinses in water, the samples were dehydrated in an ethanol series and embedded in Spurr's medium. Thin sections were stained with lead citrate and viewed in a JEOL100 electron microscope (JEOL, Tokyo, Japan).

α-Factor Halo Assay

A Δ*sst2* strain, whose growth is arrested in the presence of α-factor, was grown in YPD at 30°C to exponential phase and suspended in YPD containing 1% agar to a final OD_{600} of 3×10^{-4}. A YPD plate was overlaid with this suspension. To examine α-factor secretion, strains were grown to stationary phase in SC dropout medium and spotted (0.01 OD_{600} unit/spot) on the Δ*sst2*-covered plate. The plate was incubated at 30°C for 2 d.

In Vitro ER Vesicle-budding Assay

GPY60 was grown at 30°C in YPD to exponential phase, and microsomes were prepared as described (Wuestehube and Schekman, 1992). Purified microsomes were adjusted to 40 OD_{280} (~8 mg protein/ml) in B88 (20 mM HEPES, pH 6.8, 250 mM sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate). The preparation was frozen in liquid nitrogen and stored at −80°C.

The microsome-based α-factor packaging assay was carried out as follows based on the method described (Baker *et al.*, 1988; Rexach and Schekman, 1991; Kuehn *et al.*, 1996). [^{35}S]Prepro-α-factor was posttranslationally translocated into microsomes in the presence of 1× ATP regeneration mix (Baker *et al.*, 1988) at 10°C for 30 min. Microsomes (400 μg of protein) containing [^{35}S]gpαF were washed once with 1 ml of B88, resuspended in 50 μl of B88, mixed with 50 μl of B88 containing 4.2 M urea (final concentration, 2.1 M), and incubated at 0°C for 10 min. After addition of 1 ml of B88, microsomes were collected by centrifugation and washed twice with 1 ml of B88. Budding reactions were carried out in 50 μl of B88 containing 20 μg of urea-washed microsomes, 1× ATP regeneration mix, 0.1 mM GMP-PNP, and appropriate amounts of Sar1p, Sec13p/Sec31p, Sec23p/Sec24p, and Sec23p/Iss1p, whose concentrations are described in RESULTS. The mixture was incubated at 20°C for 30 min, unless noted otherwise, and chilled on ice for 5 min. Portions of the total reaction and the medium-speed supernatant (MSS) (12,000 × *g*, 4 min) were collected. The amount of trypsin-resistant, concanavalin A–precipitable [^{35}S]gpαF in the MSS was divided by the amount in the total fraction to determine the percentage of α-factor packaged into the vesicles.

The large-scale budding reaction was carried out as follows to isolate the vesicles derived from the ER. For each reaction, microsomes containing 2 mg of proteins were used. Microsomes were incubated with 1× ATP regeneration mix in 1 ml of B88 for 30 min at 10°C. After being washed once with 1 ml of B88, they were incubated with 2.1 M urea in 300 μl of B88 for 10 min at 0°C. After addition of 1 ml of B88, microsomes were collected by centrifugation and washed twice with 1 ml of B88. A budding reaction was carried out at 20°C for 30 or 60 min in 1 ml of B88 containing 2 mg of microsomes, 1× ATP regeneration mix, 0.2 mM GMP-PNP, 65 μg of Sar1p, 120 μg of Sec13p/Sec31p, and 35 μg of either Sec23p/Sec24p or Sec23p/Iss1p. After 5 min on ice, a 50-μl aliquot reaction mixture was taken as total, and the remaining solution was centrifuged (14,000 × *g*, 4 min) to obtain a MSS fraction. A sucrose density gradient consisting of 0.3 ml of B88 containing 70% (wt/wt) sucrose and 2.5 ml of B88 containing 15% (wt/wt) sucrose was overlaid with 750 μl of the MSS. After centrifugation at 50,000 rpm (~250,000 × *g*)

for 2 h (SW55, Beckman), the interface (~0.5 ml) between 15 and 70% sucrose was collected, and its sucrose concentration was adjusted to 55% (wt/wt) with the use of B88 containing 70% (wt/wt) sucrose. The final volume was ~0.8 ml, and 0.55 ml of this solution was placed on the bottom of a sucrose density gradient consisting of B88 containing 52.5, 50, 45, 40, 35, and 25% (wt/wt) sucrose (from the bottom to the top). The volume of each of the bottom three layers was 0.5 ml, and the volume of each of the top three layers was 1 ml. This gradient was centrifuged at 50,000 rpm (~250,000 × *g*) for 20 h (SW55, Beckman), and fractions (0.4 ml × 13) were collected from the top with a density gradient fractionator (ISCO, Lincoln, NE). Proteins in these fractions were concentrated by TCA precipitation.

Other Methods

Sarlp, Sec13p/Sec31p, and Sec23p/Sec24p were purified as described previously (Barlowe *et al.*, 1994; Yeung *et al.*, 1995; Salama *et al.*, 1997). DNA manipulation was done according to Sambrook *et al.* (1989). Yeast cells were transformed by the lithium acetate method (Ito *et al.*, 1983). Protein concentrations were determined with the Bio-Rad (Richmond, CA) protein assay kit with the use of BSA as a standard. Silver staining was carried out as described (Bloom *et al.*, 1987). Western blot analysis was performed with a nitrocellulose membrane, and ECL (Amersham) was used for detection.

RESULTS

Cloning and Sequence Analysis of SEC24

We purified the Sec23p/Sec24p complex from crude yeast cytosol and obtained peptide sequence information from tryptic fragments of Sec24p. A part of the *SEC24* gene was PCR amplified with the use of degenerate primers designed according to the peptide sequence information. Finally, a DNA fragment containing the entire *SEC24* gene was obtained by screening a *S. cerevisiae* genomic DNA library with the use of the PCR fragment as a hybridization probe. The identified ORF (YIL109C) encoded 926 amino acid residues (Figure 1), including four sequences corresponding to those obtained by sequencing of the tryptic fragments. The predicted molecular mass of 103,614 Da agrees with the estimated mass of purified Sec24p (~105 kDa). The coding sequence does not appear to contain a hydrophobic signal sequence or transmembrane domain, consistent with the observation that Sec23p/Sec24p is a cytosolic protein (Hicke *et al.*, 1992). As a final verification of identity, we constructed a hybrid of the *trpE* gene fused to the ORF YIL109C and showed that this fusion protein expressed in *Escherichia coli* could be recognized by anti-Sec24p (our unpublished data). These data confirm that *SEC24* is YIL109C.

We searched a nonredundant sequence database for similarity with the Sec24p sequence with the use of BLAST2 (http://www.ncbi.nlm.nih.gov/BLAST/) and found that human (DDBJ accession number D38555), *Caenorhabditis elegans* (SPTREMBL accession numbers Q19371 and Q23368), and *Arabidopsis thaliana* (EMBL accession number AL022537 [PID accession number e1287285]) have homologous proteins (our unpublished data).

Interestingly, a second ORF of *S. cerevisiae*, YNL049C, showed striking similarity with *SEC24*. YNL049C was first isolated in a two-hybrid interaction assay with the use of the central region of Sec16p as a bait construct and was named *ISS1* (interactor with SEC sixteen) (our unpublished data). The protein sequence of Iss1p is 62% identical to that of Sec24p (Figure 1). This sequence similarity extends throughout the length of the protein and defines two variable regions in the N-terminal part of the proteins, a glutamine-rich domain (amino acids 17–143 in Sec24p, amino acids 17–67 in Iss1p) and a charged domain (amino acids 362–372 in Sec24p, amino acids 295–327 in Iss1p).

Sec24p Depletion is Lethal and Causes a Defect in CPY Maturation and Exaggeration of the ER

The *SEC24* gene was disrupted with *LEU2* in a diploid strain. After sporulation, most tetrads contained two viable and two dead spores (Figure 2). The viable spores were all Leu^-, indicating that they retained wild-type *SEC24*. This was confirmed by Southern blot analysis (our unpublished data). *SEC24* is thus essential for cell viability.

