# Assembly of the ER to Golgi SNARE Complex Requires Uso1p

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***Abstract.*** Uso1p, a *Saccharomyces cerevisiae* protein required for ER to Golgi transport, is homologous to the mammalian intra-Golgi transport factor p115. We have used genetic and biochemical approaches to examine the function of Uso1p. The temperature-sensitive phenotype of the *uso1-1* mutant can be suppressed by overexpression of each of the known ER to Golgi v-SNAREs (Bet1p, Bos1p, Sec22p, and Ykt6p). Overexpression of two of them, Bet1p and Sec22p, can also suppress the lethality of *Δuso1*, indicating that the SNAREs function downstream of Uso1p. In addition, overexpression of the small GTP-binding protein Ypt1p, or of a gain of function mutant (*SLY1-20*) of the t-SNARE associated protein Sly1p, also confers temperature resistance. Uso1p and Ypt1p appear to function in the same process because they have a similar set of genetic interactions with the v-SNARE genes, they exhibit a synthetic lethal interaction, and they are able to suppress temperature sensitive mutants of one another when overexpressed. Uso1p acts upstream of, or in conjunction with, Ypt1p because overexpression of Ypt1p allows a *Δuso1* strain to grow, whereas overexpression of Uso1p does not suppress a *Δypt1* strain. Finally, biochemical analysis indicates that Uso1p, like Ypt1p, is required for assembly of the v-SNARE/t-SNARE complex. The implications of these findings, with respect to the mechanism of vesicle docking, are discussed.

In eukaryotic cells, newly synthesized proteins destined for secretion, or residence in organelles of the secretory pathway, must enter and transit the secretory pathway for their proper processing and targeting. This pathway consists of a number of membrane-bounded organelles, including the ER and the Golgi apparatus. The proteins are transported between these organelles via small membrane–bounded secretory vesicles (Palade, 1975), which bud from the donor membrane and are targeted to the acceptor membrane where they fuse (Orci et al., 1989). Since proper functioning of the secretory pathway is crucial for normal cell function, transport between the various compartments must be tightly regulated to ensure that vesicles fuse only with the appropriate membrane.

Both biochemical and genetic approaches have led to the identification of numerous components of the molecular machinery that mediate transport. Utilization of an in vitro intra-Golgi transport assay, which reconstitutes *cis* to *medial* Golgi transport, has allowed the purification of several cytosolic transport factors. These include *N*-ethylmaleimide sensitive factor (NSF) (Block et al., 1988) and α, β, and γ soluble NSF attachment protein (SNAP) (Clary and Rothman, 1990). The yeast homologues of NSF and α-SNAP, namely Sec18p and Sec17p, were identified genetically (Novick et al., 1980; Eakle et al., 1988; Wilson et al., 1989; Griff et al., 1992). Together, NSF, and SNAPs, which are required for the binding of NSF to the Golgi membrane (Clary et al., 1990; Weidman et al., 1989), are considered part of the general fusion apparatus (Wilson et al., 1992), which functions at several transport steps, including endosome–endosome fusion (Diaz et al., 1989), vacuolar sorting (Graham and Emr, 1991), transcytosis (Sztul et al., 1993), and synaptic vesicle fusion (Söllner et al., 1993).

Recently the molecular basis of vesicle targeting and fusion has been further illuminated. A set of integral membrane proteins, known to reside in the neuronal presynaptic terminal, was shown to function as a receptor for α-SNAP (Söllner et al., 1993*b*). This result led to the formulation of the SNAP receptor (SNARE) hypothesis, which posits that the fidelity of vesicular transport is dependent on the presence of specific molecules on the vesicle (the v-SNARE) and on the target membrane (the t-SNARE). According to this model, the SNARE molecules physically interact with one another in a specific manner, thereby assuring that a given vesicle can only fuse with the appropriate target membrane. The binding of the SNAPs and NSF to the complexed v- and t-SNAREs results in a fusion-competent complex, called the SNARE complex (Aalto et al., 1993; Söllner et al., 1993*a*,*b*; Rothman and Warren, 1994).

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1. *Abbreviations used in this paper*: 5-FOA, 5-fluoroorotic acid; CPY, carboxypeptidase Y; NSF, *N*-ethylmaleimide sensitive fusion protein; SC, synthetic complete; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.

SNAREs also function in the yeast secretory pathway (Dascher et al., 1991; Hardwick and Pelham, 1992; Aalto et al., 1993; Protopopov et al., 1993), and recently have been shown to enter into complexes analogous to those in neurons (Søgaard et al., 1994). In the ER to Golgi leg of the yeast secretory pathway, this complex includes the putative v-SNAREs Bet1p, Bos1p, Sec22p, and Ykt6p, the t-SNARE–Sed5p, the t-SNARE–associated protein Sly1p, and several other proteins (Søgaard et al., 1994). Although the small GTP-binding protein Ypt1p appears to impact on v-SNARE/t-SNARE complex formation (Lian et al., 1994; Søgaard et al., 1994), it has not been found in the SNARE complex (Søgaard et al., 1994).

An in vitro intra-Golgi transport assay that was designed to be dependent on high molecular weight proteins was used to identify and purify an additional protein, called p115, that is involved in vesicular transport (Waters et al., 1992). p115 is a peripheral membrane protein localized predominantly to the Golgi apparatus (Waters et al., 1992). p115 has also been identified as a component of transcytotic vesicles and thus was termed transcytosis-associated protein (TAP) (Sztul et al., 1993). Recently, it was demonstrated that p115 is required in vitro, along with NSF and SNAPs, for the reassembly of post-mitotic Golgi fragments into Golgi cisternae (Rabouille et al., 1995). Biochemical characterization and electron microscopy indicated that p115 exists as a parallel homo-dimer, with two globular "heads" and an extended rod-like "tail" domain (Waters et al., 1992; Sapperstein et al., 1995). Analysis of the p115 cDNA (Barroso et al., 1995; Sapperstein et al., 1995), which encodes a 108-kD protein, revealed that the "head" comprises approximately the amino-terminal two-thirds of the molecule. The "tail" comprises an approximately 250 residue coiled-coil domain followed by a small highly acidic domain at the extreme COOH terminus.

p115 is homologous to Uso1p (Barroso et al., 1995; Sapperstein et al., 1995), a yeast protein required for ER to Golgi transport (Nakajima et al., 1991). The two proteins share an overall "head-tail-acid" structural organization. Interestingly, the 206-kD Uso1p is significantly larger than p115, with a much longer coiled-coil dimerization domain accounting for most of its additional mass. In addition to their structural similarity, the two proteins share three regions of significant homology at the amino acid level: two regions in the head domain are more than 60% identical, and a third region just after the end of the predicted coiled-coil domain is 25% identical.

Several lines of evidence suggest that p115 and Uso1p function in the docking step of vesicular transport. First, the cell-free intra-Golgi transport assay used to purify p115 (Waters et al., 1992) measures only the docking and fusion stages of the vesicular transport cycle, not the formation of vesicles (Elazar et al., 1994). Second, p115 was shown to be required for the binding of transcytotic vesicles to the plasma membrane at a step before the ATP-dependent step of the transport cycle (Barroso et al., 1995). Finally, Uso1p is required in vitro for the targeting and fusion of ER-derived vesicles with purified Golgi membranes, and like p115, also functions at a step before ATP hydrolysis (Lupashin et al., 1996).

We have used genetic and biochemical approaches to further dissect the function of Uso1p in yeast vesicular transport. Multicopy suppressor analysis has revealed that *USO1* is similar to *YPT1* with respect to its genetic interactions with components of the vesicular targeting apparatus. Furthermore, we found that Uso1p, like Ypt1p, is required for assembly of the ER to Golgi v-SNARE/t-SNARE complex in vivo.

## Materials and Methods

### Reagents

Oligonucleotide primers were synthesized at the Princeton University Synthesis/Sequencing facility. Zymolyase 20T was obtained from Seikagako Kogyo Co. (Tokyo, Japan), DNA modifying enzymes and restriction endonucleases were obtained from New England Biolabs (Beverly, MA), Tran$^{35}$S-Label was from ICN Radiochemicals (Irvine, CA), protein A–Sepharose was obtained from Pharmacia LKB Nuclear (Piscataway, NJ), and 0.45-mm glass beads were from Thomas Scientific (Swedesboro, NJ).

