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Abstract. *SEC35* was identified in a novel screen for temperature-sensitive mutants in the secretory pathway of the yeast *Saccharomyces cerevisiae* (Wuestehube et al., 1996. *Genetics*. 142:393–406). At the restrictive temperature, the *sec35-1* strain exhibits a transport block between the ER and the Golgi apparatus and accumulates numerous vesicles. *SEC35* encodes a novel cytosolic protein of 32 kD, peripherally associated with membranes. The temperature-sensitive phenotype of *sec35-1* is efficiently suppressed by *YPT1*, which encodes the rab-like GTPase required early in the secretory pathway, or by *SLY1-20*, which encodes a dominant form of the ER to Golgi target-SNARE–associated protein Sly1p. Weaker suppression is evident upon overexpression of genes encoding the vesicle-SNAREs *SEC22*, *BET1*, or *YKT6*. The cold-sensitive lethality that results from deleting *SEC35* is suppressed by *YPT1* or *SLY1-20*. These genetic relationships suggest that Sec35p acts upstream of, or in conjunction with, Ypt1p and Sly1p as was previously found for Uso1p. Using a cell-free assay that measures distinct steps in vesicle transport from the ER to the Golgi, we find Sec35p is required for a vesicle docking stage catalyzed by Uso1p. These genetic and biochemical results suggest Sec35p acts with Uso1p to dock ER-derived vesicles to the Golgi complex.

PROTEIN transport through the secretory pathway occurs via transport vesicles under the direction of a large set of protein components (Rothman, 1994). The process can be divided into three stages: (*a*) vesicle budding, (*b*) vesicle docking, and (*c*) membrane fusion, with distinct sets of proteins mediating each phase. The budding step involves recruitment of coat proteins to the membrane and culminates with the release of coated vesicles (Schekman and Orci, 1996). The docking reaction is likely to require a set of integral membrane proteins on the vesicle and target membranes, termed v-SNAREs[1] and t-SNAREs (vesicle- and target membrane-soluble *N*-ethylmaleimide–sensitive fusion protein [NSF] attachment protein [SNAP] receptors, respectively), that are thought to confer specificity through their pair-wise interactions (Söllner et al., 1993*b*). Small GTP-binding proteins of the rab family also assist in the docking process (Ferro-Novick and Novick, 1993), but their precise function is not known. The fusion step ensues after docking and results in the delivery of the vesicular cargo to the next compartment in the secretory pathway.

Vesicular transport from the ER to the Golgi apparatus in the yeast *Saccharomyces cerevisiae* has been extensively characterized. Transport vesicle budding involves the assembly of the COPII coat, composed of the Sec13p/Sec31p (Pryer et al., 1993; Salama et al., 1993; Barlowe et al., 1994) and Sec23p/Sec24p heterodimers (Hicke and Schekman, 1989; Hicke et al., 1992), under the direction of an integral membrane protein, Sec12p (Nakano et al., 1988; Barlowe and Schekman, 1993), a small GTPase, Sar1p (Nakano and Muramatsu, 1989), and a multidomain protein, Sec16p (Espenshade et al., 1995; Shaywitz et al., 1997). Docking is thought to require a tethering event mediated by Uso1p (Cao et al., 1998), the yeast homologue of mammalian p115 (Barroso et al., 1995; Sapperstein et al., 1995), followed by or concurrent with the interaction of a set of ER to Golgi v-SNAREs, Bet1p, Bos1p, Sec22p (Newman and Ferro-Novick, 1987; Newman et al., 1990; Ossig et al., 1991; Shim et al., 1991; Søgaard et al., 1994) and perhaps

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1. *Abbreviations used in this paper*: CEN, centromere; COP, coat protein; CPY, carboxypeptidase Y; gp-α-factor, glycosylated pro-α-factor; GST, glutathione-S-transferase; NSF, *N*-ethylmaleimide–sensitive fusion protein; ORF, open reading frame; PGK, phosphoglycerate kinase; SC, synthetic complete medium; SNAP, soluble NSF–attachment protein; t-SNARE, target membrane–SNAP receptor; v-SNARE, vesicle-SNAP receptor; YPD, rich medium.

Ykt6p (Søgaard et al., 1994; McNew et al., 1997), with the cognate t-SNARE on the Golgi, Sed5p (Hardwick and Pelham, 1992). For some time it was thought that fusion may be initiated by disassembly of the v/t-SNARE complex (Söllner et al., 1993*a*) by yeast SNAP, Sec17p, (Griff et al., 1992) and NSF, Sec18p (Eakle et al., 1988; Wilson et al., 1989). However, this concept has been challenged by studies with a yeast system that reconstitutes homotypic vacuolar fusion, which suggests the action of Sec18p is before vesicle docking (Mayer et al., 1996; Mayer and Wickner, 1997). In addition, a prefusion role for NSF has been supported by the recent finding that liposomes bearing SNAREs alone can fuse in the absence of NSF (Weber et al., 1998).

Several proteins involved in the regulation of yeast ER to Golgi v/t-SNARE complex assembly have been identified, including Ypt1p, Uso1p, and Sly1p. Ypt1p is a member of the rab family of small GTP-binding proteins that have been identified as important components of almost every stage in the secretory pathway (Ferro-Novick and Novick, 1993). Hydrolysis of GTP by rab-like proteins has been hypothesized to provide the regulatory switch that controls the fidelity of vesicular transport (Bourne, 1988). A second protein, Uso1p (Nakajima et al., 1991), appears to function in the same pathway as Ypt1p (Sapperstein et al., 1996), and both proteins have been demonstrated to be essential for SNARE complex assembly (Søgaard et al., 1994; Sapperstein et al., 1996; Lupashin and Waters, 1997). The third protein, Sly1p, is associated with the t-SNARE Sed5p (Søgaard et al., 1994). *SLY1* is an essential gene in yeast (Dascher et al., 1991; Ossig et al., 1991), and Sly1p is required for ER to Golgi transport in vitro (Lupashin et al., 1996) and in vivo (Ossig et al., 1991). However, several lines of evidence, particularly from Sly1p homologues in other organisms, indicate that Sly1p may also function as a negative regulator of v/t-SNARE complex assembly, perhaps by preventing the association of the v- and t-SNAREs (Hosono et al., 1992; Pevsner et al., 1994; Schulze et al., 1994). A dominant allele of *SLY1*, termed *SLY1-20*, is capable of suppressing mutations in *YPT1* and *USO1*, including complete deletions (Dascher et al., 1991; Sapperstein et al., 1996). Thus, in the presence of Sly1-20p, two components required for SNARE complex assembly are no longer essential. We have proposed a model (Sapperstein et al., 1996; Lupashin and Waters, 1997) in which Ypt1p and Uso1p function to relieve the inhibitory action of Sly1p on SNARE complex assembly. In this model Sly1-20p can be thought of as a noninhibitory form of *SLY1* that renders Ypt1p and Uso1p superfluous.

We believe that the ability of *SLY1-20* to suppress defects in upstream docking regulators can be used to identify additional components involved in the regulation of vesicular docking. We have undertaken a genetic screen (to be presented elsewhere) to isolate novel components in this pathway which, when mutated, depend upon Sly1-20p for viability. In the course of this work, we discovered that two recently identified mutants, *sec34* and *sec35*, can be suppressed by *SLY1-20* and thus satisfy the criterion of our screen. These mutants were isolated in a novel screen to identify components involved in transport at any step between the ER and the *trans*-Golgi network (i.e., the Kex2p compartment) in yeast (Wuestehube et al., 1996). Both *sec34* and *sec35* accumulate the core-glycosylated form of secretory proteins at the nonpermissive temperature, indicating a block in ER to Golgi transport. Furthermore, electron microscopy indicated that both *sec34* and *sec35* accumulate numerous vesicles upon shift to the restrictive temperature (Wuestehube et al., 1996), a hallmark of genes whose protein products are involved in the docking or fusion phase of transport (Kaiser and Schekman, 1990). In this report we describe the cloning of *SEC35* and analysis of its genetic interactions with other secretory genes. Strong genetic interaction between *SEC35* and *SLY1*, *YPT1*, and *USO1* suggests that Sec35p may function in vesicle docking. To test this possibility, we devised an in vitro transport assay that depends on the addition of purified Sec35p and Uso1p. Vesicles synthesized in the absence of functional Sec35p do not fuse with the Golgi compartment and remain as freely diffusible intermediates. Upon addition of Sec35p and Uso1p, vesicles dock to the Golgi and proceed to membrane fusion. Requirements for Sec35p at the vesicle docking step correlates our genetic experiments with the biochemically distinguishable steps of vesicle docking and membrane fusion.

Materials and Methods

Strains and Microbial Techniques

The *S. cerevisiae* strains used in this work are described in Table I. The *sec35-1* strain RSY962 (Wuestehube et al., 1996) was crossed to RSY255 to obtain a *sec35-1* haploid strain with additional auxotrophies. A temperature-sensitive *ura3 leu2* segregant, GWY93, was used for subsequent analyses. The *SEC35* deletion strain, GWY128, was constructed using the γ-transformation method of one-step gene replacement (for review see Rose, 1995) in which the complete open reading frame (ORF) is replaced by sequences contained in the yeast integrating vector, pRS305 (Sikorski and Hieter, 1989). The *sec35Δ* plasmid, pSV31 (see below), was linearized with SmaI and used to transform the diploid strain, GWY30. Leu^+ transformants were purified and the deletion was confirmed by PCR using primers in *LEU2* and flanking the deletion. A *sec32-1* strain containing the *ura3-52* mutation, GWY138, was isolated as a temperature-sensitive segregant from a cross between RSY954 (*sec32-1*) and RSY271 (*sec18-1*), which could be restored to temperature resistance by the introduction of pAN109 (2 μm *BOS1*). The *sec32-1* mutation is allelic to *BOS1*, as determined by complementation analysis (Wuestehube et al., 1996). The *sec34-2* strain (RSY960) was crossed to RSY255 to generate a temperature-sensitive *sec34-2* strain with additional auxotrophies (GWY95). To create diploid strains heterozygous for *sec35-1* and *ypt1-3*, GWY93 was mated to RSY976. The strain heterozygous for *sec35-1* and *uso1-1* was generated through mating RSY962 to GWY67. The *Escherichia coli* strain used in this work was XL1-Blue (Stratagene, Inc., La Jolla, CA).