To investigate the phenotype of a strain depleted of Sec24p, we constructed haploid strain RSY875 that has a disrupted chromosomal *SEC24* locus covered by a plasmid-borne copy of *SEC24* expressed from the *GAL1* promoter. When RSY875 cells were grown to early exponential phase in MVCA (galactose) and then transferred to MVCA (glucose) to repress expression of Sec24p, cell growth slowed after 8 h and ceased after ~14 h (Figure 3A).

Using the cells grown in the glucose or galactose medium for 9, 12, and 15 h, we conducted a pulse-chase experiment to monitor the maturation of CPY as an assay for the function of the early part of the secretory pathway. Newly synthesized pro-CPY is translocated into the ER, where it is glycosylated to become the p1 form (67 kDa), and then transported to the Golgi to be modified to the p2 form (69 kDa). Mature CPY (61 kDa) is produced by proteolytic cleavage after pro-CPY enters the vacuole. In RSY875 cells grown on galactose medium, the p1 form of pulse-labeled CPY was processed to the mature form within 60 min of the chase period (Figure 3B, lanes 2, 6, and 10). In contrast, cells that had grown in glucose for 9 h processed only a portion of CPY to the mature form, and after 12 or 15 h of growth in glucose medium most of the CPY remained as the p1 form (Figure 3B, lanes 4, 8, and 12). Thus, depletion of cellular Sec24p causes a block in ER-to-Golgi transport. In the glucose-grown cells, some CPY in a 60-kDa form was detected during the pulse period (Figure 3B, lanes 3, 7, and 11, arrowheads), and this form appeared to be converted to the p1 form after the chase. This 60-kDa form of CPY likely corresponds to prepro-CPY, an ER membrane translocation precursor. An accumulation of prepro-CPY may be a consequence of a prolonged ER-to-Golgi transport defect.

We examined the morphological consequences of Sec24p depletion by electron microscopy of fixed cells. RSY875 was grown in YPGal and then transferred to either YPGal or YPD for 9 h. Cells were fixed with glutaraldehyde followed by 4% $KMnO_4$, a fixation and stain that gives contrast to membrane-derived structures (Kaiser and Schekman, 1990). Cells depleted of Sec24p by growth on glucose showed extensive ER proliferation: long sheets of ER could be seen in the cytoplasm and concentrated at the cell periphery. In a few cases, the ER sheets or tubules formed mesh-like structures (Figure 4A). The cells that contained Sec24p because of growth in galactose appeared to have normal ER morphology (Figure 4B). A control strain with a wild-type copy of *SEC24* on its chromosome showed normal morphology in

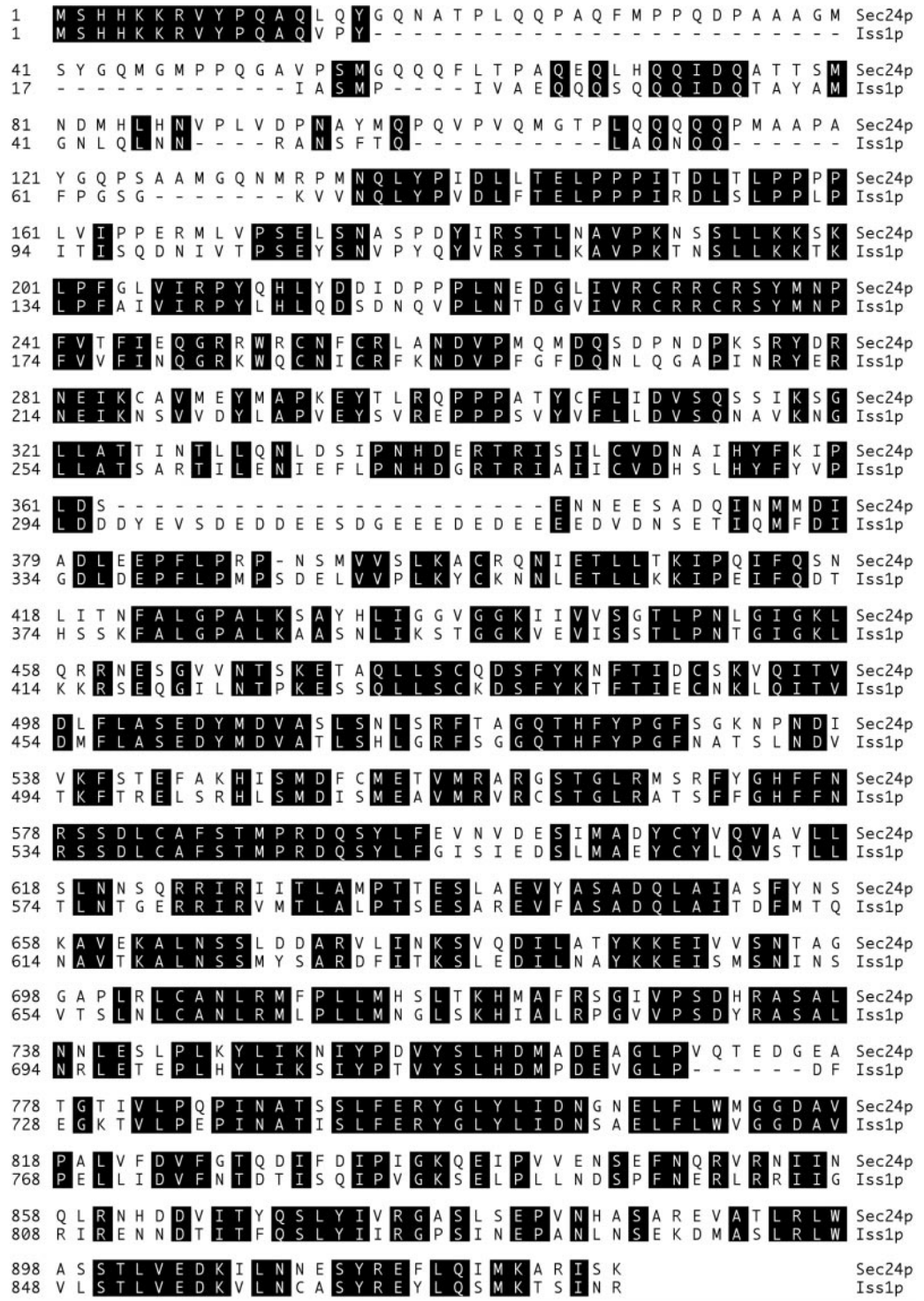

Figure 1. Sequence alignment of Sec24p (YIL109C) with Iss1p (YNL049C). The CLUSTAL method was used for alignment.

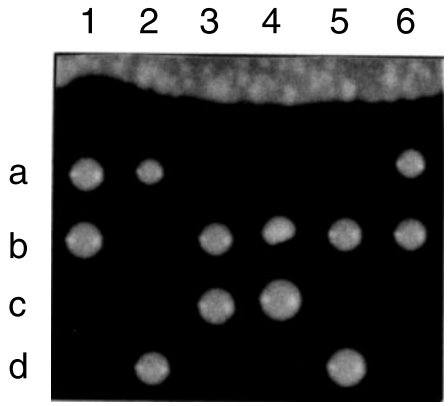

Figure 2. Tetrad analysis of a *SEC24/sec24::LEU2* diploid (RSY866). All viable spores are Leu$^-$.

both glucose and galactose media (our unpublished data). The morphological phenotype of Sec24p depletion is similar to that seen in the class I ER-to-Golgi *sec* mutants such as *sec12* (Kaiser and Schekman, 1990) and is in agreement with the in vitro observation that Sec24p is required for COPII vesicle formation (Hicke *et al.*, 1992).