Antisera against Sed5p and Bos1p were affinity purified as previously described (Søgaard et al., 1994) using anti-Sed5p and anti-Bos1p sera and the corresponding recombinant proteins (generous gifts from M. Søgaard and J. Rothman, Sloan Kettering Cancer Center), except that antibodies were coupled to protein A–Sepharose instead of Protein G beads. Antisera against Bet1p and Sec22p were obtained from R. Schekman, and the anti-

*Table I. Yeast Strains Used in This Study*

| Strain | Genotype | Source |
|---|---|---|
| MY2788 | *MAT* **a** *leu2Δ1 trp1-Δ63 ura3-52* | M. Rose |
| MY2789 | *MAT* α *leu2Δ1 trp1-Δ63 ura3-52* | M. Rose |
| GWY30 | *MAT* **a**/α *leu2Δ1/leu2Δ1 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52* | This study |
| GWY32 | *MAT* **a**/α *Δuso1::LEU2/USO1 leu2Δ1/leu2Δ1 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52* | This study |
| GWY33 | *MAT* α *uso1-1* leu2Δ1 trp1-Δ63 ura3-52* | This study |
| GWY76 | *MAT* **a**/α *Δypt1::LEU2/YPT1 leu2Δ1/leu2Δ1 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52* | This study |
| GFUI-6D | *MAT* **a** *GAL10-YPT1::HIS3 his3 leu2 trp1 ura3* | H.D. Schmitt |
| RSY255 | *MAT* **a** *leu2-3,112 ura3-52* | R. Schekman |
| RSY271 | *MAT* **a** *sec18-1 his4-619 ura3-52* | R. Schekman |
| RSY942 | *MAT* **a** *sec22-3 his4-619 lys2-801* | R. Schekman |
| RSY944 | *MAT* **a** *bet1-1 lys2-801 ura3-52* | R. Schekman |
| RSY954 | *MAT* **a** *sec32-1*‡ *leu2-3,112 lys2-801* | R. Schekman |
| RSY976 | *MAT* **a** *ypt1-3 ura3-52* | R. Schekman |
| GWY67 | *MAT* **a** *uso1-1* leu2-3,112 ura3-52 (**RSY255** background)* | This study |
| GWY71 | *MAT* α *sec18-1 uso1-1* ura3-1 his 3-11,15* | This study |

*uso1-1** is a version of the original *uso1-1* mutation (Nakajima et al., 1991; Seog et al., 1994) that contains three consecutive termination codons (amber, ochre, and opal) rather than the single amber mutation. ‡The *SEC32* gene is likely to be identical to *BOS1* (Wuestehube et al., 1995).

carboxypeptidase Y (CPY) antisera were obtained from S. Emr. HRP-conjugated secondary antibodies were obtained from Bio-Rad Labs (Hercules, CA).

## Strains, Plasmids, and Media

*S. cerevisiae* strains used in this study are listed in Table I. Yeast media were prepared as described (Sherman, 1991). The *Escherichia coli* stain XL1-Blue {*supE44 thi-1 lac endA1 gyrA96 hsdR17 relA1 (F' proAB lacI$^q$ ZΔM15 Tn10)*} (Stratagene Inc., La Jolla, CA) was used for all routine manipulations; BMH71-18 {*thi supE Δ(lac-proAB) (mutS::Tn10) (F' proAB, lacI$^q$ ZΔM15)*} (Kramer et al., 1984) was used in site-directed mutagenesis procedures where required, and MC1066 {*trpC9830 leuB6 ara$^+$ pyrF74::Tn5(Kan$^R$)*} (Casadaban et al., 1983) was used to select ligation products bearing the *LEU2* gene. Bacterial strains were grown on standard media (Miller, 1972).

The plasmids used in this study were as follows: pSFN2d (2 μm *BET1 URA3*), pJG103 (2 μm *SEC22 URA3*), pAN109 (2 μm *BOS1 URA3*), and pNB167 (2 μm *YPT1 URA3*; Bacon et al., 1989) (from S. Ferro-Novick, Yale University School of Medicine); pANY2-7 [2 μm *SAR1 URA3*; (d'Enfert et al., 1991)], p*SEC7* (2 μm *SEC7 URA3*), p*ARF1* (2 μm *ARF1 URA3*), and p*SEC21* (2 μm *SEC21 URA3*) (from R. Schekman, University of California, Berkeley, CA); pYEp*SNC1* (2 μm *SNC1 LEU2*) and pYEp*SNC2* (2 μm *SNC2 LEU2*) (from J. Gerst, Weizmann Institute of Science, Rehovot, Israel); p*SED5* (2 μm *SED5 URA3*) and p*SFT1* (2 μm *SFT1 URA3*) (from H. Pelham, MRC, Cambridge, England); p*SEC18* (2 μm *SEC18 URA3*) (from S. Emr, University of California, San Diego, CA); p*SEC17* (2 μm *SEC17 URA3*) (from C. Kaiser, MIT); and pYCP50-*SLY1-20* (CEN *SLY1-20 URA3*), p*SLY1* (2 μm *SLY1 URA3*), p*SLY41* (2 μm *SLY41 URA3*). All pSK plasmids used in this study are described below.

## Microbial Techniques

Genetic techniques were essentially as described by Rose et al. (1990). The recovery of plasmids from yeast employed an additional chloroform extraction with a subsequent ethanol precipitation. Yeast transformations were performed by the method of Elble (1992), except that 20 μl 1 M DTT was added to each transformation to increase efficiency. Each transformation reaction contained 500 μl log-phase cells, 1 μg plasmid DNA, and 3 μl 10 mg/ml sheared denatured carrier DNA. Salmon sperm carrier DNA was prepared as previously described (Schiestl and Gietz, 1989), except that the phenol chloroform extraction and subsequent ethanol precipitation were omitted. Cells were plated on synthetic complete (SC) media at varying dilutions depending on the number and density of transformants required per plate. All assessments of *uso1-1* temperature sensitivity were performed on YPD plates at 37°C since the strain is not temperature sensitive on minimal media. In all cases strains were permitted to grow for three days.

To test the ability of different genes to suppress the *Δypt1* allele by galactose shut-off in the GFUI-6D strain, the cells were transformed with each of the plasmids to be tested. The transformants were grown and purified on SC media containing 2% galactose instead of glucose to induce expression of the *YPT1* gene. The ability of the various plasmids to suppress the loss of Ypt1p was assessed by growing the strains at room temperature on SC media, which contains glucose.

## Plasmid and Strain Constructions

To generate the *Δuso1::LEU2* allele present in GWY32, the 5.6-kb BsaBI-SacI fragment containing the *USO1* gene [(Nakajima et al., 1991); GenBank accession number X54378] was subcloned from pHN169 (a kind gift of K. Yoda, University Tokyo) into Bluescript II KS$^-$ digested with SmaI and SacI to generate pSK19. The 3.8-kb internal EcoRV fragment of *USO1* was removed from pSK19 and replaced with the 2.1-kb SmaI-PvuII fragment from pMR2253 (from M. Rose, Princeton University), which contains a functional *LEU2* gene. The correct ligation product was selected by using the *E. coli* strain MC1066, which is auxotrophic for leucine and can be complemented by the presence of the *S. cerevisiae LEU2* gene. Transformations of this ligation reaction into MC1066 were plated on M9-ampicillin plates (100 μg/ml ampicillin) supplemented with uracil and tryptophan to select for the *LEU2* bearing plasmids. The resulting plasmid was called pSK21. The 4.9-kb NsiI fragment of pSK21, which contained the disrupted *USO1* gene, was isolated and transformed into GWY30 and Leu$^+$ transformants were selected. The presence of the *Δuso1* allele was confirmed by polymerase chain reaction (PCR) using primers flanking the site of the disruption (Saiki et al., 1988), and by sporulation and dissection of the resulting Leu$^+$ diploid. All 11 of the tetrads dissected segregated viable, Leu$^-$ spores in a 2:2 pattern.

The plasmid pSK47 (2 μm *USO1 URA3*) was generated from pSK19 as follows: the 5.6-kb SacI-KpnI fragment from pSK19 was isolated and subcloned into pRS426 digested with KpnI and SacI. To generate a plasmid containing the isolated *YKT6* gene, the 850-bp EcoRV-ClaI fragment from the multicopy suppressor isolate SOU197, which contains the *YKT6* gene, was subcloned into pRS426 (Christianson et al., 1992) digested with the same enzymes. The resulting plasmid was called pSK60.