Bacterial strains were grown on standard media (Miller, 1972) and transformation was performed as described (Hanahan, 1983). Transformation of yeast was performed by the method of Elble (1992) with the exception of the library transformation, which was performed by the method of Schiestl and Geitz (1989). Plasmids were isolated from yeast as described (Sapperstein et al., 1996). Yeast strains were maintained on rich media (YPD) containing 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose, or on synthetic media containing 0.67% yeast nitrogen base without amino acids, 2% glucose, and the appropriate supplements (Rose et al., 1990). Synthetic complete media (SC) contains all supplements necessary to support yeast growth. Diploid strains were sporulated at room temperature in liquid media containing 1% potassium acetate and 0.02% glucose. Unless otherwise noted, all low-temperature incubations of yeast strains were at room temperature, which was ~21°C.

Plasmids

The plasmids used in this work are shown in Table II; plasmid construction was as follows: *SEC35* (ORF YGR120c) was subcloned from a genomic library plasmid as an NsiI/BamHI fragment and ligated into the

Table I. Strains Used in This Work

Strain	Genotype	Source
RSY255	MATα *ura3-52 leu2-3,−112*	R. Schekman (University of California, Berkeley, CA)
RSY962	MAT**a** *sec35-1 lys2-801*	R. Schekman
RSY960	MAT**a** *sec34-2 lys2-801*	R. Schekman
RSY271	MATα *sec18-1 ura3-52 his4-612*	R. Schekman
RSY976	MAT**a** *ypt1-3 ura3-52*	R. Schekman
RSY942	MAT**a** *sec22-3 ura3-52 lys2-801*	R. Schekman
RSY944	MAT**a** *bet1-1 ura3-52 lys2-801*	R. Schekman
RSY954	MAT**a** *sec32-1 lys2-801 leu2-3, −112*	R. Schekman
RSY1074	MATα *sly1^{ts} ura3-1 leu2-3, −112 trp1 ade2-1*	R. Schekman
RSY277	MATα *sec21-1 ura3*	R. Schekman
NY405	MAT**a** *sec4-8 ura3-52*	P. Novick (Yale University School of Medicine, New Haven, CT)
NY420	MAT**a** *sec19-1 ura3-52*	P. Novick
SFNY314	MAT**a** *bet3-1 ura3-52 leu2-3, −112*	S. Ferro-Novick (Yale University)
CKY100	MAT**a** *sec27-1 ura3 leu2*	C. Kaiser (Massachusetts Institute of Technology, Cambridge, MA)
EGY101	MAT**a** *ret1-1 ura3 leu2 his3 trp1 suc2-Δ9*	S. Emr (University of California, San Diego, CA)
SFT1-1	MATα *URA3::sft1-1 sft1Δ::LEU2 ura3-52 leu2-3,−112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	H. Pelham (Medical Research Council, Cambridge, UK)
GWY30	MAT**a**/α *ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1 Δ63/trp1 Δ63*	This laboratory
GWY67	MATα *uso1-1 ura3-52 leu2-3, −112*	This laboratory
GWY93	MATα *sec35-1 ura3-52 leu2-3, −112*	This study
GWY128	MAT**a**/α *sec35Δ::LEU2/SEC35 ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63*	This study
GWY138	MAT**a** *sec32-1 ura3-52 his4-612 lys2-801*	This study
GWY95	MAT**a** *sec34-2 ura3-52 leu2-3, −112 lys2-801*	This study

Table II. Plasmids Used in This Work

Plasmid	Description	Source
pJG103	2 μm *SEC22 URA3*	S. Ferro-Novick
pSFN2d	2 μm *BET1 URA3*	S. Ferro-Novick
pAN109	2 μm *BOS1 URA3*	S. Ferro-Novick
pNB167	2 μm *YPT1 URA3*	S. Ferro-Novick
pGR3	2 μm *BET3 URA3*	S. Ferro-Novick
p*SED5*	2 μm *SED5 URA3*	H. Pelham
p*SFT1*	2 μm *SFT1 URA3*	H. Pelham
pNB657	2 μm *SEC19 URA3*	P. Novick
pNB142	2 μm *SEC4 URA3*	P. Novick
pYEpSNC1	2 μm *SNC1 LEU2*	J. Gerst (Weismann Institute of Science, Rehovot, Israel)
pYEpSNC2	2 μm *SNC2 LEU2*	J. Gerst
p*SLY1-LEU2*	2 μm *SLY1 LEU2*	H.D. Schmitt (Max Planck Institute, Gottingen, Germany
pSK60	2 μm *YKT6 URA3*	This laboratory
pSK47	2 μm *USO1 URA3*	This laboratory
pPI2	2 μm *VTI1 URA3*	This laboratory
pSK101	2 μm *BOS1 URA3*	This study
pSV17	2 μm *SEC35 URA3*	This study
pNB166	CEN *YPT1 URA3*	S. Ferro-Novick
pYCp50-*SLY1-20*	CEN *SLY1-20 URA3*	H.D. Schmitt
pSV11	CEN *SLY1-20 URA3*	This study
pSV15	CEN *SEC35 LEU2*	This study
pSV16	CEN *SEC35 URA3*	This study
pSV23	CEN *YGR122w LEU2*	This study
pSV42	CEN *sec35-1 URA3*	This study
pSV18	YIp *SEC35 URA3*	This study
pSV31	YIp *sec35Δ LEU2*	This study
pSV29	GST-Sec35p	This study
pSV20	His_6-Sec35p	This study
pRS424	YEp *URA3*	P. Hieter (Johns Hopkins School of Medicine, Baltimore, MD)
pRS415	YCp *LEU2*	P. Hieter
pRS416	YCp *URA3*	P. Hieter
pRS305	YIp *LEU2*	P. Hieter
pRS306	YIp *URA3*	P. Hieter
pGEX-2T	GST-fusion vector	Pharmacia Biotech, Inc., Piscataway, NJ
pQE-30	His_6-fusion vector	QIAGEN, Inc., Santa Clarita, CA

LEU2 centromere (CEN) vector, pRS415, that had been previously digested with PstI and BamHI, creating pSV15. ORF YGR122w was isolated from a genomic library plasmid by digestion with PstI and XhoI and then insertion into PstI- and XhoI-digested pRS415 to create pSV23. *SEC35* was excised from pSV15 as a KpnI/SacI fragment and ligated into three similarly digested vectors: (*a*) a *URA3* CEN vector, pRS416, to generate pSV16; (*b*) a *URA3* integrating vector, pRS306, to create pSV18; and (*c*) a *URA3* 2-μm vector, pRS424, to generate pSV17. To construct the plasmid encoding the glutathione-S-transferase (GST)–Sec35 fusion protein, pSV29, the *SEC35* ORF was amplified from pSV16 by PCR, placing a BamHI site adjacent to the sequence encoding the first amino acid of *SEC35* and an EcoRI site 3′ to the stop codon (5′ primer: 5′ cgc-gga-tcc-atg-gtc-aac-agt-cat-ag 3′; 3′ primer: 5′ ccg-gaa-ttc-gtt-ttc-tcc-caa-cta-tg 3′). The PCR product was digested with BamHI and EcoRI and then ligated into pGEX-2T (Pharmacia Biotech, Inc., Piscataway, NJ) that had also been digested with BamHI and EcoRI. The plasmid encoding the His_6-Sec35p fusion protein, pSV20, was generated by first subcloning the above PCR product into pRS416. *SEC35* was then removed from this plasmid as a BamHI/HindIII fragment and then ligated into pQE-30 (QIAGEN, Inc., Santa Clarita, CA) that had been similarly digested. To generate the plasmid used for sequencing the *sec35-1* allele, pSV42, the above primers were used to amplify the ORF using genomic DNA isolated from the *sec35-1* strain as a template. The PCR product was digested with BamHI and EcoRI and ligated into a similarly digested pRS416. The plasmid used to create the *SEC35* deletion, pSV31, was constructed in two steps. First, a 921-bp region immediately 3′ to *SEC35* was amplified by PCR, including an XbaI site adjacent to the stop codon of *SEC35* (5′ primer: 5′ gct-cta-gag-ttg-gga-gaa-aac-ttt-c 3′; 3′ primer: 5′ cgc-tgt-agt-att-ctg-agg 3′). This product was cleaved with XbaI and BamHI (at a site 826 bp from the 3′ end of *SEC35*), and ligated into a similarly digested *LEU2* integrating vector, pRS305. Second, a 1,300-bp fragment that encodes sequence 5′ to *SEC35* was isolated from pSV16 using a HindIII site just upstream of the first amino acid in *SEC35* and a second HindIII site in the polylinker of pSV16. This fragment, when ligated into the HindIII site of the plasmid generated in the first step, resulted in a construct in which sequences flanking *SEC35* are present in the polylinker of pRS305 with the region 3′ of the ORF in front of the region 5′ of the ORF. To create pSV11, *SLY1-20* was excised from pYCp50-*SLY1-20* as a SpeI/ClaI fragment (generated from a partial digest with SpeI because there is a SpeI site internal to the gene) and ligated into a similarly digested vector, pRS416. To isolate *BOS1* from the genomic fragment that contains several additional genes, the gene was liberated from pAN109 as a NruI/KpnI fragment and ligated into pRS416, which had been digested with SmaI and KpnI, generating pSK101.