Genetic Interaction between SEC24 and Other ER-to-Golgi SEC Genes

We next investigated the effect of Sec24p overproduction on the early *sec* mutants. Either pTYY122 (*SEC23* on YEp352 [2μ *URA3*]), pTYY115 (*SEC24* on YEp352), pTYY116 (*SEC23* and *SEC24* on YEp352), or vector (YEp352) was introduced into a *Sec*$^+$ strain as well as into the following *sec* mutants: *sec12-1*, *sec13-1*, *sec16-2*, *sec17-1*, *sec18-1*, *sec19-1*, *sec20-1*, *sec22-3*, *sec23-1*, *sec23-2*, *sec23-3*, and *sec23-4*. The growth of these transformants at 23 and 37°C was compared on plates. Although the temperature-sensitive phenotypes of *sec12-1*, *sec13-1*, *sec17-1*, *sec18-1*, *sec20-1*, *sec22-3*, *sec23-1*, *sec23-3*, and *sec23-4* were not suppressed by these plasmids (our unpublished data), *sec23-2* and *sec16-2* were. Overproduction of Sec24p suppressed *sec23-2* (Figure 5A), and overproduction of both Sec23p and Sec24p in the same cell suppressed *sec16-2* (Figure 5B). These genetic interactions are consistent with the binding interactions among Sec24p, Sec23p, and Sec16p that have been detected in vitro (Hicke *et al.*, 1992; Gimeno *et al.*, 1996).

Overproduction of Sec23p was toxic to both *sec12-1* and *sec13-1* (our unpublished data). Even the wild-type cells could not tolerate the overproduction of Sec23p by a multicopy plasmid with *SEC23* under the control of the *GAL1* promoter (Figure 5C). These phenomena are consistent with the fact that excess Sec23p monomer inhibits vesicle formation in vitro (Yoshihisa *et al.*, 1993). The introduction of *SAR1* on multicopy plasmid only partially mitigated the growth inhibition by overexpression of *SEC23* (our unpublished data).

A temperature-sensitive allele of *SEC24*, *sec24-1*, was isolated from a collection of random temperature-sensitive *S. cerevisiae* mutants by screening for secretion defects at 37°C. The *sec24-1* mutant is unable to grow at temperatures >27°C and accumulates the ER (p1) form of the secretory marker protein CPY (Chitouras, Frand, and Kaiser, unpublished observations). We crossed *sec24-1* to different *sec* mutant strains to test for possible synthetic lethal interactions. Because Sec24p is required for COPII vesicle formation in vitro, we expected to find strong synthetic lethal interactions between *sec24-1* mutations and other vesicle-formation mutations (*sec12-4*, *sec13-1*, *sec16-2*, and *sec23-1*), which was the case (Table 3). In addition, we detected significant synthetic lethal interactions between *sec24-1* and mutations in v-SNARE genes (*sec22-3* and *bet1-1*) and other mutants defective in vesicle fusion (*sec17-1* and *sec18-1*) (Table 3). Such interactions with docking or fusion mutations were not observed for *sec12*, *sec13*, *sec16*, or *sec23* mutations (Kaiser and Schekman, 1990), suggesting that Sec24p may have a unique function among the COPII genes in the packaging of SNARE proteins, or in the docking or fusion of ER-derived vesicles with the Golgi apparatus (Peng *et al.*, 1999).

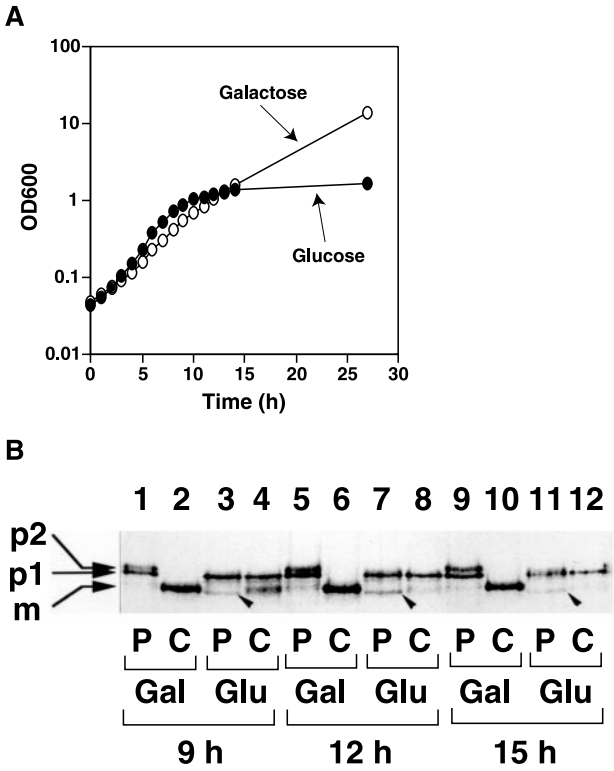

Figure 3. Secretion defects caused by depletion of Sec24p. (A) RSY875, carrying *SEC24* expressed from the *GAL1* promoter, was grown to log phase. After washing, the culture was divided into two portions and transferred to either fresh MVCA (galactose) or MVCA (glucose) at 30°C, and growth was monitored. To keep exponential growth, cultures were diluted 10-fold after 10 h. (B) A culture of RSY875 was transferred to MV-lowS (galactose) (lanes 1, 2, 5, 6, 9, and 10) or MV-lowS (glucose) (lanes 3, 4, 7, 8, 11, and 12). After 9, 12, or 15 h of cultivation at 30°C, 3.0-OD_{600}-unit cells were pulse labeled with [^{35}S]methionine for 10 min (lanes marked with P) and then chased for 60 min with an excess of unlabeled methionine (lanes marked with C). CPY was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. p1, p2, and m represent p1 precursor (67 kDa), p2 precursor (69 kDa), and mature form (61 kDa) of CPY, respectively. Bands marked with arrowheads (60 kDa) may represent untranslocated precursors.

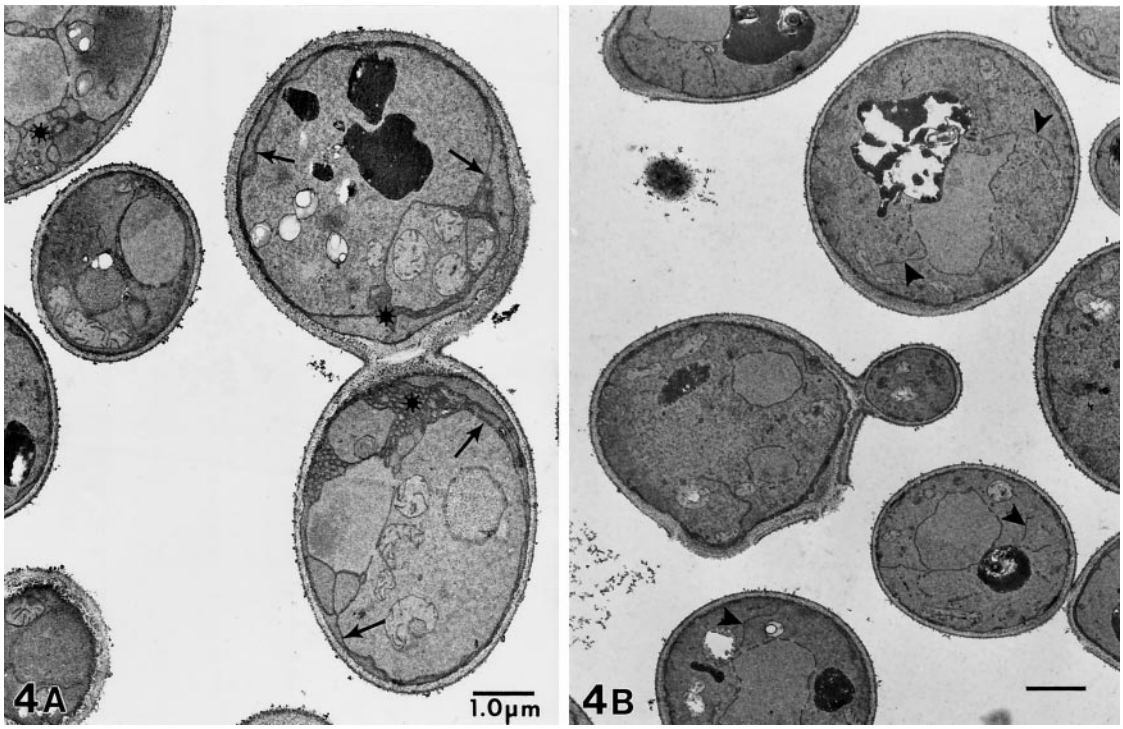

Figure 4. Electron microscopy of RSY875 cells grown for 9 h in YPGlu (A) or YPGal (B). (A) Glucose-grown Sec24p-depleted cells show amplification of ER membranes (arrows) and occasional tubules or meshwork structures (asterisks). (B) Cells in YPGal have normal ER morphology (arrowheads). Bars, 1 μm.