The *uso1-1** mutant allele utilized throughout these studies bears three consecutive nonsense mutations (amber, ochre, and opal) in the *USO1* gene instead of the single amber mutation present in the *uso1-1* allele (Seog et al., 1994); the mutant proteins encoded by *uso1-1* and *uso1-1** are identical. *uso1-1** was generated in pSK19 by site-directed mutagenesis using the unique site elimination method (Deng and Nickoloff, 1992) and confirmed by DNA sequence analysis with the dideoxy method (Sanger et al., 1977). To introduce the mutation into the genome, *uso1-1** was subcloned into the YIp-*URA* vector pRS306 (Sikorski and Hieter, 1989) on a KpnI-SacI fragment. The resulting plasmid, pSK20, was linearized with BspEI, which cuts in *uso1-1**, and transformed into both MY2789 (wt), RSY255 (wt), RSY271 (*sec18-1*). Transformants were selected on SC-Ura media, purified once on the same media, and then patched onto a YPD plate. After growth at room temperature for 2 d, the YPD patch plates were then replica plated onto 5-fluoroorotic acid (5-FOA) media (Rose et al., 1990) to force the loss of the *URA3* gene and either the wild-type *USO1* allele or the *uso1-1** allele. After 2 d at room temperature, cells from the patches on 5-FOA media were streaked for single colonies on 5-FOA plates. The presence of the *uso1-1** mutation in individual colonies from MY2789 and RSY255 was determined by patching colonies from these plates to YPD plates and incubation at 37°C; colonies that had acquired temperature sensitivity were considered to have acquired the *uso1-1** mutation. The resulting *uso1-1** strains were GWY33 (from MY2789) and GWY67 (from RSY255). Transformation of these strains with a CEN-based plasmid bearing the *USO1* gene restored temperature resistance. Since the *sec18-1* mutation in RSY271 also causes temperature sensitivity, to detect the presence of the introduced mutation in the *sec18 uso1-1** double mutant (GWY71) it was necessary to first transform the strains with a *SEC18* plasmid (provided by T. Graham, Vanderbilt University). In this way the presence of the *uso1-1** mutation was detectable.

## Multicopy Suppressor Screen

For the multicopy suppressor screen of *uso1-1*, we used a modified version of the mutation, called *uso1-1** that contains three consecutive stop codons (see above), to eliminate a potential background of nonsense suppressors. For the screen, 29,000 transformants of a yeast genomic YEp24 library (Carlson and Botstein, 1982) were grown on SC-Ura plates. Since the average insert size for this library is >10 kb, we screened >19 genome equivalents. Each of these plates was serially replica plated to two YPD plates. The replica YPD plates were then incubated for 3 d at 37°C. Serial replica plating was employed because we have found that when the *uso1-1* strain is incubated at the restrictive temperature revertant colonies arise at a frequency of ~$10^{-5}$. Under the plating conditions used for this screen it is sometimes difficult to distinguish between plasmid-dependent temperature-resistant colonies and colonies that have revertants growing in them. Therefore, from these plates we chose those transformants that were able to grow on both plates; 355 transformants met this criterion. Cells from each temperature-resistant transformant were patched onto SC-Ura media to check that each of the isolates bore plasmid DNA. All but one were able to grow on the selective media. Cells from each isolate were then retested for temperature-resistance on YPD at 37°C. Of the 354 isolates tested, 305 still exhibited a temperature-resistant phenotype. These were divided into two groups, strong and weak, based on the degree to which each was able to confer temperature resistance. Plasmids from the 173 strains in the strong suppressor group were isolated and retransformed into GWY33. Of the resulting strains, 132 exhibited plasmid-linked suppression. To identify known genes in the panel of suppressors we employed Southern blotting (Sambrook et al., 1989). 1 μg of the plasmid DNA isolated from each candidate was applied to BA35 nitrocellulose (Schleicher & Schuell, Keene, NH) using a Schleicher & Schuell Manifold II. Eight identical filters were generated, one for each probe to be tested. Radiolabeled probes were made by the random-primer labeling method (Feinberg and Vogelstein, 1984) with DNA fragments generated by PCR with the following oligonucleotide primers: *USO1:* USK2 5′CTTGTTCACGACTTCGAGTG3′ and USK8 5′GTGAATCGTTCA-

CTACTGCC3'; *BET1:* BET1-F 5'GAGGAACAGATGGGAGCT3' and BET1-R 5'CACTATAGGGCAAAAGG3'; *BOS1:* BOS1-F 5'GGTCCATCTCTGCAACTC3' and BOS1-R 5'CGCGATCCAAAAGACTAG3'; *SEC7:* SEC7-F 5'CAATCCGTCTGTGAAACC3' and SEC7-R 5'TATGGGTAGACCTTGGGG3'; *SEC22:* SEC22-F 5'GACACCACAGTCTGCCAC3' and SEC22-R 5'GAAGTTGATCTTTTGCGC3'; *SEC17:* SEC17-F 5'GGTGTTCCTTCATCGGGT3' and SEC17-R 5'CGCAAAATTCGGATCTTC3'; *SLY1:* SLY1-F 5'GGCTGTGGAGGAAATTGC3' and SLY1-R 5'CCCAGTTTCTCAGCGATG3'; *SLY41:* SLY41-F 5'CCCTGACGGTATCCTTCC 3' and SLY41-R 5'CTGGAAAGCAAGCATTGC3'; and *YPT1:* YPT1-F 5'GAGGTTTTCGGACGACAC3' and YPT1-R 5'CCGGTGTTGGTTAAACTC3'. PCR reactions were performed with Taq Polymerase (Boehringher Mannheim, Indianapolis, IN) under standard conditions (Saiki et al., 1988). Each fragment was gel purified from low melting point agarose (SeaPlaque; FMC, Rockland, ME) with β-agarase (New England Biolabs, Beverly, MA). The filters were hybridized in Church buffer (Church and Gilbert, 1984) overnight at 65°C with $3 \times 10^6$ cpm of probe per ml of hybridization buffer. Washes were done in 0.1 × SSC/0.1% SDS at 65°C. Filters were subjected to autoradiography with Kodak (Rochester, NY) XAR-5 film at −80°C for 6 h.

This hybridization scheme revealed that 18 of the plasmids contained known genes, leaving a collection of 114 suppressors bearing unknown genes. Restriction digestion with HindIII and EcoRI revealed that a number of plasmids were present more than once leaving a collection of 88 distinct plasmids. The ends of the genomic inserts in 48 of these plasmids have been sequenced. Double-stranded plasmid DNA sequencing was performed using Sequenase 2.0 (Amersham, Arlington Heights, IL) according to the manufacturer's instructions with the primers YEP24F-5'CTACTTGGAGCCACTATCG3' and YEp24R-5'GTGATGTCGGCGATATAGG3'.

### *Metabolic Labeling and Immunoprecipitation of CPY*

Yeast strains were grown at 22°C in SC media lacking methionine, or methionine and uracil, to an $OD_{600nm}$ of 0.5. For each timepoint, 1 $OD_{600}$ unit of cells was harvested by centrifugation, resuspended in 250 μl of the same media. Pulse-labeling was initiated immediately after the shift to the restrictive temperature of 38°C by the addition of 50 μCi of Tran$^{35}$S-label. The chase period was initiated by the addition of methionine and cysteine to a final concentration of 1 mM each, and terminated by transferring the cells to a tube on ice containing 5 μl of 50× termination mix (500 mM $NaN_3$, 5 mg/ml cycloheximide). After a minimum of 10 min on ice, the cells were pelleted, the supernatant aspirated, and 70 μl of bead buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 2 mM EDTA, 1% SDS) and ~170 mg of glass beads were added to each sample (equivalent to 5/6 of the final volume). Cells were lysed by vortexing for five 1-min intervals, with 1 min on ice between each burst. 180 μl of bead buffer were added to each tube, the tube vortexed, and the entire lysate transferred to a fresh tube. The samples were then incubated at 100°C for 4 min and centrifuged (13,000 *g*) for 5 min. The supernatants were transferred to fresh tubes and 700 μl of immunoprecipitation dilution buffer (60 mM Tris, pH 7.4, 190 mM NaCl, 6 mM EDTA, 1.25% Triton X-100) was added. Protein extract from a *S. cerevisiae Δprc1* strain (from M. Rose), which is deleted for the gene encoding CPY, was also added to a final concentration of 1 mg/ml to act as competitor (Scidmore et al., 1993). Samples were vortexed and centrifuged for 10 min. The supernatants were transferred to fresh tubes and 0.6 μl anti-CPY antisera and 20 μl of a 50% slurry of protein A–Sepharose in dilution buffer were added to each tube. The samples were rotated overnight at 4°C, or for 2 h at room temperature. Bead-bound immune complexes were pelleted and washed once with urea buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% SDS, 2 M urea), twice with immunoprecipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% SDS), and once with final wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA). Immune complexes were released from the beads by the addition of 20 μl of 2× Laemmli sample buffer (Laemmli, 1970) and incubation at 100°C for 4 min. In all cases, the entire supernatant was subjected to SDS-7% PAGE. Gels were fixed in 25% isopropanol/10% acetic acid for 20 min, soaked in 0.7 M sodium salicylate, pH 7.0 for 20 min, dried, and then subjected to autoradiography with Kodak XAR-5 film at −80°C.