Cloning of SEC35

To clone *SEC35*, GWY93 was transformed with two YCp50-based *URA3* libraries containing inserts of 10–15 kb and 15–20 kb (A1 and B1, respectively) (Rose et al., 1987). Temperature-resistant transformants were selected directly by incubation at 37.5°C for 2 d on SC-URA media. After confirming the temperature resistance of each colony by retesting on YPD media at 37.5°C, plasmids were isolated from each temperature-resistant transformant and used to retransform the *sec35-1* strain. Plasmids that conferred temperature resistance were subjected to restriction digest analysis to eliminate duplicate isolates and to identify overlapping fragments. Five plasmids from each library were end-sequenced by the dideoxy chain termination method (Sanger et al., 1977) using primers that flank the insert site in YCp50 (YEp24-F and YEp24-R; Sapperstein et al., 1996), and the resulting sequences were used to search the *Saccharomyces* Genome Database with the BLAST algorithm (Altschul et al., 1990). A 9-kb region was common to all the library clones and contained two uncharacterized ORFs, YGR120c and YGR122w, and the gene encoding an ammonium transporter, *MEP1* (Marini et al., 1994). Both novel ORFs were subcloned individually into pRS415, generating pSV16 and pSV23 (refer to plasmid construction).

For integrative mapping, pSV18 was digested at a unique site within *SEC35* (SnaBI) and used to transform GWY93. The resulting temperature-resistant strain was mated to a wild-type strain of the opposite mating type, and the diploid was sporulated and subjected to tetrad analysis.

Sequencing of the sec35-1 Allele

The UV-generated *sec35-1* allele was sequenced by the Princeton University sequencing facility (Princeton, NJ) from plasmid pSV42 (see above). The ORF was sequenced using primers homologous to the polylinker of pRS416 (T3: 5′ aat-taa-ccc-tca-cta-aag-gg 3′; and KS: 5′ tcg-agg-tcg-acg-ggt-atc 3′).

Suppression Analysis

To test for suppression of the *sec35-1* strain, GWY93 was transformed with the following plasmids (see Table II): pSV16, pSV11, pSK60, pSK47, pJG103, pSFN2d, pSK101, pNB166, pNB167, pGR3, pSED5, pSFT1, pNB657, pNB142, pPI2, p*SLY1-LEU2*, pYEpSNC1, and pYEpSNC2. Purified transformants were grown to stationary phase at 21°C in synthetic media. Suppression was tested by spotting the saturated culture and five 10-fold dilutions onto YPD plates followed by incubation for 4 d at 37.5°C. Suppression of the secretory mutants shown in Table I by pSV17 (2 μm of *SEC35 URA3*) was tested by streaking purified transformants for single colonies on YPD plates followed by incubation at 37.5°C. Suppression of the *bet3-1* strain was assessed at 30°C.

To test the suppression of the *SEC35* deletion strain, the *sec35Δ*/*SEC35* heterozygous deletion strain (GWY128) was transformed with pSV16, pSV11, and pNB167. Purified transformants were sporulated and dissected on YPD plates. Suppression of the deletion was assessed after 5 (pSV16) or 7 (pSV11 and pNB167) d of growth at 21°C.

Metabolic Labeling and Immunoprecipitation of Carboxypeptidase Y

Strains were grown at room temperature in SC media lacking methionine (SC-MEI) or SC-URA-MET media to an OD_{595} of 0.3–0.6. For each strain, one OD unit of cells was pelleted, resuspended in 0.5 ml of the same media in which it was grown, and then preshifted to 39°C for 45 min. This incubation and all those subsequent were conducted at 39°C in a shaking water bath. Cells were radiolabeled for 10 min with 50 μCi Tran^{35}S-label (ICN Radiochemicals, Irvine, CA), followed by a 20-min chase, initiated by the addition of a one twenty-fifth volume of chase mix (250 mM cysteine, 250 mM methionine). The reactions were stopped by addition of an equal volume of 20 mM sodium azide and a one-fiftieth volume of 10 mg/ml cycloheximide, and then placed on ice for 10 min. Cell lysis and immunoprecipitation of carboxypeptidase Y (CPY) was performed as previously described (Sapperstein et al., 1996). Antibodies against CPY were provided by S. Emr (University of California, San Diego, CA).

Purification of Recombinant Proteins from E. coli

To purify GST–Sec35p, the fusion protein was expressed in XL1-Blue from pSV29. After growth of a 500-ml bacterial culture at 37°C to an OD_{595} of 1.0, expression of the fusion protein was induced by the addition of isopropylthiogalactoside to 0.5 mM. After a 4-h induction period, cells were harvested (7,000 *g* for 10 min at 4°C in a rotor [model SA600; Sorvall Inc., Newtown, CT]), resuspended in 25 ml PBS, and then lysed by sonication. To prevent nonspecific protein–protein interaction, Triton X-100 (Sigma Chemical Co., St. Louis, MO) was added to 1%, and the lysate was rocked at 4°C for 30 min. After removal of debris and insoluble protein by centrifugation (12,000 *g* for 10 min at 4°C in a rotor [model SA600; Sorvall Inc.]), the lysate was incubated with 2 ml of glutathione–Sepharose beads (Pharmacia Biotech, Inc., Piscataway, NJ) with gentle end-over-end mixing for 2 h at 4°C. The beads were washed twice with 25 ml PBS/1% Triton X-100 and loaded into a 2-ml column. The fusion protein was eluted with 5 ml of 20 mM reduced glutathione in 50 mM Tris-Cl, pH 8.0.

To purify His_6-Sec35p, a 500-ml culture of XL1-Blue transformed with pSV20 was grown, induced, and then processed as described above. The clarified bacterial sonicate containing 1% Triton X-100 was incubated with 2 ml Ni^{2+}-NTA-agarose (QIAGEN, Inc., Santa Clarita, CA) for 2 h at 4°C with end-over-end mixing. The beads were washed twice with 25 ml PBS/1% Triton X-100 and then twice with 25 ml wash buffer (50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 10% glycerol; QIAGEN, Inc., Santa Clarita, CA). The beads were transferred to a 2-ml column and His_6-Sec35p was eluted with 250 mM imidazole in wash buffer. For use in the in vitro assay (see below), His_6-Sec35p was further purified by size-exclusion chromatography to ensure that it was monomeric: 200 μl of a sample containing 5 mg of protein was chromatographed through a 24-ml Superose 12 column (model HR10/30; Pharmacia Biotech, Inc.) at 0.3 ml/min in 25 mM Hepes/KOH, pH 7.0, 150 mM KOAc, 1 mM DTT.

Antibody Production and Purification

To generate antigen for antibody production, Sec35p was excised from the purified GST–Sec35p fusion protein by cleavage with thrombin (Boehringer Mannheim, Indianapolis, IN) during dialysis against 50 mM Tris-Cl, pH 8.0, for 12 h at 4°C. The GST moiety and any uncleaved fusion protein was removed by passage of the material over a 2-ml glutathione–Sepharose column and collecting the Sec35p-containing column flow-through. Recombinant Sec35p was used for the preparation of polyclonal antibodies in rabbits by standard protocols (Harlow and Lane, 1988) at the Princeton University (Princeton, NJ) animal facility. A Sec35p affinity column, generated by coupling 20 mg of His_6-Sec35p (purified as described above) to 3.5 ml cyanogen bromide-activated Sepharose according to the manufacturer's specifications (Pharmacia Biotech, Inc.), was used to affinity purify antibodies to Sec35p (Harlow and Lane, 1988).

Preparation of Protein Extracts and Immunoblotting

Total yeast protein extracts were prepared as described (Ohashi et al., 1982). Samples were separated by SDS-12% PAGE, transferred to nitrocellulose, and then probed with the appropriate primary antibodies according to standard protocols (Harlow and Lane, 1988). Affinity-purified antibodies against Sec35p were made as described above, antibodies against Sed5p were generated and affinity purified as described previously (Lupashin and Waters, 1997), and monoclonal antibodies against phosphoglycerate kinase (PGK) were from Molecular Probes, Inc. (Eugene, OR). Horseradish peroxidase–conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA) were used at a dilution of 1:3,000. Incubations with both primary and secondary antibodies were for 1 h at room temperature. Immunoblots were developed with the chemiluminescent detection kit (Amersham Corp., Arlington Heights, IL).

Subcellular Fractionation and Extractions

A 250-ml culture of RSY255 was grown in YPD media to an OD_{595} of 0.5–1.0, washed in distilled water, and then resuspended at 30 OD/ml in ice-cold buffer 88 (20 mM Hepes/KOH, pH 7.0, 150 mM KOAc, 5 mM $Mg[OAc]_2$) containing 1 mM DTT, 1 mM PMSF, 5 mM 1,10-phenanthroline, 2 μM pepstatin A, 2 μg/ml aprotinin, and 0.5 μg/ml leupeptin. One-half the sample volume of acid-washed glass beads (0.45-mm diameter; Thomas Scientific, Swedesboro, NJ) was added to the mixture, which was then vortexed six times for 20 s with 1-min incubations on ice between each burst. To remove unlysed cells, the crude yeast lysate was centrifuged at 1,000 *g* for 3 min at 4°C in a rotor (model SA600; Sorvall, Inc.). The supernatant was diluted to 5 mg/ml in buffer 88 and used for several manipulations. First, to examine the total membrane-associated pool of Sec35p, the S1 supernatant was layered over a 14% sucrose cushion (in buffer 88), centrifuged at 175,000 *g* for 60 min at 4°C in a rotor (model

TLA100.2; Beckman Instruments, Palo Alto, CA), and separated into the S175 and P175 fractions. Second, the S1 supernatant was centrifuged at 10,000 *g* for 15 min at 4°C in a rotor (model SA600; Sorvall, Inc.) to generate the supernatant (S10) and pellet (P10) fractions. The S10 was layered over a 14% sucrose cushion (in buffer 88) and then centrifuged at 175,000 *g* to generate the supernatant (S175) and pellet (P175) fractions. Finally, to perform protein extractions, 0.5 ml of the S1 supernatant was mixed with an equal volume of either buffer 88 or one of the three extraction solutions (2% Triton X-100 in buffer 88, 2M NaCl in buffer 88, and 200 mM Na_2CO_3 in H_2O) and then incubated on ice for 30 min. The extracts were then layered over a 14% sucrose cushion adjusted to the final concentration of the extraction solution (1% Triton X-100 in buffer 88, 1 M NaCl in buffer 88 or 100 mM Na_2CO_3 in H_2O, respectively), centrifuged at 175,000 *g* for 60 min, and then separated into the S175 and P175 fractions. For all 175,000 *g* centrifugation steps, 0.8 ml of sample was layered over a 0.2 ml sucrose cushion and, after centrifugation, the supernatant fractions were removed in a total of 0.9 ml, thus distributing the cushion between the supernatant and pellet fractions.