ISS1 Is Not Essential for Growth or Transport of CPY and Gas1p

Next, we examined the function of the *SEC24* homologue *ISS1*. We used *HIS3* to disrupt one copy of the *ISS1* gene in a diploid strain. On sporulation, the heterozygous diploid yielded four spores from most tetrads. Two of the spores were His^+, and the other two were His^-. The presence of the *iss1-Δ1::HIS3* disruption in the His^+ strain was verified by

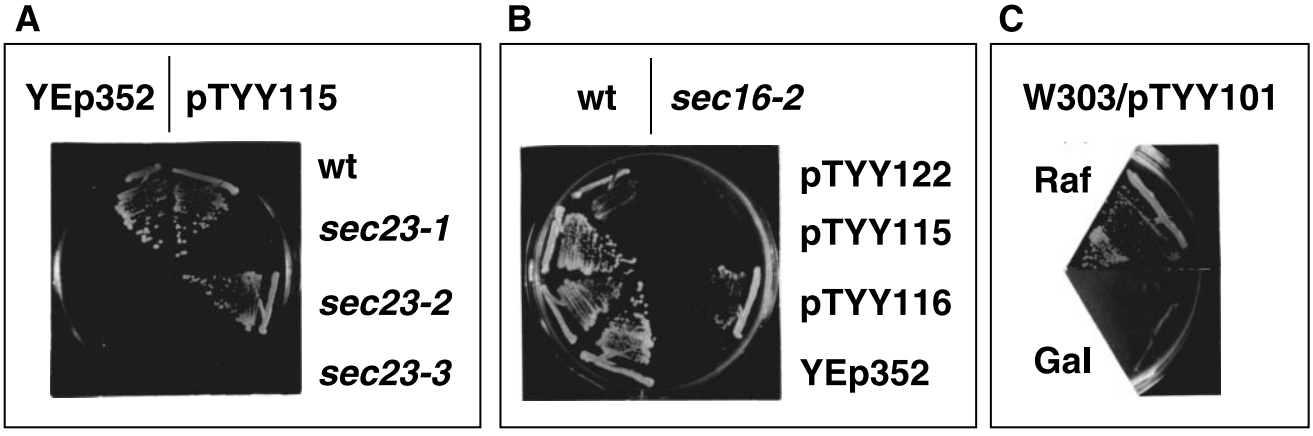

Figure 5. Effect of Sec24p and/or Sec23p overproduction on yeast growth. (A) The growth of wild-type (wt), *sec23-1*, *sec23-2*, and *sec23-3* strains with either pTYY115 (2 μm *SEC24*) or YEp352 (2 μm without *SEC24*) at 37°C. pTYY115 suppressed temperature-sensitive *sec23-2*. (B) Wild-type and *sec16-2* strains with either pTYY122 (2 μm *SEC23*), pTYY115 (2 μm *SEC24*), pTYY116 (2 μm *SEC23 SEC24*), or YEp352 were streaked on a MVCA (glucose) plate and incubated at 37°C for 3 d. Only pTYY116 suppressed *sec16-2*. (C) RSY612 (pTYY101 [2 μm *Gal1p–SEC23*]) precultured on MVCA (raffinose) was streaked on either MVCA (raffinose; Raf) or MVCA (galactose; Gal). This strain did not grow on galactose plates, whereas RSY612 with vector plasmid pCG109 grew on both raffinose and galactose plates (our unpublished data).

Table 3. Genetic interactions of *iss1* and *sec24* mutants

Genotype	Incubation temperature		
	28°C	30°C	33°C
sec24-1	++	−	−
sec24-1 iss1-Δ1::TRP1	++	−	−
SNAREs			
sec22-3	++	±	−
sec22-3 iss1-Δ1::TRP1	Double mutants not viable at 25°C		
sec22-3 sec24-1	Double mutants not viable at 25°C		
bet1-1	+++	+++	+++
bet1-1 iss1-Δ1::TRP1	+++	−	−
bet1-1 sec24-1	Double mutants not viable at 25°C		
Vesicle fusion			
sec17-1	+++	++	±
sec17-1 iss1-Δ1::TRP1	+++	++	±
sec17-1 sec24-1	−	−	−
sec18-1	+++	−	−
sec18-1 iss1-Δ1::TRP1	+++	−	−
sec18-1 sec24-1	Double mutants not viable at 25°C		
Vesicle formation			
sec12-4	+++	−	−
sec12-4 iss1-Δ1::TRP1	+++	−	−
sec12-4 sec24-1	Double mutants not viable at 25°C		
sec13-1	+++	+	−
sec13-1 iss1-Δ1::TRP1	+++	+	−
sec13-1 sec24-1	Double mutants not viable at 25°C		
sec16-2	++	−	−
sec16-2 iss1-Δ1::TRP1	++	−	−
sec16-2 sec24-1	Double mutants not viable at 25°C		
sec23-1	+++	−	−
sec23-1 iss1-Δ1::TRP1	+++	−	−
sec23-1 sec24-1	Double mutants not viable at 25°C		

Growth of single colonies on YPD after 24–48 h. +++, growth comparable to wild type; −, no growth.

PCR with the use of primers to the 5′-noncoding region of *ISS1* and *HIS3* coding sequences. The *iss1-Δ1::HIS3* haploids grew normally at 24, 30, and 37°C. Therefore, *ISS1* is not essential for cell growth.

The rate of maturation of CPY and Gas1p in the *iss1-Δ1::HIS3* strain was examined by a pulse-chase experiment. Gas1p is first modified in the ER to form a 105-kDa GPI-anchored precursor with *N*- and *O*-linked core oligosaccharides. Then it is modified to a 125-kDa mature form in the Golgi and delivered to the plasma membrane. After a 7-min pulse, TKY10 (*ISS1*) and TKY12 (*iss1-Δ1::HIS3*) were chased for up to 60 min. The maturation rate of CPY in TKY12 was almost the same as that in TKY10 (Figure 6A). Gas1p maturation was slightly slower in TKY12 than in TKY10, but the effect was moderate (Figure 6B). These data indicate that *ISS1* is not essential for the transport of these proteins.

Suppression of SEC24 Mutations by ISS1

Because Iss1p is similar to Sec24p in both sequence and protein–protein interactions, we tested whether Iss1p could substitute for Sec24p during ER-to-Golgi transport. An initial indication of functional overlap came from the observation that the expression of *ISS1* from a high-copy-number (2μ) plasmid could restore growth of *sec24-1* at temperatures up to 36°C (our unpublished data). We further tested the ability of *ISS1* overexpression to suppress a chromosomal deletion of *SEC24*. RSY866, a diploid strain heterozygous for a *SEC24* gene disruption (*sec24::LEU2*), was transformed with pTKY4 (2μ plasmid with *ISS1* gene). Sporulation of this diploid often yielded tetrads with four viable spores. In the tetrads with four viable spores, two of the spore clones were Leu^+ and the other two were Leu^-. The absence of the *SEC24* gene in Leu^+ spore clones was confirmed by Western blot analysis for Sec24p. Thus, *sec24::LEU2* can be suppressed by increased dosage of the *ISS1* gene. We could not obtain a *sec24::LEU2* haploid strain with the use of pRH200 (*CEN ISS1*) instead of pTKY4 (2 μm *ISS1*) (our unpublished data).