### *Immunoprecipitation of Sed5p Protein Complexes*

Experiments were performed essentially as described (Søgaard et al., 1994), except for minor modifications. In brief, *S. cerevisiae* RSY255 (wild-type), RSY271 (*sec18-1*), GWY67 (*uso1-1**), GWY71 (*sec18-1 uso1-1**), and GWY85 (*sec18-1 uso1-1**/p*USO1*) were grown to mid-logarithmic phase in either SC or SC-Ura media supplemented with 1% casamino acids (Difco, Detroit, MI) at 25°C. Cells were harvested, spheroplasted, and incubated in YPD-sorbitol for 1 h at either the permissive (24°C) or restrictive temperature (37°C). The isolated spheroplasts were lysed in buffer D (Søgaard et al., 1994) and debris was removed by centrifugation. The extracts were collected and frozen in aliquots in liquid nitrogen. Spheroplast detergent extracts (60 μg of protein) were diluted in buffer E (Søgaard et al., 1994) and rotated overnight with 20 μl of affinity-purified anti-Sed5p antibodies coupled to protein A–Sepharose with dimethylpimelimidate (Harlow and Lane, 1988). Beads were washed four times with buffer E, and eluted twice with 0.15 ml of 0.1 M glycine (pH 2.4). The eluates were pooled, precipitated with 10% (wt/wt) trichloroacetic acid, and resuspended in Laemmli sample buffer. Proteins were resolved by SDS-12% PAGE and electrophoretically transferred onto nitrocellulose membrane. Membranes were probed with 1,000-fold diluted, affinity-purified anti-Sec22p and anti-Bet1p antiserum. Immunoblots were developed using chemiluminescent detection (Renaissance; DuPont-NEN, Boston, MA).

## *Results*

### *Ypt1p and the ER to Golgi v-SNAREs Can Suppress a Uso1p Defect*

We undertook a genetic analysis of *USO1* to determine how Uso1p fits into the molecular framework of proteins already known to function in the yeast secretory pathway, and to potentially identify new genes involved in secretion. We began by searching for genes that could suppress the temperature-sensitive phenotype of the yeast *uso1-1* mutant when overexpressed. This strain harbors a nonsense mutation in the *USO1* gene that causes the Uso1p protein to be truncated after approximately one-fourth of its coiled-coil domain (Seog et al., 1994). To identify multicopy suppressors of *uso1-1*, a total of 29,000 transformants were replica-plated to YPD at the restrictive temperature of 37°C (see Materials and Methods for details). From these, 355 temperature-resistant colonies were isolated. After retesting the temperature-sensitive phenotype, 305 of the isolates were still resistant. Plasmids from the 173 transformants exhibiting the strongest suppression were isolated and retransformed into the *uso1-1* strain. Upon retransformation, 132 plasmids were able to confer temperature resistance in a plasmid dependent manner.

Since it had been previously demonstrated that Uso1p functions in ER to Golgi transport (Nakajima et al., 1991), we investigated whether any of the plasmid-linked suppressors contained genes known to function in this portion of the secretory pathway. To this end, all suppressor plasmids were hybridized with probes corresponding to a panel of genes known to function in ER to Golgi or intra-Golgi transport. The probes were *BET1*, *BOS1*, *SEC7*, *SEC17*, *SEC22*, *SED5*, *SLY1*, *SLY41*, and *USO1*. This hybridization procedure revealed that six of the plasmids contained the *USO1* gene itself (data not shown). In addition, the collection contained six plasmids bearing *BET1*, five bearing *SEC22*, and one bearing *BOS1*. Interestingly, *BET1*, *BOS1*, and *SEC22* encode proteins that are localized to ER-derived vesicles and are putative v-SNARE molecules (Lian and Ferro-Novick, 1993; Rexach et al., 1994). Furthermore, all three proteins appear to be components of the yeast ER to Golgi v-SNARE/t-SNARE complex (Søgaard et al., 1994). The other 114 plasmids did not hybridize with any of the probes (data not shown).

The remaining collection of 114 suppressor plasmids

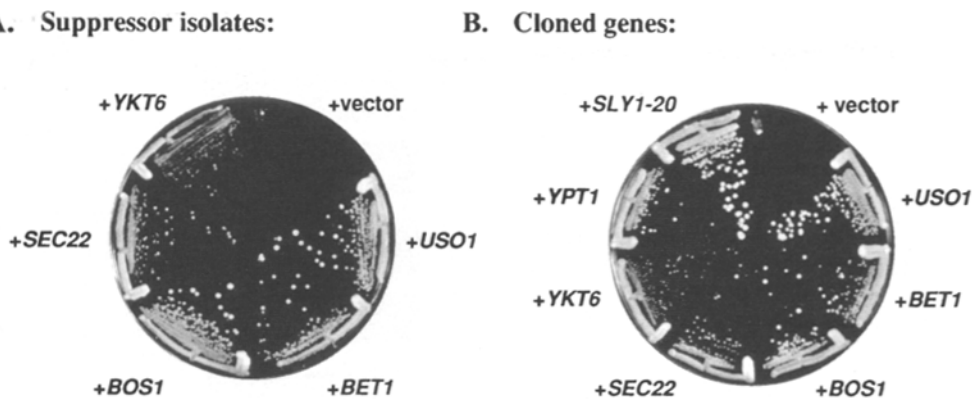


*Figure 1.* Suppression of the temperature-sensitive growth phenotype of the *uso1-1* mutant. (*A*) Suppression of *uso1-1* by plasmids isolated in the multicopy suppressor screen. (*B*) Suppression of *uso1-1* by the individual suppressor genes, each isolated from their genomic background. All plasmids were 2 μm except for p*SLY1-20* which was a CEN plasmid. Plates were incubated for 3 d at 37°C.

was subjected to restriction mapping to identify duplicates; 27 plasmids were eliminated in this manner. Of the remaining 88 plasmid-linked suppressors, we sequenced the ends of the inserts from the 48 strongest suppressors. In this manner we hoped to potentially identify the genomic region borne by these plasmids and thus, the candidate open reading frames responsible for the suppression phenotype. Sequence analysis revealed that several regions of the genome were represented multiple times among this group. One of these regions, a segment from chromosome XI, contains the *YKT6* gene. Ykt6p is also a member of the yeast ER to Golgi SNARE complex (Søgaard et al., 1994), and based on its homology to Sec22p, Ykt6p is considered to be a v-SNARE.

Therefore, overexpression of each of four different plasmids, each encoding a putative ER to Golgi v-SNARE (Bet1p, Bos1p, Sec22p, and Ykt6p,), was able to suppress the temperature-sensitive phenotype of the *uso1-1* mutant (Fig. 1 *A*). Multicopy *BET1* and *BOS1* are able to suppress the temperature-sensitive growth phenotype as well as the *USO1* gene itself (Fig. 1 *A*). In comparison to these genes, *SEC22* and *YKT6* appear to suppress this phenotype somewhat more weakly (Fig. 1 *A*).

The remaining group of suppressor plasmids have been found to contain 13 distinct chromosomal regions. Two of the inserts, however, cannot be localized to a sequenced chromosomal region since they do not align with any sequences currently in the yeast sequence database. We are currently focusing our attention on the only two suppressors which have demonstrated an ability to suppress the *uso1-1* transport defect.