In Vitro ER to Golgi Transport Assay

Yeast semi-intact cells from either wild-type (RSY255) or the *sec35-1* strain (RSY962) were prepared from log phase cultures of strains grown at 23°C and stored frozen at −70°C (Baker et al., 1988). Before assays, a tube of cells was quickly thawed and washed three times in buffer 88 to remove cytosol, with each wash followed by centrifugation at 12,000 rpm in a refrigerated centrifuge (model 5417; Eppendorf Scientific, Inc., Hamburg, Germany). [^{35}S]-pre-pro-α-factor was posttranslationally translocated into ER membranes of semi-intact cells at 10°C as previously described (Baker et al., 1988). After washing semi-intact cells with buffer 88, vesicle docking and transport assays were performed at the indicated temperatures (Barlowe, 1997; Cao et al., 1998). Briefly, the addition of purified COPII proteins buds ER-derived vesicles from semi-intact cells that are freely diffusible and remain in the supernatant fraction after centrifugation at 14,000 rpm. Protease-protected glycosylated [^{35}S]pro-α-factor ([^{35}S]gp-α-factor) contained in these vesicles was quantified after solubilization and precipitation with concanavalin A–Sepharose. For transport assays, COPII proteins in addition to purified Uso1p, LMA1 and His_6-Sec35p were added as indicated in the figure legends, and then the amount of Golgi-modified [^{35}S]gp-α-factor was measured by immunoprecipitation with anti-α1,6-mannose–specific antibodies. The data presented is the average of duplicate determinations and the error bars represent the range.

Results

Cloning of SEC35

SEC35 was cloned by complementation of the temperature-sensitive phenotype of the *sec35-1* strain. The *sec35-1* strain was transformed with a CEN-based library, and temperature-resistant transformants were selected directly by incubation at the restrictive temperature. Greater than 50 genome equivalents were screened and 32 temperature-resistant colonies were obtained. To test plasmid linkage of the temperature-resistant phenotype, plasmids were isolated from each of the temperature-resistant colonies and used to retransform the *sec35-1* strain. The ends of the genomic inserts of 10 plasmids that restored temperature resistance were sequenced, and the resulting nucleotide sequences were used to search the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/). Each of the plasmids contained a fragment of chromosome VII, and a 9-kb region bearing two uncharacterized ORFs and a gene encoding an ammonium transporter, *MEP1* (Marini et al., 1994), was common to all of them. Each of the two novel ORFs was subcloned into a CEN-plasmid and tested for its ability to complement the temperature-sensitive phenotype of the *sec35-1* strain. Only one of the two ORFs, designated YGR120c in the *Saccharomyces* Genome Database, restored growth at 37.5°C to wild-type levels.

To determine whether YGR120c encoded authentic Sec35p or a low copy number suppressor, integrative mapping was performed. A plasmid bearing YGR120c and *URA3* was cleaved at a unique site within the coding sequence of YGR120c to direct integration into its genomic locus in the *sec35-1* strain. The resulting Ura$^+$ temperature-resistant strain was mated to a wild-type strain and the diploid was sporulated and dissected. Of the 24 tetrads examined, all segregants were temperature resistant. Since the temperature-sensitive phenotype did not segregate away from the integrated plasmid that was marked by *URA3*, the cloned gene is very tightly linked to the temperature-sensitive *sec35-1* mutation. Therefore, integrative mapping confirms that YGR120c is *SEC35*.

Sequence of Sec35p and Sec35-1p

The predicted protein sequence encoded by *SEC35* is shown in Fig. 1 *a*. Sec35p is composed of 275 residues and has a calculated mass of 31.8 kD and a pI of 4.56. Sec35p lacks any hydrophobic stretch of sufficient length to act as a transmembrane domain, nor does it contain consensus sequences for lipid modification; thus, it is predicted to be a cytosolic or peripheral membrane protein. Sec35p shows no significant propensity to form coiled coils as predicted by the PairCoil algorithm (Berger et al., 1995), although the COILS program (Lupas et al., 1991) does identify two regions of ~20 amino acids with greater than 70% probability of forming coiled coils. Database searches reveal no homologues of Sec35p.

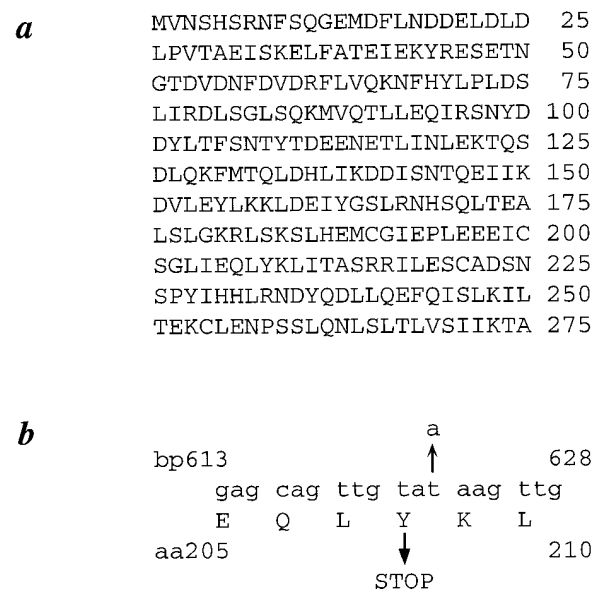

Figure 1. Predicted amino acid sequence of Sec35p and Sec35-1p. (*a*) Protein sequence of Sec35p. *SEC35* was sequenced as part of the Yeast Genome Sequencing project and corresponds to ORF YGR120c, GenBank/EMBL/DDBJ accession number Z72905. (*b*) Sequence of wild-type *SEC35* from bp 613 to 628 with the transversion mutation at bp 624 (*t* to *a*) and corresponding codon change (*tyrosine* to *ochre*) present in the *sec35-1* allele indicated with *arrows*.

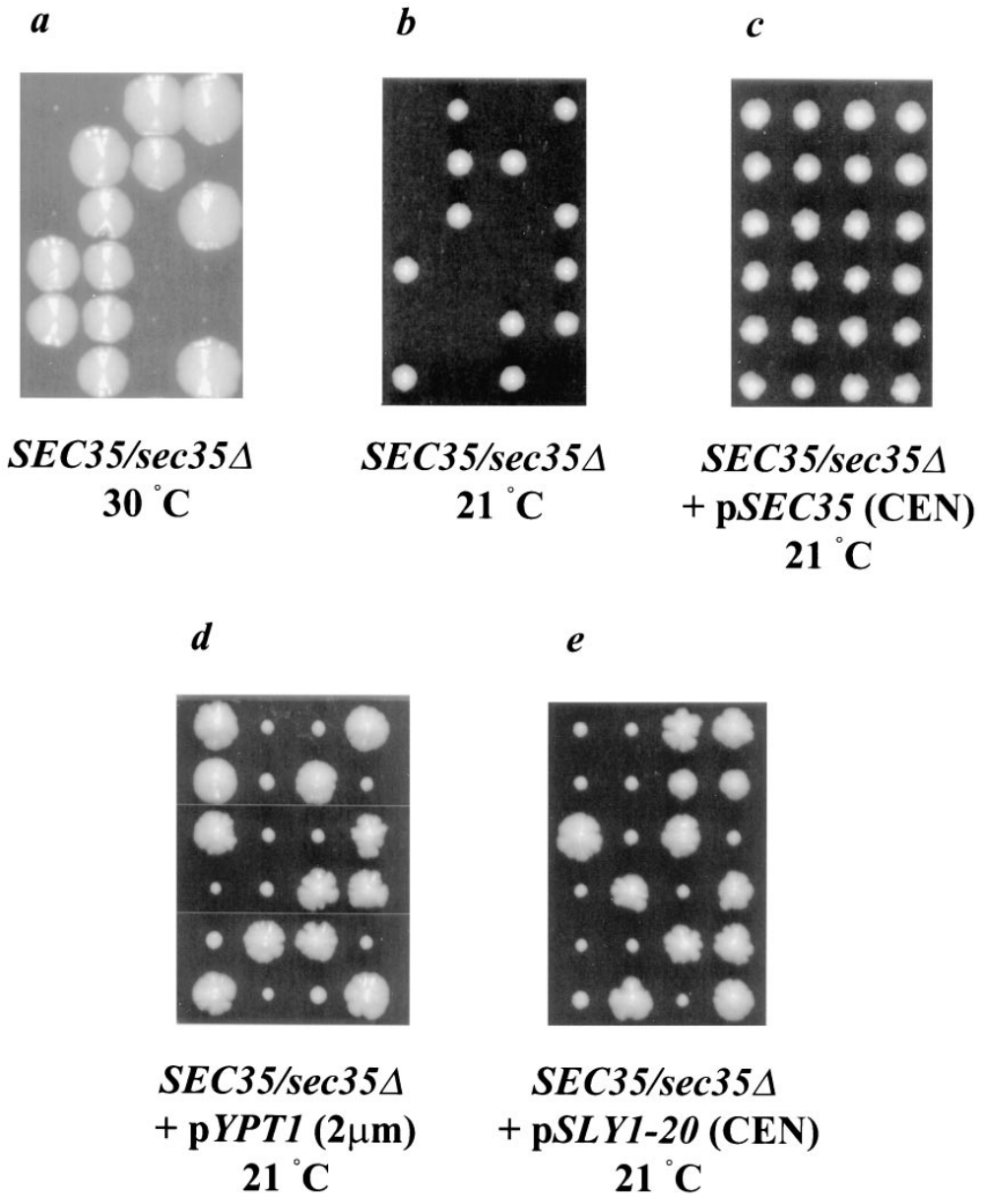

Figure 2. A haploid strain containing a deletion of *SEC35* exhibits a severe growth defect at 30°C and is inviable at 21°C; the cold-sensitive lethality can be rescued by the expression of *SEC35*, low levels of *SLY1-20*, or high levels of *YPT1*. The *sec35Δ/SEC35* heterozygous deletion strain alone (*a* and *b*), or transformed with p*SEC35 (CEN)* (*c*), p*YPT1 (2μm)* (*d*), or p*SLY1-20 (CEN)* (*e*) was sporulated and dissected. The resulting tetrads were incubated at 30°C for 7 d (*a*) or at 21°C for 5 d (*b* and *c*) or 7 d (*d* and *e*) on YPD plates. In *c–e*, only tetrads containing four viable spores are shown; tetrads with two or three viable spores were assumed to result from tetrads in which one or both of the *sec35Δ* haploid spores failed to receive the suppressing plasmid.