We also found that *ISS1* tagged with a His_6-encoding sequence can suppress *SEC24* disruption (Figure 7A), indicating that the His_6-tagged version of Iss1p is functional in vivo. Immunoblot analysis showed that all four strains derived from one tetrad produced Iss1p-His_6 as well as Sec23p and that Sec24p was absent from the two Leu^+ spores (Figure 7B). A strain with the chromosomal disruption

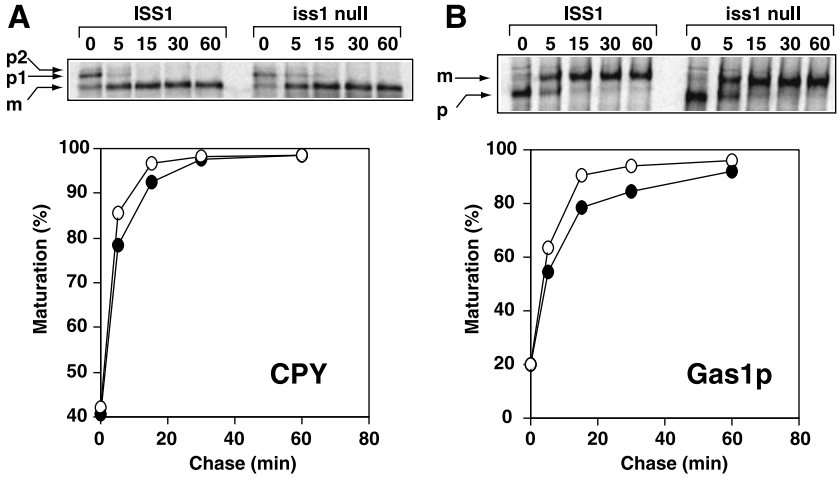

Figure 6. Effect of *ISS1* deletion on maturation of CPY (A) and Gas1p (B). After pulse-chase analysis of CPY or Gas1p, the intensities of radioactive bands were quantified with a PhosphorImager (Molecular Dynamics). Maturation was calculated as follows: CPY maturation (%) = m/(p1 + m) × 100; Gas1p maturation (%) = m/(p + m) × 100, where p indicates precursor and m indicates mature form. (○) *ISS1* strain; (●) *iss1* null strain.

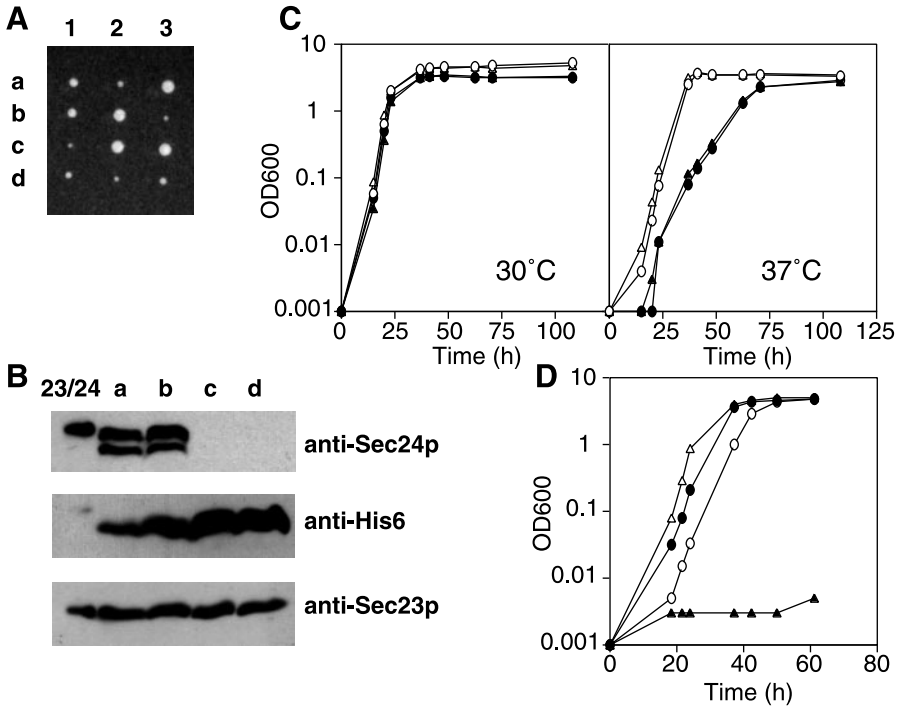

Figure 7. Complementation of *sec24* disruption by *ISS1*. (A) Tetrad analysis of RSY866 (*SEC24/sec24::LEU2* [pTKY6 (2 μm His_6-tagged version of *ISS1*)]). Four viable spores were obtained. Two smaller colonies in each set grew on SC-Leu, indicating the disruption of *SEC24* (our unpublished data). (B) Western blot analysis of TKY5 (*MATa SEC24* [pTKY6]) (a), TKY6 (*MAT*α *SEC24* [pTKY6]) (b), TKY7 (*MATa sec24:: LEU2* [pTKY6]) (c), and TKY8 (*MAT*α *sec24:: LEU2* [pTKY6]) (d). Sec23p/Sec24p-His_6 (0.15 μg) (Yeung *et al.*, 1995) was loaded onto the lane indicated by 23/24. The cells were grown in SC-Ura to stationary phase and disrupted with glass beads in HSLB containing protease inhibitors and 2-mercaptoethanol. After centrifugation at 14,000 $\times$ *g* for 10 min, protein in the supernatant was concentrated by TCA precipitation. The precipitated protein (100 μg) was analyzed by immunoblotting with the use of either anti-Sec24p, anti-His_6, or anti-Sec23p antibody. The slower mobility of Sec24p in lane 23/24 is probably due to the presence of His_6 tag, which was attached to Sec24p in the cells analyzed here. In lanes a and b, a degradation product of Sec24p is also seen. (C) Growth curve of TKY5, TKY6, TKY7, and TKY8 in SC-Ura at 30 and 37°C. (○) TKY5; (△) TKY6; (●) TKY7; (▲) TKY8. (D) TKY22 (*sec24:: LEU2* [pTKY11 (*Gal1p–ISS1*)]) and TKY23 (*SEC24* [pTKY11 (*Gal1p–ISS1*)]) were cultured in SC-Ura (2% galactose) and then transferred to either SC-Ura (2% galactose) or SC-Ura (2% glucose). Growth at 30°C was monitored. (○) TKY23 in galactose medium; (△) TKY23 in glucose medium; (●) TKY22 in galactose medium; (▲) TKY22 in glucose medium.

sec24::LEU2 suppressed by pTKY4 (2 μm *ISS1*) grew more slowly than the wild-type strain at 37°C, but the growth was indistinguishable from that of the wild-type strain at 24 and 30°C (Figure 7C).

The apparent indispensability of Iss1p overexpression in cells with the chromosomal disruption *sec24::LEU2* was confirmed by a galactose shut-off experiment. TKY22 (*sec24::LEU2* [pTKY11 (*GAL1p–ISS1*)]) grew well when Iss1p expression was induced by growth in the galactose medium, but the growth of this strain was severely inhibited when Iss1p expression was repressed by growth on glucose medium (Figure 7D).

We next examined whether protein secretion was fully restored in strains with the chromosomal disruption *sec24::LEU2* suppressed by pTKY4 (2 μm *ISS1*). A convenient test for secretion of α-factor is to assay the extent of growth inhibition of a *MAT***a** *sst2*Δ strain by a halo assay. Clear halos of equal diameter formed around both a *MAT*α *sec24::LEU2* (pTKY4 [2μ *ISS1*]) strain and the *MAT*α control strain, showing that the secretion of α-factor was normal (Figure 8A).

We also examined the maturation kinetics of CPY and Gas1p in the *sec24::LEU2* (pTKY4 [2μ *ISS1*]) strain by a pulse-chase experiment. Maturation of CPY occurred in the *sec24::LEU2* (pTKY4 [2μ *ISS1*]) cells at almost the same rate as in *SEC24* control cells (Figure 8B). Maturation of Gas1p occurred more slowly, but after a 60-min chase, >90% of Gas1p was converted to the mature form (Figure 8B). These data indicate that transport of CPY and Gas1p from the ER to the Golgi can be achieved in the Sec24p-depleted cells if there is sufficient Iss1p.