To confirm the results of the multicopy suppression screen and to extend our analysis, we examined whether a number of genes previously shown to function in the yeast secretory pathway could suppress the *uso1-1* mutation. For this analysis, isolated genes, rather than genomic fragments, were employed. In agreement with the results of the multicopy suppressor screen, we found that *BET1*, *BOS1*, *SEC22*, and *YKT6* were each able to suppress the temperature-sensitive phenotype of the *uso1-1* mutant (Fig. 1 *B*). Again, *BOS1* and *BET1* are somewhat better suppressors of *uso1-1* than either *YKT6* or *SEC22*.

Although *YPT1*, which encodes the small rab-like GTP-binding protein Ypt1p, was not recovered in the multicopy suppressor screen, it was found to be a good suppressor of *uso1-1* (Fig. 1 *B*). Similarly, *SLY1-20*, which contains an activating mutation in *SLY1* (Dascher et al., 1991), was able to suppress the *uso1-1* mutation. For all experiments involving suppression by *SLY1-20* we used a CEN plasmid; suppression by this construct was identical to that observed for a 2 μm *SLY1-20* plasmid (data not shown). Interestingly, *SLY1-20* was originally identified as a mutation that enables the *SLY1* gene to compensate for the reduced levels of Ypt1p found in cells after galactose shut-off of a *GAL10-YPT1* expression plasmid (Dascher et al., 1991). Wild-type *SLY1*, however, which encodes a t-SNARE–associated protein (Søgaard et al., 1994), has no effect on the temperature sensitivity of either the *uso1-1* or the *ypt1-3* mutant (data not shown). We also found that multicopy *ARF1*, *SAR1*, *SEC7*, *SEC17*, *SEC18*, *SEC21*, *SED5*, *SFT1*, *SNC1*, and *SNC2* were unable to suppress the *uso1-1* strain.

The suppression data are summarized in Table II. The temperature-sensitive phenotype of the *uso1-1* mutant was not suppressed by overexpression of "fusion components" (Sec17p or Sec18p), by overexpression of Golgi to plasma membrane v-SNAREs (Snc1p or Snc2p), by overexpression of a putative intra-Golgi v-SNARE (Sft1p), or by the

*Table II. Genes Tested for Their Ability to Suppress the Temperature-sensitive Growth Defect of uso1-1 Cells*

| | Gene | Function of gene product |
|---|---|---|
| Do Suppress: | | |
| | *USO1* | ER-Golgi docking/fusion |
| | *BOS1* | ER-Golgi v-SNARE |
| | *BET1* | ER-Golgi v-SNARE |
| | *SEC22* | ER-Golgi v-SNARE |
| | *YKT6* | Putative ER-Golgi v-SNARE |
| | *YPT1* | ER-Golgi docking/fusion regulator |
| | *SLY1-20* | Gain of function mutation in *SLY1* |
| Do Not Suppress: | | |
| | *SED5* | ER-Golgi t-SNARE |
| | *SLY1* | ER-Golgi t-SNARE–associated protein |
| | *SEC17* | SNAP |
| | *SEC18* | NSF |
| | *SNC1* | Golgi-PM v-SNARE |
| | *SNC2* | Golgi-PM v-SNARE |
| | *SFT1* | Putative intra-Golgi v-SNARE |
| | *SEC7* | ER through Golgi factor |
| | *ARF1* | Low MW GTPase involved in COPI budding |
| | *SEC21* | COPI subunit |
| | *SAR1* | COPII-associated GTP binding protein |

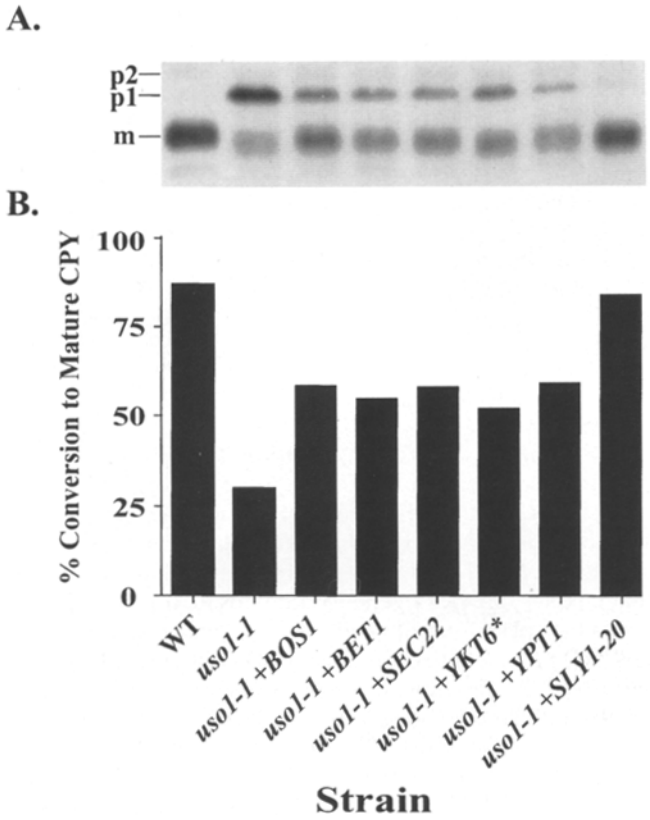


*Figure 2.* Suppression of the *uso1-1* transport defect. (*A*) Autoradiograph of carboxypeptidase Y (CPY) immunoprecipitations from wild-type cells, *uso1-1* cells, or *uso1-1* cells transformed with each of the suppressor plasmids. All plasmids were 2 μm except for p*SLY1-20* which was a CEN plasmid. Cells were shifted to the restrictive temperature of 38°C, pulse labeled for five minutes, and allowed to chase for 20 min at the same temperature. CPY was isolated from cell extracts by immunoprecipitation and resolved by SDS-PAGE. The different forms of CPY are indicated on the left. (*p1*) The core-glycosylated form, ER form; (*p2*) the outer-chain glycosylated Golgi form, and m: the mature, vacuolar form. (*B*) Quantitation of the ability of the suppressor genes to restore maturation of CPY in *uso1-1* cells. All three species of CPY (p1, p2, and mature) were quantitated by Phosphorimager analysis with background subtraction. The percentage of mature CPY was then calculated for each lane as follows: % conversion to mature = mature CPY/(p1 CPY + p2 CPY + mature CPY) × 100. In three independent experiments, the relative ability of each of the suppressors to restore transport was similar. *The *YKT6* gene in this strain is in the genomic background in which it was isolated from the multicopy suppressor screen.

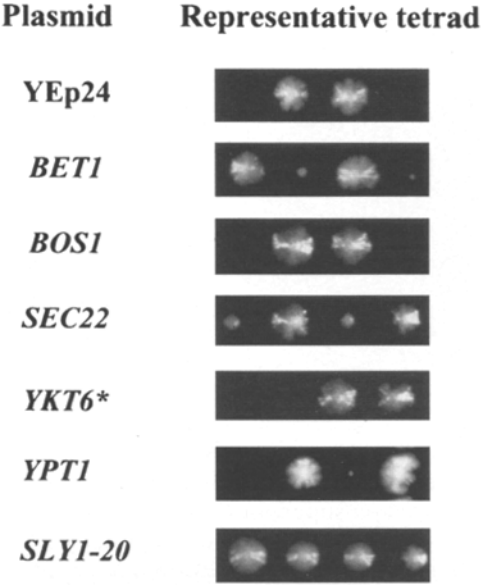


*Figure 3.* Suppression of the inviability of *Δuso1*. A heterozygous *USO1/Δuso1::LEU2* diploid strain (GWY32) was transformed with plasmids bearing each of the genes to be tested. All of the plasmids used were 2 μm, except for the *SLY1-20* plasmid, which was a CEN plasmid. The resulting strains were sporulated and dissected onto YPD plates. In all cases, except for *YPT1*, the dissected tetrads were allowed to grow for 6 d at room temperature. After 6 d, tetrads from the *YPT1* bearing strain displayed 2:2 segregation. However, when the segregants were allowed to grow for longer, small Leu$^+$ colonies became apparent. The tetrad shown here contains a single small colony and was photographed after 9 d incubation at room temperature. The genotype of all viable colonies was tested for available markers and mating type to ensure that the segregation of each was as anticipated. In all cases a minimum of eight tetrads were scored.

overexpression of several vesicle budding components (Arf1p, Sar1p, Sec21p). Therefore, all of the factors able to suppress *uso1-1* appear to be specific for the targeting/docking step of ER to Golgi transport.