Sequencing of the *sec35-1* allele revealed a single base pair change at base pair 624 (t to a) resulting in the conversion of a tyrosine codon to an ochre stop codon (Fig. 1 *b*). The *sec35-1* allele thus encodes a truncated protein with a predicted mol wt of 24 kD. Because the strain containing this truncation displays no growth defect at temperatures from 21° to 30°C, the carboxy-terminal 68 amino acids of the protein are not crucial to the function of the protein in this temperature range.

Deletion of SEC35 Results in a Severe Growth Defect

To determine whether *SEC35* is an essential gene, the entire ORF was replaced in a diploid strain by the sequences in a *LEU2* integrating plasmid (refer to Materials and Methods). After sporulation of the heterozygous deletion strain, tetrads were dissected on YPD plates and incubated at 30°C. Upon incubation for 7 d, tetrads were observed to contain two distinct sizes of colonies, with each tetrad containing two large and two (or, less frequently, one) very small segregants (Fig. 2 *a*). The small segregants were all Leu^+, indicating the presence of the disruption (marked by *LEU2*), whereas the large segregants were all Leu^-. Thus, *SEC35* is not an essential gene although the small colony size of the *sec35Δ* haploid indicates a severe growth defect in the absence of *SEC35*.

In contrast, when dissected tetrads were incubated at 21°C a $2^+:2^-$ segregation for viability was observed (Fig. 2 *b*). All viable spores were Leu^- and thus *SEC35* is an essential gene at this temperature. Examination of the terminal phenotype of the *sec35Δ* haploid segregants revealed microcolonies of ~25 cells and thus spores deleted for *SEC35* can germinate and undergo four or five cell divisions before terminating vegetative growth. Transformation of the *sec35Δ/SEC35* heterozygous diploid strain with a plasmid encoding *SEC35* before sporulation and dissec-

tion complemented the cold-sensitive lethality of the *sec35Δ* haploid, resulting in $4^+:0^-$ segregation for viability (Fig. 2 *c*). Of the tetrads with four viable spores, the deletion (marked by *LEU2*) segregated $2^+:2^-$, and the *sec35Δ* haploid colonies were invariably Ura^+ due to their reliance on the plasmid for viability.

Multicopy Suppression Analysis

To gain insight into the function of Sec35p we undertook a systematic analysis of its genetic interactions with other genes involved in secretion. Since the *sec35-1* mutant accumulates vesicles and is likely to be involved in vesicular docking or fusion, we first examined the interaction of *SEC35* with other genes known to be involved in this step. To do this, we tested the ability of several known genes to act as multicopy suppressors of the *sec35-1* temperature-sensitive growth defect. As shown in Fig. 3, the temperature sensitivity of *sec35-1* could be suppressed by overexpression of *YPT1* from a 2-μm plasmid, or by low-copy expression of the dominant mutation of *SLY1*, termed *SLY1-20* (Ossig et al., 1991). *YPT1* expressed from a low-copy (CEN) plasmid could also suppress the temperature-sensitive growth defect, although to a slightly lower level than the high-copy plasmid (data not shown). Thus, *SEC35* displays a strong genetic interaction with two genes, *YPT1* and *SLY1*, whose protein products are implicated in the regulation of vesicle docking (Rexach and Schekman, 1991; Segev, 1991; Lian et al., 1994; Søgaard et al., 1994; Lupashin et al., 1996; Lupashin and Waters, 1997). In contrast, high-copy expression of a third gene with a potential role in the regulation of vesicle docking, *USO1* (Sapperstein et al., 1996), was incapable of suppressing *sec35-1*. Overexpression of the wild-type allele of *SLY1* was also unable to suppress the growth defect of the *sec35-1* strain, emphasizing the unique function of the *SLY1-20* allele. To determine the specificity of the suppression of *sec35-1* by *YPT1*, we also tested *SEC4*, a yeast *YPT1* homologue involved in the docking stage of Golgi to plasma membrane transport (Goud et al., 1988). *SEC4*, however, was unable to suppress the growth defect when expressed from a 2-μm plasmid (data not shown), indicating that the suppression of *sec35-1* by *YPT1* is specific.

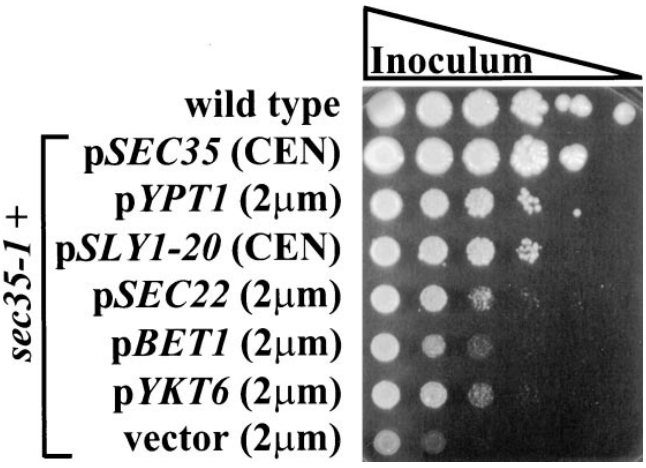

Figure 3. Suppression of the temperature-sensitive growth phenotype of the *sec35-1* strain. Wild-type and *sec35-1* strains containing the indicated plasmids were grown to stationary phase in synthetic media and used for a series of 10-fold dilutions. The stationary phase culture and five serial dilutions of each strain were spotted onto YPD plates and grown for 4 d at 37.5°C.

The *sec35-1* strain could also be suppressed, although to a much lesser extent, by overexpression of three v-SNAREs involved in docking vesicles to the *cis*-Golgi, Sec22p, Bet1p, and Ykt6p (Newman and Ferro-Novick, 1987; Ossig et al., 1991; Søgaard et al., 1994; McNew et al., 1997) (Fig. 3). A fourth v-SNARE, Bos1p, (Shim et al., 1991; Newman et al., 1992), however, was incapable of suppressing the *sec35-1* defect (data not shown). Interestingly, when the genes encoding the two v-SNAREs required for Golgi to plasma membrane transport, *SNC1* and *SNC2* (Gerst et al., 1992; Protopopov et al., 1993), were tested for high-copy suppression of the *sec35-1* temperature sensitivity, *SNC2* was found to suppress the defect as well as, if not better than, each of the ER to Golgi v-SNAREs. In contrast, *SNC1* had no effect (data not shown).

A number of other genes were also tested for the ability to suppress *sec35-1* when expressed from 2-μm plasmids, including those encoding the ER to Golgi t-SNARE *SED5* (Hardwick and Pelham, 1992), the putative intra-Golgi retrograde SNARE *SFT1* (Banfield et al., 1995), the multifunctional v-SNARE *VTI1* (Fischer von Mollard et al., 1997; Lupashin et al., 1997), a novel ER to Golgi transport factor *BET3* (Rossi et al., 1995), and the GDP dissociation inhibitor *SEC19*, also known as *GDI1* (Garrett et al., 1994). None of these genes improved the growth of *sec35-1* at the restrictive temperature.

Because *SLY1-20* and high-copy *YPT1* strongly suppressed the temperature-sensitive allele of *SEC35*, we next tested whether they were capable of suppressing the cold-sensitive lethality of the genomic deletion. The heterozygous deletion strain was transformed with plasmids encoding either high levels of *YPT1* or CEN levels of *SLY1-20*, sporulated, and then subjected to tetrad analysis. After incubation at room temperature for 7 d, the majority of the tetrads contained three or four viable spores (Fig. 2, *d* and *e*), rather than the $2^+:2^-$ segregation evident without the plasmid (Fig. 2 *c*). The *sec35Δ* haploid colonies (marked by *LEU2*) were without exception Ura^+, and thus were viable by virtue of the suppressing plasmid. Suppression of the deletion is not complete, however, as indicated by the small size of the haploid colonies bearing the deletion compared with the wild-type segregants in each tetrad. The ability of *SLY1-20* and high-copy *YPT1* to suppress the deletion of *SEC35* implies that their protein products function either downstream from, or in a parallel pathway with, Sec35p.

If Sec35p and Ypt1p were functioning in parallel pathways, one might expect the genetic interactions to be reciprocal in nature, and thus a temperature-sensitive allele of *YPT1* might be suppressed by high level expression of *SEC35*. To test this hypothesis, the *ypt1-3* strain was transformed with a 2-μm plasmid encoding *SEC35* and then suppression was tested at the restrictive temperature. Although the levels of Sec35p expressed from the plasmid were ~20× the genomic level (see Fig. 6), a high level of Sec35p was incapable of compensating for the temperature sensitivity of *ypt1-3* (data not shown). This result im-

plies that Ypt1p may function downstream of Sec35p, but the possibility of a parallel pathway cannot be completely ruled out. The ability of 2-μm–expressed *SEC35* to suppress temperature-sensitive mutations in many other genes required for ER to Golgi transport was also tested. High levels of Sec35p were expressed in each of the following strains but were unable to suppress the growth defect at the restrictive temperatures: *sec22-3*, *bos1-1*, *bet1-1* (ER to Golgi v-SNAREs), *sec18-1* (yeast NSF), *uso1-1* (ER to Golgi docking factor), *sly1^{ts}* (t-SNARE–associated protein), *bet3-1* (novel ER to Golgi factor), *sft1-1* (putative retrograde t-SNARE), *sec19-1* (GDP-dissociation inhibitor), *sec4-8* (Golgi to plasma membrane rab-like GTPase), *sec21-1* (γ-COP), *sec27-1* (β′-COP), *ret1-1* (α-COP), and *sec34-2* (novel ER to Golgi factor).