Genetic Interactions between ISS1 and v-SNARE Mutants

To learn more about the function of Iss1p in the secretory pathway, we tested for synthetic lethal interactions between *iss1-*Δ*2::TRP1* and a panel of secretion mutants (Table 3). Synthetic lethal interactions have been helpful for detecting the step in the secretory pathway at which a gene product acts, because synthetic lethal interactions usually occur only between two genes that affect the same step of the pathway (Newman *et al.*, 1987; Salminen and Novick, 1987; Rothblatt *et al.*, 1989; Kaiser and Schekman, 1990; Gimeno *et al.*, 1996). Surprisingly, *iss1-*Δ*2::TRP1* did not affect mutants defective in vesicle formation (*sec12-4*, *sec13-1*, *sec16-2*, and *sec23-1*) or vesicle fusion (*sec17-1* and *sec18-1*) but showed strong synthetic lethal interactions with mutants defective in v-SNARE proteins required for ER-to-Golgi transport (*sec22-3* and *bet1-1*) (Table 3).

We also examined the corresponding effects of *ISS1* overexpression on v-SNARE mutations. *ISS1* on either a low- or high-copy-number plasmid was transformed into a variety of *sec* and *bet* mutant strains, and the transformants were tested for their temperature sensitivity. *ISS1* overexpression suppressed the growth defect of both *sec22* alleles tested (Figure 8C) but had no effect on the growth of any of the other mutants, with the exception of *sec24-1*. The ability of *ISS1* overexpression to suppress *sec22* is consistent with the synthetic lethality of *iss1 sec22* double mutants. Thus, both *ISS1* and *SEC24* are implicated in vesicle docking or fusion by their genetic interactions.

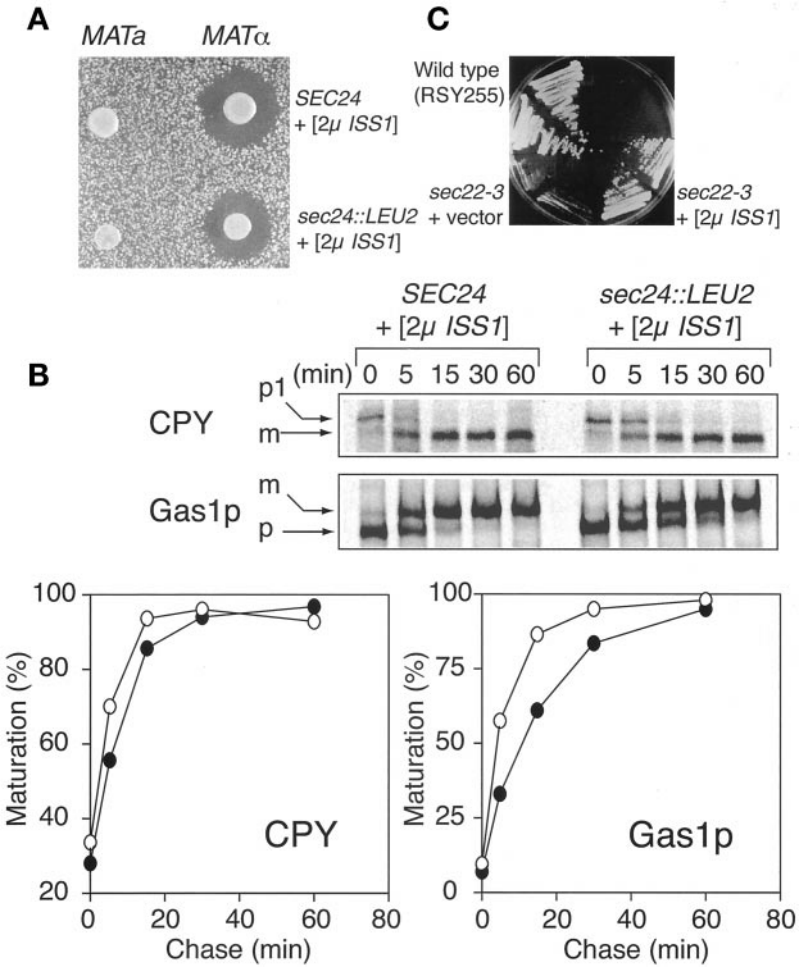

Figure 8. *ISS1* overexpression suppresses *sec24::LEU2 and sec22-3*. (A) Halo assay for α-factor secretion comparing *MATα* control strain TKY6 (*MATα SEC24* [pTKY6 (2μ *ISS1*)]) with TKY8 (*MATα sec24::LEU2* [pTY6 (2μ *ISS1*)]). (B) Pulse-chase analysis of CPY and Gas1p transport in TKY6 (*SEC24* [pTKY6 (2μ *ISS1*)]) and TKY8 (*sec24::LEU2* [pTKY6 (2μ *ISS1*)]). Cells were labeled for 7 min and chased for the times indicated. Maturation was calculated as for Figure 6. (○) TKY6; (●) TKY8. (C) Suppression of *sec22-3* by 2μ *ISS1*. CKY70 (*sec22-3*) carrying either *ISS1* on a 2μ plasmid or a control plasmid was grown for 3 d at 30°C. Duplicate transformants are shown.

Purification of Iss1p as a Complex with Sec23p

Iss1p was purified from RSY620 harboring pTKY7, which expresses a His_6-tagged version of the *ISS1* gene under the control of the *GAL1* promoter, and pTKY9, which expresses *SEC23* under the control of the *GAL1* promoter. A high-speed supernatant fraction prepared from the lysate was loaded onto a Ni-NTA agarose column and eluted with a stepwise gradient of imidazole-containing buffers. The peak fractions, eluted with 500 mM imidazole, contained two proteins (Figure 9, lane 6), one of which (100 kDa) was identified as His_6-tagged Iss1p and the other of which (85 kDa) was identified as Sec23p by Western blotting with either His_6 antibody or Sec23p antibody (our unpublished data). The coelution of Iss1p with Sec23p indicated that these proteins assemble into a complex (when a control extract from cells expressing Iss1p without the His_6 tag was used, Sec23p did not bind to the column). An extract from 25 g (wet weight) of cells yielded 1.5 mg of the Sec23p/Iss1p complex. We found that glycerol was necessary to stabilize purified Sec23p/Iss1p; in buffers that lacked glycerol, the purified complex lost ~80% of its activity in 5 d at 4°C, whereas >80% of its activity remained under the same conditions in the presence of 20% glycerol.

Sec23p/Iss1p Drives Formation of COPII-like Vesicles from the ER

The α-factor halo assay (Figure 8A) with *sec24*-disrupted cells suggested that Iss1p could substitute for Sec24p in α-factor trafficking in vivo and suggested that Iss1p might also substitute for Sec24p in the incorporation of gpαF into COPII vesicles in vitro (Barlowe *et al.*, 1994; Bednarek *et al.*, 1995). We examined this activity with the use of purified Sec23p/Iss1p and found that 20–25% of gpαF was captured in vesicles budded from ER membranes incubated with Sar1p, Sec13p/Sec31p, and Sec23p/Iss1p (without Sec23p/Sec24p) (Figure 10). The packaging of gpαF into vesicles required added GTP or GMP-PNP; in the absence of these nucleotides, <5% of gpαF was released into the vesicle fraction (our unpublished data). The amount of gpαF packaged increased with increasing concentrations of Sec23p/Iss1p (Figure 10A) and increasing incubation times (Figure 10B). Together, these results indicate that Sec23p/Iss1p, like Sec23p/Sec24p, drives the formation of gpαF-containing vesicles from the ER.