### *Restoration of ER to Golgi Transport in the uso1-1 Mutant*

Since overexpression of the ER to Golgi v-SNAREs, Ypt1p, and Sly1-20p suppressed the growth defect of *uso1-1* at the restrictive temperature, we investigated whether this ability correlated with an ability to suppress the secretory defect of this mutant strain. To do so we examined the processing of the well-characterized vacuolar protease CPY. In wild-type cells the earliest glycosylated form of CPY, called p1 CPY, is associated with the ER (Stevens et al., 1982). p1 CPY is then further glycosylated in the Golgi to generate the p2 form, which is subsequently proteolytically processed in the vacuole to generate mature CPY.

To monitor the ability of each of these strains to process CPY to its mature form a pulse-chase analysis was performed using a wild-type strain, the *uso1-1* strain, and *uso1-1* strains bearing plasmids containing the different suppressor genes. Cells were grown at the permissive temperature (24°C), then shifted to the restrictive temperature (38°C) and immediately pulse-labeled for 5 min with Tran$^{35}$S-label. After a 20-min chase at the restrictive temperature, the cells were lysed, and CPY was immunoprecipitated and analyzed by SDS-PAGE and fluorography (Fig. 2). Whereas wild-type cells had processed 85% of the radiolabeled CPY to the mature form, *uso1-1* cells had matured only 30% of the CPY; the balance of the CPY was in the p1 form indicating that it had not exited the ER (Fig. 2). This observation is consistent with the previously reported accumulation of the ER form of invertase in the *uso1-1* mutant (Nakajima et al., 1991). Compared to the *uso1-1* strain, the overexpression of each of the v-SNAREs or Ypt1p resulted in generation of twice the amount of

mature CPY, ~60%. Notably, expression of Sly1-20p was able to suppress the transport defect almost completely (Fig. 2). These results suggest that the ability of the *BET1*, *BOS1*, *SEC22*, *YKT6*, and *SLY1-20* to suppress the *uso1-1* growth defect stems directly from their ability to restore, at least partially, ER to Golgi transport.

### *Suppression of a Deletion of USO1*

To determine whether the proteins encoded by the *uso1-1* suppressors were able to bypass the requirement for Uso1p, we tested whether any of them suppress the lethality of a *USO1* deletion. The ability of one gene to suppress a complete deletion of another suggests that the function of the suppressor is downstream of the missing gene product. To test this, a heterozygous *USO1/Δuso1* diploid, in which the disrupted *Δuso1* allele is marked with the *LEU2* gene (*Δuso1::LEU2*), was transformed with each of the *uso1-1* multicopy suppressor plasmids. The resulting strains were sporulated and dissected. Representative tetrads are shown in Fig. 3.

The *USO1/Δuso1::LEU2* strains containing *BOS1* or *YKT6* on 2-μm plasmids yielded two viable Leu$^-$ spores and two inviable spores (Fig. 3), indicating that overexpression of these genes was unable to suppress the *Δuso1 null* allele. Microscopic examination of the inviable spores revealed the presence of microcolonies containing two or more cells, indicating that these spores were able to germinate and divide at least once. Therefore, their inability to grow into visible colonies was due to a vegetative growth defect. In contrast, for tetrads resulting from the dissection of diploids containing *SEC22* or *BET1* on 2 μm plasmids we observed two normal sized Leu$^-$ colonies and two slowly growing Leu$^+$ colonies (Fig. 3). Therefore, two of the v-SNAREs weakly suppress the *Δuso1* allele, indicating that they function are downstream of Uso1p.

In addition to two of the v-SNAREs, the *Δuso1* allele could also be suppressed by overexpression of Ypt1p. However, this suppression was somewhat weaker than that observed for *BET1* or *SEC22*. Whereas small Leu$^-$ colonies were detected in the *BET1* and *SEC22* containing strains after 5 d of growth at room temperature, colonies were not observed for the *YPT1* bearing strain until after one week (Fig. 3). Furthermore, the frequency of these small Ura$^+$Leu$^-$ colonies was low; in 23 tetrads only five such colonies were observed after 9 d at room temperature.

Finally, tetrad dissection of *USO1/Δuso1::LEU2* strain containing p*SLY1-20* produced normal-sized Leu$^+$ colonies (Fig. 3). Therefore, *SLY1-20*, which is the best suppressor of the *uso1-1* temperature sensitivity and the *uso1-1* transport defect, is also the best suppressor of a complete deletion of *USO1*. Taken together, these results indicate the v-SNAREs Sec22p and Bet1p, the t-SNARE associated protein Sly1p, and the rab-like GTP-binding protein Ypt1p function downstream of Uso1p.

Interestingly, the multicopy suppression of the *Δuso1* allele follows a pattern similar to that observed for the *Δypt1* allele. Specifically, *BET1*, *SEC22*, and *SLY1-20*, are also able to suppress the functional loss of Ypt1p (Dascher et al., 1991). This led to their designation as *SLY* genes (for suppressor of loss of Ypt1p function); *SEC22* is allelic to *SLY2*, and *BET1* is allelic to *SLY12* (Dascher et al., 1991).

### *The uso1-1 Suppressors Are ypt1-3 Suppressors*

All but one of the multicopy suppressors of *uso1-1* have previously been demonstrated to interact genetically with *YPT1* (Dascher et al., 1991; Newman et al., 1990). To examine this observation in a systematic fashion we determined the ability of each of the *uso1-1* suppressors to allow growth of the temperature-sensitive *ypt1-3* strain (Rexach et al., 1994) at the restrictive temperature. As shown in Fig. 4, all of the *uso1-1* suppressors can also suppress the temperature-sensitive phenotype of the *ypt1-3* strain. *SLY1-20* is the strongest *ypt1-3* suppressor, followed closely by *SEC22* and *BOS1*. *BET1* and *YKT6* are somewhat weaker suppressors, and *USO1* is the weakest. There are two notable differences between the patterns observed for suppression of the *ypt1-3* and *uso1-1* temperature-sensitive alleles. First, *SEC22* is a better suppressor of *ypt1-3* than of *uso1-1*. Second, whereas *YPT1* is a strong suppressor of *uso1-1*, *USO1* is a very weak suppressor of *ypt1-3*.

### *Genetic Interactions of USO1 and YPT1*

The finding that the same set of genes can suppress either *ypt1-3* or *uso1-1* temperature sensitivity, as well as the observation that the same subset of genes can suppress a deletion of either *USO1* or *YPT1*, suggests that Uso1p and Ypt1p may function in the same process. This is supported by the ability of *YPT1* and *USO1* to suppress temperature sensitive alleles of each other. Based on these findings we analyzed the genetic interaction between *USO1* and *YPT1* in more detail.

Although *USO1* had not been identified as a suppressor of *Δypt1* in the original *SLY* screen (Dascher et al., 1991), we tested whether *USO1* is able to suppress a loss of Ypt1p. We examined this in two ways. First, we assessed the ability of multicopy *USO1* to suppress reduced levels of Ypt1p, which was the method employed in the original *SLY* screen. For this purpose, we used a haploid strain that contains *YPT1* under the control of the *GAL10* promoter. Due to the essential nature of the *YPT1* gene, this

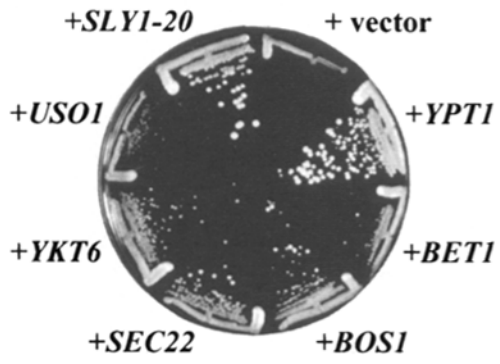


*Figure 4.* Suppression of *ypt1-3* by the same panel of suppressor genes shown in Fig. 1 *A*. Cells were plated on YPD and permitted to grow for 3 d at 37°C. All plasmids were 2 μm except for p*SLY1-20* which was a CEN plasmid.