Suppression of the ER to Golgi Transport Block of the sec35-1 Strain by SLY1-20 and High-Copy YPT1

To confirm that the suppression of the temperature-sensitive growth defect of the *sec35-1* strain by *SLY1-20* and *YPT1* was due to a direct effect on secretion, we tested whether expression of either gene could suppress the ER to Golgi transport defect. Thus, we examined the ability of CPY to transit from the ER to the vacuole in the mutant strain expressing low levels of *SLY1-20* or high levels of *YPT1* (Fig. 4). In wild-type cells, CPY is translocated into the ER where it is glycosylated, producing a 67-kD form termed p1 (Stevens et al., 1982). The oligosaccharides are extended in the Golgi generating the p2 form. Finally, upon transport to the vacuole, CPY is proteolytically processed to the 61-kD mature form. To examine progress of CPY through the secretory pathway, we performed a pulse-chase analysis of CPY maturation. Strains were shifted to the restrictive temperature for 45 min, labeled with Tran^{35}S-label for 10 min, and then chased for 20 min with an excess of unlabeled cysteine and methionine. CPY was then immunoprecipitated, separated by SDS-PAGE, and then visualized by autoradiography. Wild-type and *sec18-1* strains served as controls for the mature and p1 forms of CPY, respectively. Whereas the *sec35-1* strain accumulated the p1 form of CPY, as shown previously (Wuestehube et al., 1996), the expression of Sly1-20p or Ypt1p was capable of suppressing the ER to Golgi defect (Fig. 4), resulting in the procession of the majority of CPY to its mature form. Nevertheless, since a significant amount of the p1 form of CPY did accumulate, neither of these proteins was capable of full suppression of the *sec35-1* defect. A plasmid bearing the wild-type *SEC35* gene, however, was capable of completely restoring the secretory block and all CPY was found in the mature form.

sec35-1 Displays a Synthetic Interaction with ypt1-3 and uso1-1

To further define the genetic interactions of *SEC35* we tested whether the *sec35-1* strain displayed a synthetic lethal interaction with mutant alleles of other known genes in the secretory pathway. Thus, the *sec35-1* strain was mated individually to several strains each containing a temperature-sensitive mutation in one of the known secretory genes and the resulting diploids were sporulated and dissected. Due to the suppression of *sec35-1* by *YPT1*, we

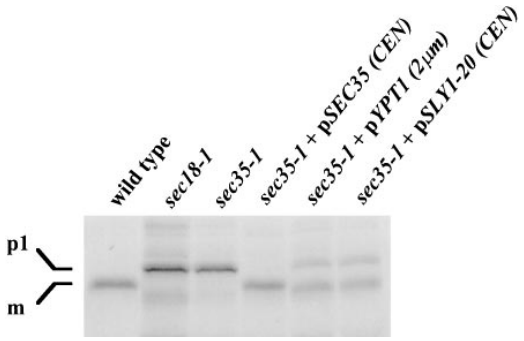

Figure 4. Suppression of the ER to Golgi transport defect of *sec35-1* by expression of *SLY1-20* or high levels of *YPT1*. Wild-type and *sec18-1* strains, as well as the *sec35-1* strain with and without the indicated plasmids, were grown at 21°C to mid-logarithmic phase and then shifted to 39°C for 45 min. Cells were labeled at 39°C for 10 min with Tran^{35}S-label and then chased for 20 min at the same temperature. CPY was immunoprecipitated from each strain, resolved by SDS-7%PAGE, and then visualized by autoradiography. The migration of the ER precursor (*p1*) and mature (*m*) forms of CPY are indicated on the left.

first tested whether the mutant alleles of the two genes would display a synthetic interaction. Incubation of dissected tetrads from the diploid strain heterozygous for both *sec35-1* and *ypt1-3* at 30°C, a temperature permissive for both mutations, resulted in a pattern of viable spores consistent with a synthetic lethal interaction between the two alleles (Fig. 5 *a*): tetrads consisting of four viable spores segregated 0^+:4^- for temperature sensitivity (parental ditypes), tetrads consisting of two viable spores segregated 2^+:0^- for temperature sensitivity (nonparental ditypes), and tetrads consisting of three viable spores segregated 1^+:2^- for temperature-sensitivity (tetratypes). In all cases the inviable progeny must be the haploid segregant bearing both the *sec35-1* and the *ypt1-3* mutation.

Although we failed to see a genetic interaction between *SEC35* and *USO1* through multicopy suppression analysis,

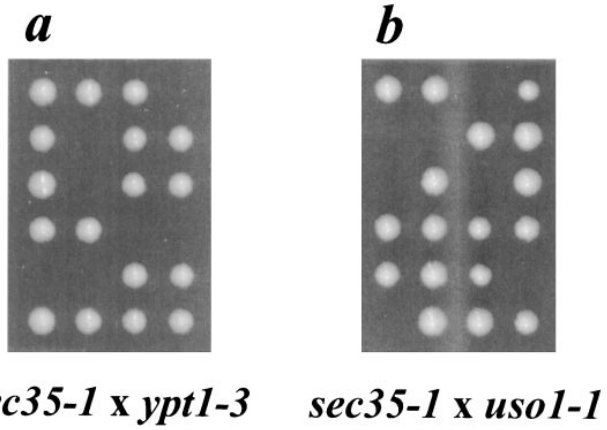

Figure 5. The *sec35-1* allele exhibits a synthetic interaction with *ypt1-3* and *uso1-1*. The *sec35-1* strain was mated to a strain bearing the *ypt1-3* allele (*a*) or the *uso1-1* allele (*b*), and the resulting diploid strains were sporulated, dissected on YPD plates, and then incubated at 30°C for 3 d. Six representative tetrads of each dissection are shown.

we tested whether mutations in the two genes displayed a synthetic interaction. Analysis of tetrads from a diploid strain heterozygous for both *sec35-1* and *uso1-1* revealed a pattern of viability identical to that seen with the segregants from the diploid heterozygous for both *sec35-1* and *ypt1-3* (Fig. 5 *b*). This synthetic lethal interaction is the first genetic interaction that has been observed between *SEC35* and *USO1*. Interestingly, when tetrads dissected from diploid strains heterozygous for both *sec35-1* and *ypt1-3* or *sec35-1* and *uso1-1* were incubated at 21°C, all spores were viable (data not shown). The interaction between *sec35-1* and both *ypt1-3* and *uso1-1* is best described as a conditional synthetic lethality.

Localization of Sec35p

To initiate a biochemical analysis of Sec35p, recombinant Sec35p was used to generate anti-Sec35p antibodies in rabbits. The polyclonal serum was affinity purified on bacterially expressed His_6-Sec35p and used to probe total yeast protein extracts. As shown in Fig. 6, the affinity-purified anti-Sec35p antibody specifically recognizes a protein of ~35 kD, close to the predicted mol wt of 31.8 kD. This band was absent in a strain deleted for *SEC35* (expressing *SLY1-20* from a CEN plasmid to suppress the *sec35Δ* growth defect), and is thus specific for Sec35p. As expected, Sec35p is highly overexpressed in a strain bearing a 2-μm *SEC35* plasmid. In the temperature-sensitive *sec35-1* strain incubated at either the permissive or the restrictive temperatures, the truncated 24-kD protein predicted from the sequence of the *sec35-1* allele is not detected, indicating that the mutation greatly decreases the stability of the protein. However, upon longer exposure of the immunoblot the fragment of Sec35p was visible, migrating with an apparent mol wt of 27 kD. This truncated Sec35p is more abundant at the permissive temperature than at the restrictive temperature (data not shown).

We next tested whether there existed a membrane-associated pool of Sec35p, as might be predicted for a protein required for membrane traffic. Wild-type yeast were lysed with glass beads and centrifuged at 1,000 *g* for 3 min to remove unbroken cells, yielding the low-speed supernatant S1. The S1 supernatant was then centrifuged at 175,000 *g* for 60 min and separated into pellet (P175) and supernatant (S175) fractions that should contain the membrane-bound and soluble proteins, respectively. The P175 and S175 fractions were resolved by SDS-PAGE and immunoblotted with affinity-purified antibodies against Sec35p, the *cis*-Golgi protein Sed5p, and the cytosolic protein PGK. As shown in Fig. 7 *a*, approximately two-thirds of Sec35p is found in the P175 fraction, and thus a significant proportion of the protein may be membrane-associated. As expected of an integral membrane protein, Sed5p localizes to the P175 fraction, whereas the cytosolic protein PGK remains in the supernatant fraction. Although we consistently observe a fraction of Sec35p in the P175 pellet, the proportion of pelletable protein is variable, ranging from about one-third to two-thirds of the total Sec35p (data not shown).

The localization of Sec35p was then examined through subcellular fractionation. The S1 fraction described above was centrifuged at 10,000 *g* for 15 min, yielding the P10

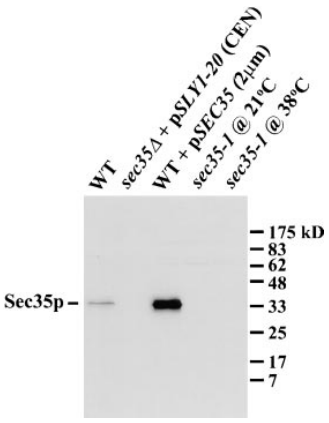

Figure 6. Characterization of the affinity-purified anti-Sec35p antibody. Extracts were made by glass bead lysis from logarithmically growing strains: wild-type, *sec35Δ* transformed with p*SLY1-20 (CEN)*, wild-type transformed with p*SEC35* (*2μm*), and *sec35-1*. All strains were incubated at 21°C before lysis except for the *sec35-1* strain, which was maintained at 21°C or shifted to 38°C for 1 h before lysis. Extracts (0.2 OD_{595} per lane) were separated by SDS-12%PAGE and immunoblotted with affinity-purified anti-Sec35p antibodies. The migration of mol wt markers is shown on the right.