To compare the cargo molecules in the vesicles produced with Sec24p and Iss1p, we carried out the budding reaction

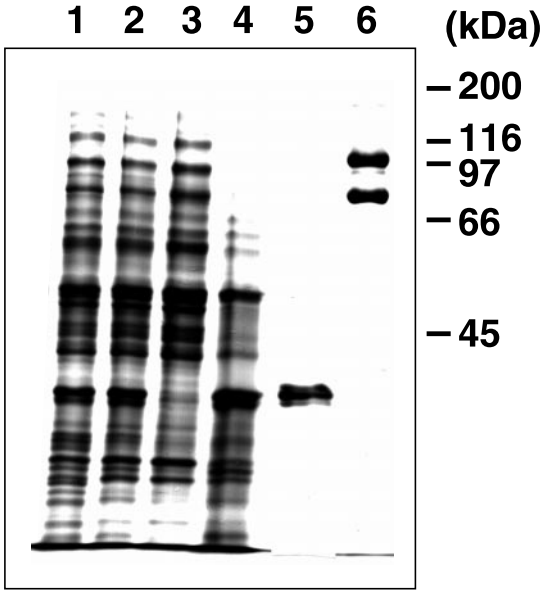

Figure 9. Purification of Sec23p/Iss1p from RSY620 (pTKY7 [2 μm *GAL1p–ISS1* (His6-tagged)], pTKY9 [2μ *GAL1p–SEC23*]). Proteins in the cell extract and eluted from the Ni-NTA agarose column were separated on SDS-PAGE (10%) and stained with Coomassie brilliant blue R-250. Lane 1, cell lysate (5 μg); lane 2, high-speed supernatant (5 μg); lane 3, flow through from the column (5 μg); lane 4, B-II eluate (5 μg); lane 5, B-III eluate (2 μg); lane 6, B-IV (500 mM imidazole) eluate (1 μg).

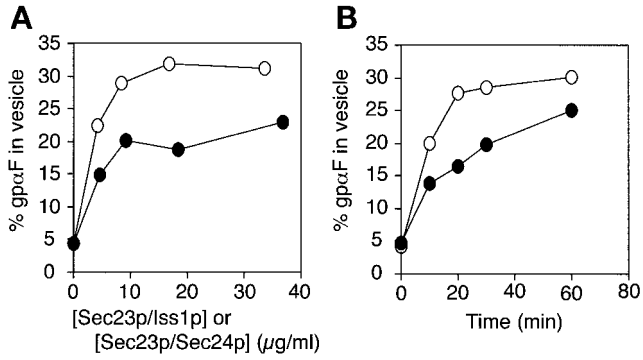

Figure 10. Packaging of gpαF precursor into vesicles produced from microsomes. (A) Microsomes (containing 23 μg of proteins) were incubated for 30 min at 20°C with 64 μg/ml Sar1p, 144 μg/ml Sec13p/Sec31p, and indicated concentrations of either Sec23p/Iss1p (●) or Sec23p/Sec24p (○) in the presence of 0.1 mM GMP-PNP in 50 μl of B88. The percentage of gpαF packaged into the vesicles was determined as described in MATERIALS AND METHODS. (B) Experiment carried out under the same conditions as described for A except that the fixed concentration of Sec23p/Iss1p (9.2 μg/ml) or Sec23p/Sec24p (8.4 μg/ml) was used and that the reaction was conducted for the times indicated. (○) Reaction with Sec23p/Sec24p; (●) reaction with Sec23p/Iss1p.

on a large scale in the presence of Sec23p complexes containing Sec24p or Iss1p and isolated the vesicles on a sucrose density gradient. As shown in Figure 11A, Sec23p was present in the fractions whose sucrose concentration was ~38% when the budding reaction was carried out in the presence of Sec23p/Iss1p or Sec23p/Sec24p. Sec23p was not found in these fractions when the budding reaction was carried out in the absence of GMP-PNP (Figure 11A) or without Sec23p/Iss1p and Sec23p/Sec24p (our unpublished data). Thus, Sec23p/Iss1p produces vesicles from the ER in the presence of Sar1p, Sec13p/Sec31p, and GMP-PNP, and the densities of these vesicles are similar to those of regular COPII vesicles produced by Sec23p/Sec24p. Like COPII vesicles, the vesicles produced with Iss1p contained SNARE molecules (Sec22p and Bet1p) and did not contain the ER-resident protein Sec61p (Figure 11B). Silver staining of the proteins showed that the vesicles produced with Iss1p contained Iss1p (100-kDa band) as well as Sec31p, Sec23p, Sec13p, and Sar1p. The vesicles produced with Sec24p contained Sec24p (105-kDa band) instead of Iss1p. Other abundant proteins in the Iss1p-coated vesicles were indistinguishable from those in the Sec24p-coated regular COPII vesicles (Figure 11C).

DISCUSSION

Sec24p Plays an Essential Role in COPII Vesicle Formation In Vivo

Here we have shown that Sec24p is indispensable for protein transport from the ER in vivo; deletion of the *SEC24* gene is lethal, and depletion of Sec24p causes exaggeration of the ER as well as a defect in CPY exit from the ER (Figures 2–4). A homology search revealed that humans, *C. elegans*, and *A. thaliana* have *SEC24* homologues. Thus, Sec24p probably performs a similar role in vesicle formation from the ER in all eukaryotes.

Gimeno *et al.* (1996) showed that Sec24p and Sec23p bind to adjacent regions of Sec16p: Sec23p binds to a C-terminal region and Sec24p binds to a more central region of Sec16p. The finding that the temperature sensitivity of *sec16-2* is suppressed by the combined overproduction of Sec24p and Sec23p suggests that interaction between these proteins is important for budding events in vivo, although the requirement of Sec16p is not absolute in vitro (Matsuoka *et al.*, 1998). It is also known that Sec31p interacts with Sec24p (Shaywitz *et al.*, 1997). Therefore, it is probable that Sec24p serves as an essential structural unit of coat structure.

GTP hydrolysis by Sar1p renders the COPII coat unstable (Barlowe *et al.*, 1994), and the GTPase activity of Sar1p is activated by Sec23p (Yoshihisa *et al.*, 1993). Accordingly, the timing of GTP hydrolysis by Sar1p as activated by Sec23p may be regulated in some way to prevent premature release of the coat subunits before budding is complete. It is interesting to speculate that Sec24p may participate in the regulation of Sar1p GTPase activation. Although previous experiments indicated that Sec24p does not affect the GTPase-activation activity of Sec23p in solution (Yoshihisa *et al.*, 1993), in the context of an intact coat, e.g., one that includes Sec16p (Espenshade *et al.*, 1995), Sec24p may impede the interaction of Sec23p with Sar1p until vesicle fission has been completed. Because overproduction of Sec23p impairs cell growth (Figure 5C) and excess Sec23p inhibits the budding reaction in vitro (Yoshihisa *et al.*, 1993), it may be that an appropriate balance of Sec24p and Sec23p is important for efficient budding.