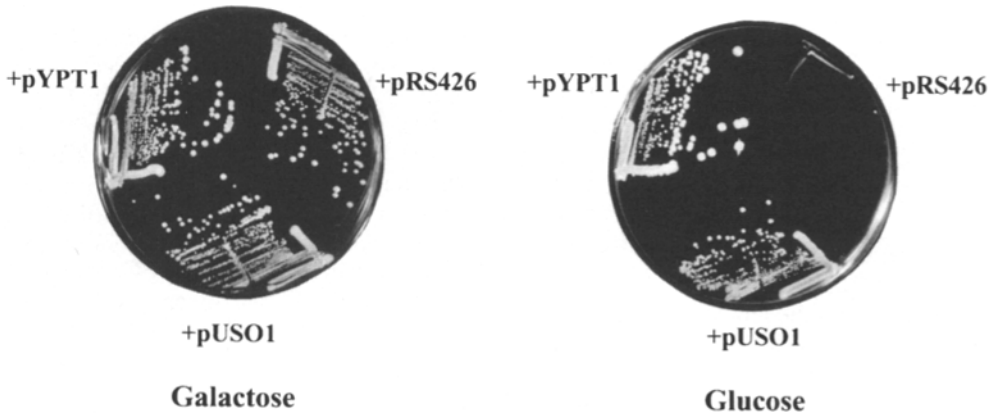


*Figure 5.* Overexpression of Uso1p can suppress reduced levels of Ypt1p. All sectors contain GFUI-6D cells, which contains *GAL10-YPT1::HIS3* at the *YPT1* locus. These cells also contain either the 2 μm vector (pRS426; Christianson et al., 1992), pSK47 (2 μm *USO1 URA*), or pNB167 (2 μm *YPT1 URA*). (*left*) On galactose media, which permits expression of Ypt1p. (*right*) On glucose media, which represses expression of Ypt1p.

strain is viable only on galactose media, which induces the expression of *YPT1*. In contrast, cells grown on glucose media express low, but detectable, levels of Ypt1p (Ossig et al., 1995). We transformed this strain with either 2 μm p*YPT1*, 2 μm p*USO1*, or the 2 μm vector alone and tested the ability of each strain to grow on galactose media and glucose media. As expected, all three strains were able to grow equally well on media containing galactose, when Ypt1p is expressed (Fig. 5, *left*). On glucose media, however, the strain containing the vector alone was unable to grow, whereas the strains containing the *YPT1* plasmid or the *USO1* plasmid were able to grow (Fig. 5, *right*). This result indicates that overexpression of *USO1* is able to compensate for reduced levels of wild-type Ypt1p, a result consistent with suppression of *ypt1-3* by *USO1*.

To test the ability of *USO1* to suppress the complete loss of Ypt1p, a heterozygous *YPT1/Δypt1::LEU2* diploid was transformed with 2 μm *USO1*, 2 μm *YPT1*, or the 2-μm vector alone, and then sporulated and dissected. Upon dissection, all tetrads from the strains containing either the vector alone or the *USO1* plasmid contained only two viable Leu$^-$ spores (data not shown) indicating that *USO1* cannot suppress a *Δypt1* allele. Thus, although high levels of Uso1p can compensate for low levels of Ypt1p activity, some Ypt1p function is required for this suppression by Uso1p to occur. This suggests that Uso1p exerts its function through, or in conjunction with, Ypt1p.

We also determined whether mutant alleles of the *USO1* and *YPT1* exhibit a synthetic lethal interaction with one another. Both *uso1-1* and *ypt1-3* are able to grow well at 24 and 30°C, but exhibit a temperature-sensitive growth phenotype at 34°C or higher. To test the phenotype of the double mutant, we crossed the strains bearing the individual *uso1-1* and *ypt1-3* mutant alleles. Diploids resulting from the *uso1-1/ypt1-3* cross were sporulated, dissected, and then tested for temperature sensitivity and for the presence of the *uso1-1* and *ypt1-3* alleles by complementation. Colonies derived from this dissection are shown in Fig. 6 (*left*). In cases where four spores grew at 24°C, all were inviable at 37°C, indicating that each spore contained a single mutant allele (parental ditype); this was confirmed by complementation. In several cases, three spores were able to grow at 24°C, yet only one of the three spores was viable at 37°C (tetratype). The three viable spores always consisted of one wild-type, one *uso1-1*, and one *ypt1-3*; by inference, the inviable spore was the *uso1-1 ypt1-3* double mutant. Finally, some tetrads yielded a 2 viable:2 inviable segregation pattern at both 24 and 37°C (non-parental ditypes); complementation indicated that the viable segregants were wild-type, and by inference, the inviable spores were the *uso1-1 ypt1-3* double mutants.

To be certain that the synthetic lethal interaction was due exclusively to the interaction of *uso1-1* and *ypt1-3* we transformed the *uso1-1/ypt1-3* diploid with either *USO1* or *YPT1* 2 μm plasmids and performed tetrad analysis of the resulting strains (Fig. 6, *right*). As expected, all spores from the transformed strain were able to grow at 24°C since all of the double mutant segregants were covered by either p*YPT1* or p*USO1*. However, when these colonies were replica plated to 5-FOA media, which forces cells to

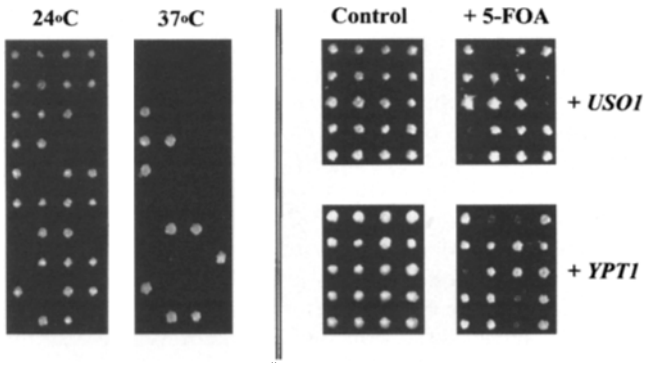


*Figure 6. uso1-1* and *ypt1-3* exhibit a synthetic lethal interaction. (*left*) The two mutant strains were crossed and the resulting diploid was sporulated and dissected. The tetrads were permitted to grow at room temperature, and then were replica-plated to YPD at 37°C to determine which colonies contained temperature-sensitive alleles. (*right*) To verify that the synthetic lethal phenotype observed was due to mutations in the *USO1* and *YPT1* genes, the *USO1/uso1-1 ypt1-3/YPT1* diploid was transformed with either p*USO1 URA3* (pSK47) or p*YPT1 URA3* (pNB167). The resulting strains were sporulated and dissected. The dissected tetrads were permitted to grow on selective media (*control*). They were then replica plated to 5-FOA plates to force the loss of the plasmids. Plates were incubated at 24°C for 3 d. In a separate experiment the genotype of all temperature-sensitive segregants was confirmed by complementation. In all cases the genotype of these spores was either *uso1-1* or *ypt1-3*.

lose the plasmids, the synthetic phenotype was uncovered (Fig. 6, *right*). This synthetic lethal interaction underscores the existence of a strong genetic interaction between *USO1* and *YPT1* and suggests that Uso1p and Ypt1p may function in the same step of ER to Golgi transport.

### *Uso1p Is Required for Assembly of the SNARE Complex*

Taken together, our genetic data suggest that Uso1p and Ypt1p function at the same step in vesicular transport upstream of the v-SNAREs, and that Uso1p may act through, or in concert with Ypt1p. Previously, it has been suggested that Ypt1p is required for assembly of the ER to Golgi SNARE complex because v-SNARE/t-SNARE complexes were not demonstrable in *ypt1-3* cells (Søgaard et al., 1994). Furthermore, it has been proposed that Ypt1p mediates SNARE complex assembly through specific activation of the v-SNAREs Bos1p and Sec22p (Lian et al., 1994). Based on the similar suppression profiles of the *USO1* and *YPT1* genes, we were interested to determine whether Uso1p is also required for assembly of the ER to Golgi SNARE complex. To address this question, wild-type, *uso1-1*, *sec18-1*, and *uso1-1 sec18-1* cells (with or without a 2 μm *USO1* plasmid) were grown to mid-logarithmic phase and spheroplasted. The cells were then incubated for 1 h at either the permissive (24°C) or restrictive (38°C) temperature. After incubation, the cells were lysed and the t-SNARE Sed5p was immunoprecipitated with affinity-purified antibodies. A control reaction without anti-Sed5p antibodies was performed as well. The immunoprecipitates were then analyzed for their Bet1p and Sec22p content (v-SNAREs) by immunoblotting with specific antibodies (Fig. 7). In wild-type cells neither Bet1p nor Sec22p was associated with Sed5p at either temperature (*top*), although these proteins were detected in the supernatant of the immunoprecipitation (*bottom*) indicating that they were present. The fact that the v-SNAREs are not associated with Sed5p in wild-type cells is expected since the v-SNARE/t-SNARE complex should exist only transiently in wild-type cells. Likewise, no complex accumulated in *sec18-1* cells grown at the permissive temperature. However, in agreement with previously published data (Søgaard et al., 1994), *sec18-1* cells accumulated the Sed5p/Sec22p/Bet1p complex at the restrictive temperature. Presumably, complex accumulation occurs at the restrictive temperature because the mutant version of Sec18p, which is the yeast equivalent of NSF, cannot disassemble the complex as a prelude to membrane fusion (Söllner et al., 1993*a*,*b*). The *uso1-1* strain incubated at either the permissive or restrictive temperature did not accumulate the complex. However, since the *uso1-1* strain contains wild-type Sec18p, even if the complex could assemble in these cells, it would not accumulate. Therefore, to determine whether Uso1p was required for assembly of the complex, we tested a *uso1-1 sec18-1* double mutant. In this strain, if the v-SNARE/t-SNARE complex formed it would accumulate at the restrictive temperature, due to the absence of Sec18p activity. The absence of complex formation in the double mutant would indicate a defect in its formation. Indeed, we found that *uso1-1 sec18-1* double mutant cells failed to accumulate the Sed5p/Sec22p/Bet1p complex at the restrictive temperature. Identical results were obtained when the immunoprecipitations were performed with anti-Bos1p antibodies (data not shown). As a final control to confirm that the inability to form a v-SNARE/t-SNARE complex was due to the *uso1-1* mutation we tested a *uso1-1 sec18-1* double mutant strain containing a