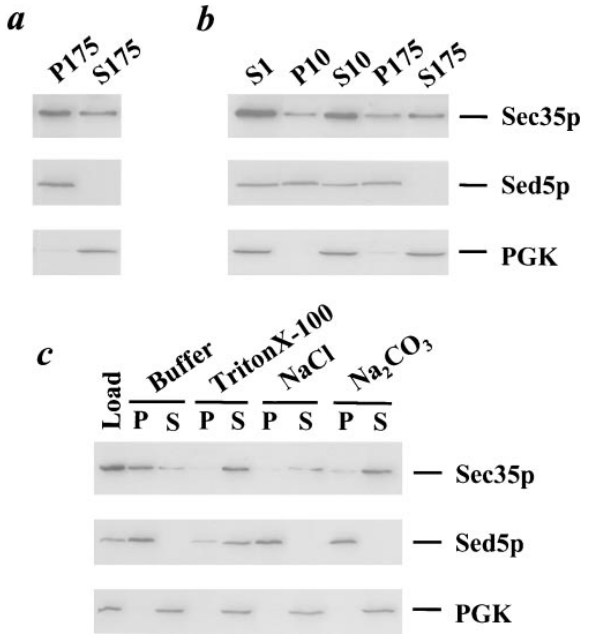

Figure 7. Sec35p is a peripheral membrane protein. (*a*) Sec35p partitions into soluble and membrane-associated pools. A wild-type strain was grown to logarithmic phase, lysed with glass beads, and then centrifuged at 1,000 *g* for 3 min to generate the S1 supernatant fraction. The S1 supernatant was centrifuged at 175,000 *g* to obtain the S175 supernatant and P175 pellet fractions. (*b*) The membrane-associated portion of Sec35p fractionates similarly to the Golgi protein, Sed5p. The S1 supernatant was centrifuged at 10,000 *g* to generate the S10 supernatant and P10 pellet fractions, and the S10 was subsequently centrifuged at 175,000 *g* to obtain the S175 supernatant and P175 pellet fractions. (*c*) Sec35p behaves as a peripheral membrane protein. The S1 supernatant was incubated with buffer 88, 1% Triton X-100, 1 M NaCl, or 100 mM Na_2CO_3, pH 11, centrifuged at 175,000 *g*, and separated into supernatant and pellet fractions. (*a–c*) Aliquots of each fraction were separated by SDS-12%PAGE and immunoblotted with affinity-purified anti-Sec35p (*top panel*), anti-Sed5p (*center panel*), or anti-PGK (*bottom panel*) antibodies. The samples loaded in each lane were derived from equivalent amounts of starting material.

pellet and S10 supernatant fractions, and the S10 was further centrifuged at 175,000 *g* for 60 min to generate the P175 pellet and S175 supernatant fractions. With this series of differential centrifugation steps, all organelles of the secretory pathway should be contained in one or more of the pellet fractions: the ER and vacuole are located primarily in the P10 fraction, whereas the Golgi partitions between the P10 and P175 fractions (Walworth et al., 1989; Nakayama et al., 1992). To determine the localization of Sec35p, the supernatant and pellet fractions were resolved by SDS-PAGE and immunoblotted with affinity-purified antibodies. As shown in Fig. 7 *b*, although a portion of Sec35p once again remains in the S175, the membrane-associated pool of Sec35p partitions between the P10 and P175 fractions. The fractionation pattern of the membrane-associated Sec35p is similar to that of the *cis*-Golgi marker Sed5p, which also localizes to the P10 and P175 fractions. Nonetheless, because of the large pool of soluble Sec35p and the labile nature of its membrane association in vitro (data not shown), we have been unable to unequivocally determine its precise subcellular localization.

Finally, to determine whether the membrane association of Sec35p is as an integral or peripheral membrane protein, we attempted to extract the protein from the membrane using detergent, high salt, or high pH. Although both integral and peripheral membrane proteins should be extracted by detergent that solubilizes the membranes, the hallmark of a peripheral membrane protein is the ability to be extracted by salt or high pH, conditions which often disrupt protein–protein interactions. The S1 supernatant was thus incubated for 30 min on ice in either buffer, 1% Triton X-100, 1 M sodium chloride, or 100 mM sodium carbonate, pH 11, centrifuged at 175,000 *g* for 60 min, and then separated into pellet and supernatant fractions. The fractions were then resolved by SDS-PAGE and immunoblotted with antibodies against Sec35p, Sed5p, and PGK. Whereas Sed5p and PGK behaved as expected for an integral membrane protein or a cytosolic protein, respectively (Fig. 7 *c*), Sec35p was extractable by Triton X-100, NaCl, and Na_2CO_3 and thus behaves as a peripheral membrane protein.

Sec35p Is Required for ER to Golgi Vesicle Docking

Our genetic and biochemical experiments suggest that Sec35p functions during the docking stage of ER to Golgi transport perhaps through peripheral association with membranes. To directly test this idea, we used a cell-free assay that measures distinct stages of ER to Golgi transport by following the radioactively labeled precursor of the secretory protein α-factor, glycosylated pro-α-factor ([^{35}S]gp-α-factor). Transport in semi-intact yeast cells may be reproduced in vitro with washed membranes, purified COPII proteins, Uso1p, a protein complex called LMA1 (Xu et al., 1997), and ATP (Barlowe, 1997). COPII-coated vesicles that have budded from the ER are freely diffusible and then dock to Golgi membranes upon addition of Uso1p. Sec18p and LMA1 are not required for the docking phase, but are required for efficient fusion of vesicles (Barlowe, 1997). These soluble proteins represent some, but not all, of the genetically defined components for fusion of ER-derived vesicles. Other proteins that are peripherally bound to membranes (such as Sly1p and Ypt1p) or are integral membrane proteins (such as Sed5p, Bet1p and Bos1p) have required schemes designed to selectively inactivate their function in vitro such that reconstitution experiments can be performed (Cao et al., 1998). To inactivate Sec35p in vitro, we prepared membranes from the temperature-sensitive *sec35-1* strain and measured reconstituted ER to Golgi transport at restrictive temperatures.

In Fig. 8, semi-intact yeast cell membranes prepared from a wild-type strain and the *sec35-1* strain were incubated with purified transport factors at 23° or 29°C. Wild-type membranes show a modest decrease in transport at 29°C, whereas transport in the *sec35-1* semi-intact membranes is reduced to near background levels (Fig. 8, *A* and *B*, compare *open bars*). This inhibition is specific for Sec35p function because the addition of purified His_6-Sec35p (1.2 μg) restored transport in mutant membranes to near wild-type levels at 29°C (Fig. 8, *A* and *B*, compare *hatched bars*). The restoration of transport was maximal with 1.2 μg of His_6-Sec35p, although stimulation could be observed with as little as 0.24 μg (data not shown). Because no stimulation of transport was observed when His_6-Sec35p was added to wild-type reactions, it is likely that ample levels of Sec35p are present on washed membranes. However, we have established a specific requirement for Sec35p in cell-free transport of [^{35}S]gp-α-factor from the ER to the Golgi when membranes are prepared from the *sec35-1* strain and reactions performed at 29°C.

To further characterize Sec35p function in distinct transport stages, we assayed vesicle docking in mutant *sec35-1* semi-intact cells. For this assay, docking is measured as the amount of COPII-generated vesicles that attach to Golgi membranes in the presence of Uso1p. The docked vesicles

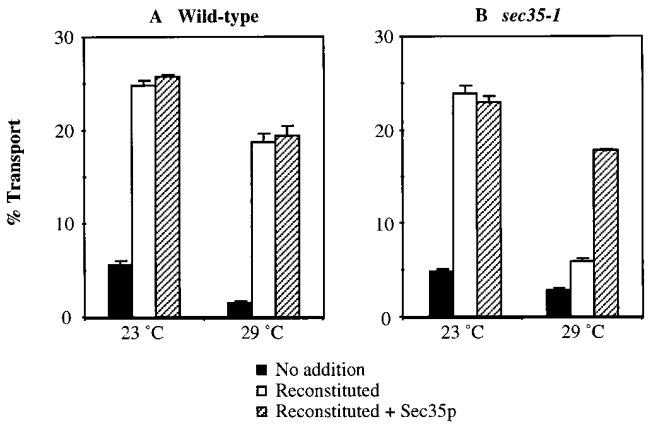

Figure 8. Sec35p is required for ER to Golgi transport in vitro. Semi-intact yeast cell membranes prepared from wild-type (*A*) or *sec35-1* (*B*) strains were incubated at 23° or 29°C for 60 min. Reactions contained an ATP regeneration system alone (*No addition*, *solid bars*), COPII, Uso1p, and LMA1 (*Reconstituted*, *open bars*), or COPII, Uso1p, LMA1 and His_6-Sec35p (*Reconstituted + Sec35p*, *hatched bars*). Outer-chain modified forms of [^{35}S]gp-α-factor were immunoprecipitated with anti–α1,6-mannose–specific antibodies and the percent transport reflects the amount of total [^{35}S]gp-α-factor that has acquired Golgi-specific outer-chain modifications.

sediment with semi-intact cells after a brief centrifugation step (Barlowe, 1997). As seen in Fig. 9, wild-type and *sec35-1* semi-intact membranes bud nearly equivalent amounts of ER-derived vesicles when purified COPII proteins are added at 23°and 29°C (Fig. 9, *A* and *B*, *wide hatched bars*). Uso1p addition docked this intermediate and reduced the level of freely diffusible vesicles in wild-type reactions but docking was not efficient in reactions containing *sec35-1* membranes at the restrictive temperature (Fig. 9, *A* and *B*, compare *open bars*). Finally, the addition of purified His_6-Sec35p restored docking efficiency in *sec35-1* membranes at 29°C and did not alter the level of docking in wild-type reactions (Fig. 9, *A* and *B*, *narrow hatched bars*). In summary, ER to Golgi transport in *sec35-1* membranes is prevented because vesicles fail to dock.