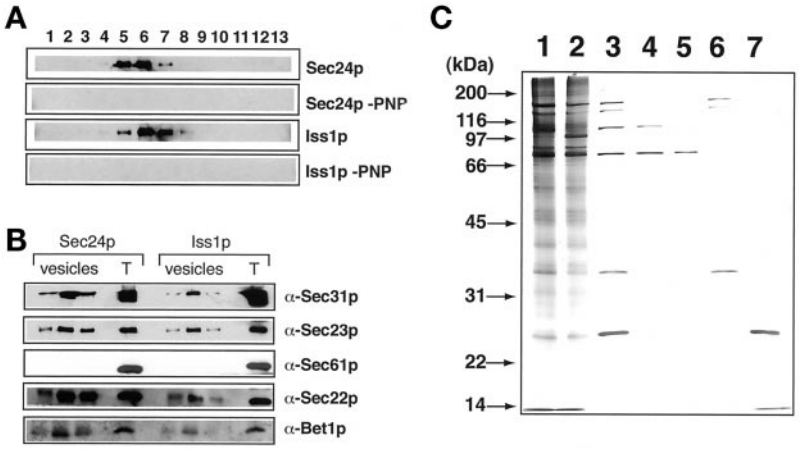

Figure 11. Characterization of COPII-like vesicles produced with Iss1p. (A) Budding reactions were carried out in the presence of Sec23p/Sec24p or Sec23p/Iss1p with or without GMP-PNP as described in MATERIALS AND METHODS. Vesicles (0.55 ml) obtained from each budding reaction were subjected to flotation in a sucrose density gradient (55–25% stepwise gradient). Proteins in each fraction were precipitated, and one-fifth of each precipitate was analyzed by immunoblotting with Sec23p antiserum. Sucrose concentrations of the fractions containing Sec23p were as follows. Fractions obtained with Sec24p: fraction 5, 36.1%; fraction 6, 38.4%; fraction 7, 40.5%. Fractions obtained with Iss1p: fraction 5, 36.0%; fraction 6, 38.2%; fraction 7, 40.3%. Sec24p-PNP and Iss1p-PNP indicate results obtained in the absence of GMP-PNP. (B) Vesicle fractions were obtained by flotation in a sucrose density gradient. Three successive fractions constituting the Sec23p peak were analyzed by immunoblotting of antisera specific for Sec31p, Sec61p, Sec22p, and Bet1p. Fractions were prepared as described for A. Lane T contains total budding reaction mixture (1 μl). (C) Silver staining of the vesicle fractions obtained with Sec24p (lane 1) and Iss1p (lane 2). Lane 3, COPII mix (50 ng of Sar1p, 50 ng of Sec23p/Sec24p, and 50 ng of Sec13p/Sec31p); lane 4, Sec23p/Sec24p (50 ng); lane 5, Sec23p/Iss1p (50 ng); lane 6, Sec13p/Sec31p (50 ng); lane 7, Sar1p (50 ng).

Iss1p Replaces the Essential Role of Sec24p

The *S. cerevisiae* genomic sequence revealed a gene (*ISS1*) with striking similarity to *SEC24* (Figure 1). We found that deletion of *ISS1* does not have a significant effect on yeast growth or the maturation kinetics of CPY and Gas1p (Figure 6). We found that on overexpression, Iss1p can fulfill the essential function of Sec24p (Figure 7). Strains with a chromosomal disruption of *SEC24* that were suppressed by overexpression of *ISS1* grew slowly at 37°C, indicating that Iss1p itself or interactions between Iss1p and other proteins may be unstable at high temperature. Because disruption of *SEC24* in an otherwise wild-type strain is lethal, the endogenous level of Iss1p expressed from the chromosomal gene must not be sufficient to carry out the essential function of Sec24p (Figures 2 and 3).

Genetic Interactions of SEC24 and ISS1

Tests for genetic suppression and synthetic lethality revealed a possible connection between the functions of *SEC24* and *ISS1* and SNARE proteins involved in COPII vesicle function. First, the loss-of-function mutants *iss1* and *sec24* were found to exacerbate mutations in the SNARE genes *SEC22* and *BET1* (Table 3). Second, overexpression of *ISS1* partially suppressed mutations in *SEC22* (Figure 8C). These genetic interactions of *sec24* mutants with vesicle-docking mutants were particularly surprising, because none of the other mutants in COPII components shows interactions with vesicle-docking mutants (Kaiser and Schekman, 1990).

Four mechanisms can be postulated by which Sec24p and Iss1p could affect vesicle docking or fusion. First, Sec24p and Iss1p may be required for loading of the v-SNAREs Sec22p, Bet1p, and, possibly, Bos1p into vesicles during vesicle formation. Consistent with this idea is the observation that Bet1p and Bos1p are concentrated into prebudding complexes by the COPII proteins Sar1p, Sec23p, and Sec24p (Springer and Schekman 1998). It is conceivable that Sec24p and Iss1p similarly direct incorporation of docking factors into COPII-coated vesicles by binding to their cytosolic domains. To date, attempts to coprecipitate v-SNARE proteins with either Sec24p or Iss1p have been unsuccessful. A second possibility is that Sec24p and Iss1p may be required for the formation of retrograde transport vesicles that recycle integral membrane docking factors to the ER. If formation of these vesicles is blocked, Sec22p and other docking factors will be depleted from the ER, ultimately causing a defect in vesicle docking. Interestingly, mutants in coatomer components that block recycling (*sec21*, *sec26*, and *sec27*) have multiple genetic interactions with docking mutants similar to *iss1* and *sec24* mutants (Newman and Ferro-Novick, 1987; Duden *et al.*, 1994). If Iss1p and Sec24p in fact participate in retrograde transport, it would be expected that mutants in these proteins cause missorting of recycled cargo proteins. However, we have been unable to detect a sorting defect in *iss1* mutants. In particular, deletion of *iss1* does not cause missorting of Kar2p, an ER protein that undergoes recycling, and does not affect the recycling of a KKXX-containing fusion protein (our unpublished data). A third possibility is that Sec24p and Iss1p participate in the fusion reaction itself. Recently, Peng *et al.* (1999) detected strong and specific interaction between Sec24p and Sed5p, a t-SNARE that marks the docking site for COPII vesicles targeted to the *cis*-Golgi compartment. It is possible that Sec24p and Iss1p participate in the formation of complexes between v-SNAREs and t-SNAREs. Finally, a role for Sec24p and Iss1p in vesicle docking or fusion could reflect a requirement for these proteins in regulated disassembly of the COPII coat, possibly stimulating GTP hydrolysis on Sar1p. More detailed biochemical studies will be required to resolve these possibilities.

Cargo Recruitment by Sec24p-coated Vesicles and Iss1p-coated Vesicles

Recently, it was shown that Sar1p and Sec23p/Sec24p cooperate to recruit cargo molecules into ER-derived vesicles (Aridor *et al.*, 1998; Kuehn *et al.*, 1998; Springer and Schekman, 1998). This suggests a direct interaction between these

coat proteins and cargo molecules or adaptor molecules that bind to the cargo molecules. Because Iss1p and Sec24p differ, we considered the possibility that Sec23p/Iss1p serves to recruit a subset of cargo molecules different from those recruited by Sec23p/Sec24p. We found that purified Sec23p/Iss1p replaces Sec23p/Sec24p to drive vesicle formation from the ER in vitro (Figures 10 and 11). However, we did not see a clear difference in cargo composition between Sec24p- and Iss1p-coated vesicles (Figures 10 and 11). Therefore, at least the abundant cargo proteins as well as α-factor and SNAREs (Sec22p and Bet1p) can be recruited into the vesicles by both Sec24p and Iss1p in collaboration with the other COPII components. We cannot exclude the possibility that Iss1p recruits a different subset of cargo molecules whose amounts are too small to be detected by silver staining and that are not essential to cell viability under the conditions we used.

Sec24p and Iss1p may display quantitative rather than qualitative differences in the recognition of cargo molecules. In the titration experiments and kinetic studies measuring the packaging of α-factor precursor, we found that purified Sec23p/Iss1p was consistently less active than Sec23p/Sec24p (Figure 10). Of course, Sec23p/Iss1p may simply be inherently less active than Sec23p/Sec24p as a coat promoter. However, in *sec24* null mutant cells suppressed by overexpression of *ISS1*, we found a measurable difference in the rates of maturation of CPY and Gas1p compared with wild-type cells (Figure 8). This result is most consistent with qualitative differences in cargo or cargo receptor recognition by Iss1p and Sec24p.

Pagano *et al.* (1999) and Roberg *et al.* (1999) reported the cloning and characterization of a more distant (23% identical) homologue of Sec24p. Pagano *et al.* found that this homologue (which they call Sec24C), when deleted, reduces the secretion of a subset of prominent proteins detected in the cell culture supernatant. Pagano *et al.* also report the cloning and deletion of *ISS1* (which they call Sec24B). Deletion of this gene has no effect on the spectrum of proteins secreted into the culture supernatant. Roberg *et al.* (1999) discovered *SEC24C* by a screen for mutations lethal in a *sec13-1* mutant strain. They call this gene *LST1* and demonstrate that *lst1* null cells are sensitive to low pH because of a deficit in transport of the major plasma membrane ATPase, Pma1p, out of the ER. Roberg *et al.* document a complex of Lst1p and Sec23p and suggest that this dimer may perform a specialized function in the packaging of Pma1p into COPII vesicles.

Together, our data and the recent reports suggest that Sec24p and its homologues may define the selectivity of cargo protein sorting. The signal that interacts with Sec24p, Iss1p (Sec24B), and Lst1p (Sec24C), and the binding pocket within these coat subunits, remain to be defined.

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