Discussion

sec35-1 is a novel secretory mutant identified in a genetic screen for temperature-sensitive mutants that accumulate unprocessed α-factor internally (Wuestehube et al., 1996). The *sec35-1* strain is defective in the transport of both soluble and membrane proteins from the ER to the Golgi apparatus. In addition, numerous vesicles accumulate at the restrictive temperature, suggesting that there is a defect in vesicle docking or fusion (Wuestehube et al., 1996). We have cloned *SEC35* and demonstrated that it encodes a novel cytosolic protein of 32 kD. The cloned gene can suppress the temperature-sensitive growth defect, as well as the ER to Golgi secretory defect, of the *sec35-1* strain; it is authentic *SEC35* as shown by integrative mapping.

SEC35 displays a strong genetic interaction with two genes whose protein products are involved in the regulation of vesicle docking: the small GTP-binding protein *YPT1* and the t-SNARE–associated protein *SLY1*. The temperature-sensitive growth defect of the *sec35-1* strain is

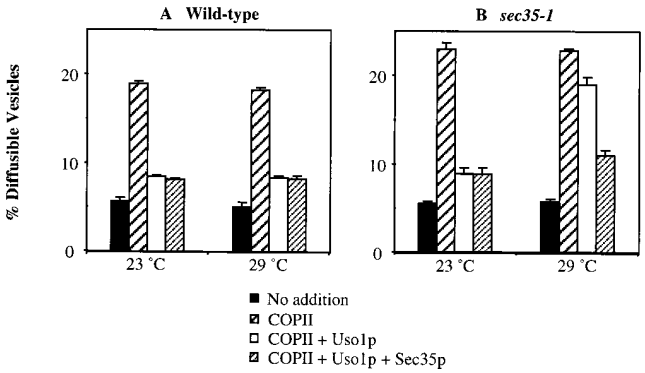

Figure 9. Sec35p is required for vesicle docking. Semi-intact yeast cell membranes prepared from wild-type or *sec35-1* strains were incubated at 23° or 29°C for 30 min. Reactions contained an ATP regeneration system alone (*No addition*, *solid bars*), COPII (*COPII*, *wide hatched bars*), COPII and Uso1p (*COPII + Uso1p, open bars*), or COPII, Uso1p, and His_6-Sec35p (*COPII*, *Uso1p + Sec35p*, *narrow hatched bars*). Freely diffusible vesicles containing [^{35}S]gp-α-factor were separated from semi-intact membranes by centrifugation at 18,000 *g* for 3 min. The percent diffusible vesicles reflects the amount of total concanavalin A precipitable [^{35}S]gp-α-factor contained in the 18,000 *g* supernatant fluid.

suppressed by either high levels of *YPT1* or low levels of a dominant allele of *SLY1*, termed *SLY1-20*. Suppression of the growth defect is due to a restoration of secretory pathway function, since a vacuolar protein, CPY, which accumulates in its ER-modified form in the *sec35-1* strain, is transported to the vacuole in the presence of *SLY1-20* or high-copy *YPT1*. Neither gene can completely replace the function of *SEC35*, however, since only partial restoration is obtained.

High levels of *YPT1* or low levels of *SLY1-20* can also rescue the cold-sensitive lethality of the *SEC35* deletion strain. This suppression of the *sec35Δ* strain indicates that both Ypt1p and Sly1p function downstream of, or in a parallel pathway with, Sec35p. However, overexpression of *SEC35* fails to suppress the temperature-sensitive growth defect of either the *ypt1-3* or the *sly1^{ts}* strains, contrary to what might be expected if Sec35p operates in a parallel pathway. Thus, it seems more likely that Ypt1p and Sly1p function downstream of Sec35p. Furthermore, since Ypt1p acts upstream of the SNAREs and Sly1p to regulate SNARE complex assembly (Søgaard et al., 1994; Lupashin and Waters, 1997), Sec35p is likely to impinge on this process as well.

The interpretation that Sec35p is involved in regulating SNARE complex assembly is supported by the result that overexpression of a number of v-SNAREs involved in docking vesicles to the *cis*-Golgi (Sec22p, Bet1p, and Ykt6p) are capable of suppressing the temperature-sensitive growth defect of the *sec35-1* strain, albeit to a lesser extent than *YPT1* or *SLY1-20*. This suppression may be due to a mass action effect, in which increasing the amount of one v-SNARE can drive v/t-SNARE complex formation to an extent sufficient to allow a low level of growth. Intriguingly, the *sec35-1* strain can also be suppressed by overexpression of one of the Golgi to plasma membrane v-SNAREs, *SNC2*. This suppression pattern suggests that Sec35p may function at more than one step in the secretory pathway. However, overexpression of the Golgi to plasma membrane Ypt1p-like GTPase, *SEC4*, is unable to suppress *sec35-1*. Attempts to address the effect of Sec35p at multiple stages by preloading the secretory pathway of the *sec35-1* strain with labeled CPY and shifting to the nonpermissive temperature were inconclusive, since the temperature-sensitive defect of the *sec35-1* strain is slow relative to the rate of secretion (data not shown).

Multicopy suppression analysis failed to identify a genetic interaction between *SEC35* and the third gene whose protein product has a role in the regulation of SNARE complex assembly, *USO1*. However, mutations in *SEC35*, *USO1*, and *YPT1* exhibit a very similar pattern of suppression since each is suppressed by both *SLY1-20* and ER to Golgi v-SNAREs (Sapperstein et al., 1996). In addition, the three genes are related through synthetic lethal interactions: the *sec35-1* allele is conditionally lethal in combination with either *uso1-1* or *ypt1-3*, and *uso1-1* displays a synthetic lethal interaction with *ypt1-3* (Sapperstein et al., 1996). These data suggest that the functions of Sec35p, Uso1p, and Ypt1p in ER to Golgi transport are intimately associated.

At the ER to Golgi step of the secretory pathway it is likely that v/t-SNARE complex formation occurs during the docking phase of the reaction, before membrane fu-

sion. This interpretation is based on the observation that Sec18p (NSF), which is required to disassemble v/t-SNARE complexes (Söllner et al., 1993*a*,*b*) allows docked vesicles to fuse in vitro (Barlowe, 1997). Because our genetic analysis indicates that Sec35p functions the docking step, we tested this hypothesis directly using an in vitro assay that monitors individual stages of ER to Golgi transport. In this cell-free assay, vesicle budding from washed semi-intact cells requires the addition of COPII components, whereas docking is dependent upon both COPII proteins and Uso1p. Membranes isolated from the *sec35-1* strain and incubated at the restrictive temperature were found to be competent for vesicle formation but defective in Uso1p-dependent vesicle docking. Thus, Sec35p appears to be required for the docking of ER-derived vesicles.

Sec35p was not identified as one of the cytosolic components required for vesicle docking in wild-type semi-intact cells (Barlowe, 1997). In addition, purified Sec35p does not stimulate docking in wild-type cells and thus sufficient Sec35p is present in the cytosol-free membranes to provide its essential function. These results indicate that Sec35p is associated with membranes. Indeed, we find that a significant portion of Sec35p behaves as a peripheral membrane protein, potentially associating with the Golgi. The above in vitro data from wild-type cells implies that the membrane-associated pool of Sec35p may be the functional form of this protein since neither the removal of soluble Sec35p nor the addition of recombinant Sec35p affects docking. It is possible, however, that either the soluble fraction of Sec35p is not removed with the washes or that very little Sec35p, soluble or membrane-associated, is required for function.

Recent results from a number of laboratories suggest the following outline for ER to Golgi transport. First, vesicle budding occurs under the direction of COPII proteins resulting in a freely diffusible vesicle (Barlowe et al., 1994). Docking then occurs in two stages: in the first stage, the vesicle is "tethered" to the Golgi in a reaction that requires Uso1p and Ypt1p (Cao et al., 1998) while the second stage may be a SNARE-dependent event characterized by an assembled v/t-SNARE complex spanning the vesicle and target membrane, as proposed in the original SNARE hypothesis (Söllner et al., 1993*b*). In this case, the role of Uso1p and Ypt1p in the regulation of SNARE-complex assembly (Sapperstein et al., 1996; Lupashin and Waters, 1997) may equate to a dependence on the tethered state for SNARE-dependent docking. After docking, Sec18p would act to disassemble the v/t-SNARE complex and allow the reaction to proceed towards fusion. What is perhaps least clear in this scenario for ER to Golgi transport is the exact role of Sec18p in facilitating progression towards fusion. For example, it is possible that Sec18p acts to disassemble v/t-SNARE complexes within (not across) the vesicle and/or target membranes of the tethered intermediate. This disassembly would allow the freed v- and t-SNAREs to enter into new v/t-SNARE complexes that span the vesicle and target membranes. Fusion could then ensue without a further requirement for Sec18p, as suggested by the recent work on vacuole fusion in yeast (Mayer et al., 1996; Mayer and Wickner, 1997) and the reconstitution of membrane fusion with SNAREs alone (Weber et al., 1998). The essential difference in the models is that the first (tethering → SNARE-dependent docking → Sec18p function → fusion) a vesicle-target membrane-spanning v/t-SNARE complex is disassembled by Sec18p, allowing fusion, whereas in the second model (tethering → Sec18p function → SNARE-dependent docking → fusion) the vesicle-target membrane-spanning v/t-SNARE complex is, in effect, generated by Sec18p and proceeds spontaneously to fusion. Regardless of the precise role of Sec18p in generating or consuming a SNARE-docked state, our genetic and biochemical results clearly place Sec35p as acting in the Uso1p-dependent docking phase of ER to Golgi vesicle docking.

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