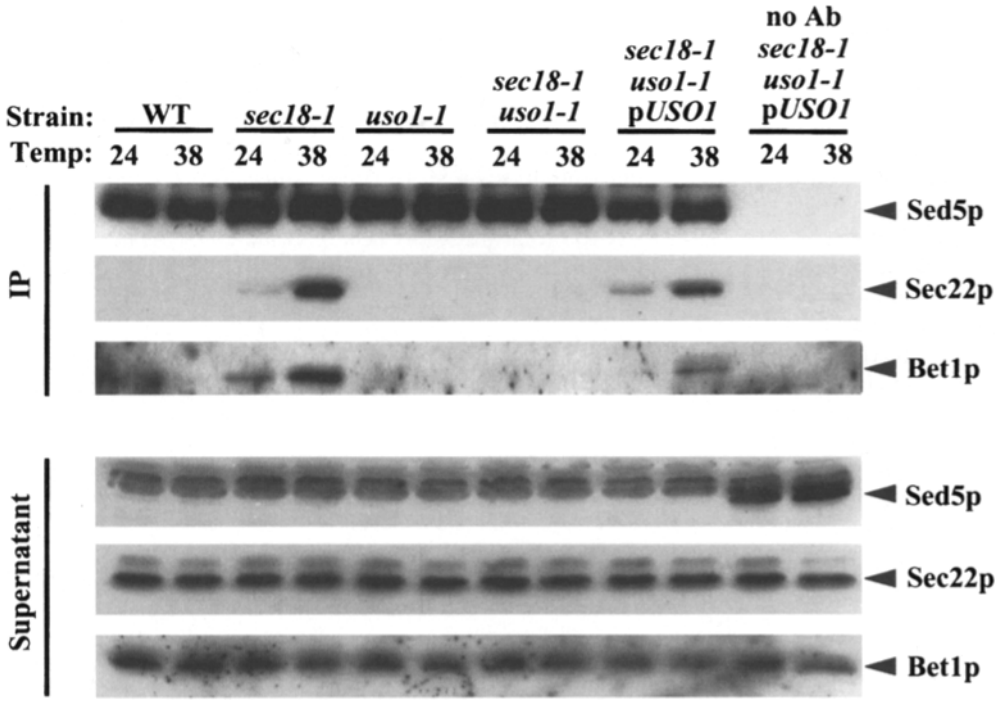


*Figure 7.* Uso1p is required for assembly of the ER to Golgi SNARE complex. Wild-type (RSY255), *sec18-1* (RSY271), *uso1-1* (GWY67), *uso1-1 sec18-1* (GWY71), or *uso1-1 sec18-1*/p*USO1* (GWY71 + pSK47 (2 μm *USO1 URA3*)) spheroplasts were incubated at 24 or 38°C for 1 h, and then lysed with Triton X-100. 60 μg of each detergent extract was immunoprecipitated with anti-Sed5p antibodies covalently coupled to protein A–Sepharose beads (20 μl). Immunoprecipitated proteins were loaded on a SDS-12% PAGE gel. The separated polypeptides were transferred to nitrocellulose and analyzed by Western blotting using affinity-purified antibodies against Sed5p, Sec22p and Bet1p (*top*). One-half of the supernatant from each immunoprecipitation reaction was also subjected to PAGE and immunoblotted (*bottom*). Bound antibodies were visualized using chemiluminescence.

*USO1* plasmid. In this strain, the SNARE complex once again accumulated at the restrictive temperature. Therefore, functional Uso1p is required for formation of the ER to Golgi v-SNARE/t-SNARE complex.

## Discussion

Uso1p was identified in a novel screen for secretion mutants and has been shown to be required for ER to Golgi transport (Nakajima et al., 1991). It is homologous to the mammalian transport factor p115 (or TAP), which is required for the docking or fusion step of intra-Golgi transport (Elazar et al., 1994; Waters et al., 1992), for binding of transcytotic vesicles to the plasma membrane (Barroso et al., 1995), and for the reassembly of the Golgi apparatus from dispersed Golgi fragments after mitosis (Rabouille et al., 1995). The precise biochemical function of neither p115 nor Uso1p has been determined. To further elucidate how Uso1p, and possibly p115, function in membrane trafficking, we undertook an analysis of Uso1p function in yeast.

We performed a biochemical analysis to determine whether assembly of the ER to Golgi v-SNARE/t-SNARE complex is affected in *uso1-1* mutant cells. To do this we used a yeast strain that has a temperature-sensitive mutation in the *SEC18* gene, which encodes the yeast homologue of NSF (Wilson et al., 1989). The Sec18p ATPase acts to disassemble the v-SNARE/t-SNARE complex (Söllner et al., 1993*a*,*b*) thereby allowing membrane fusion to ensue. By immunoprecipitation of the t-SNARE Sed5p, and examination of whether the v-SNAREs Bet1p and Sec22p are associated with it, we have found, in agreement with the results of Søgaard et al. (1994), that Bet1p and Sec22p are not associated with Sed5p in wild-type cells; this is most likely due to the transient nature of the targeting complex in the presence of active Sec18p. In contrast, when the same experiment is performed in a *sec18-1* mutant strain at the restrictive temperature, where Sec18p is inactive, Bet1p and Sec22p coimmunoprecipitate with Sed5p. Preparation of a double mutant strain containing both the *sec18-1* and *uso1-1* mutations allowed us to perform an epistasis experiment to determine whether functional Uso1p is required for assembly of the v-SNARE/t-SNARE complex. Indeed, we found that the presence of the *uso1-1* allele prevented the accumulation of the SNARE complex that is normally evident in a *sec18-1* strain at the restrictive temperature. SNARE complex assembly was restored by complementing the *uso1-1* mutation with *USO1* on a plasmid. From these results we conclude that functional Uso1p, like Ypt1 (Søgaard et al., 1994), is required for assembly of the ER to Golgi v-SNARE/t-SNARE complex.

Although the v-SNARE/t-SNARE complex is unable to form in the absence of Uso1p function, Uso1p does not appear to be a member of the SNARE complex. Using a version of the *sec18-1 uso1-1* strain that contained a 2-μm plasmid encoding epitope-tagged Uso1p, we were unable to detect Uso1p in the complex which accumulates at the restrictive temperature (data not shown). This is consistent with the absence of Uso1p in the yeast ER to Golgi SNARE complex (Søgaard et al., 1994), as well as with the absence of p115 in the mammalian SNARE complex (Söllner et al., 1993*b*).

Genetic analysis of *USO1* and its relationship to other genes involved in ER to Golgi transport (summarized in Table III) has provided insight into how Uso1p may facilitate SNARE complex assembly. We have found that overexpression of each of the known yeast ER to Golgi v-SNAREs (Bet1p, Bos1p, Sec22p, and Ykt6p) confers temperature resistance, as well as partial restoration of ER to Golgi transport, to *uso1-1* mutant cells. The simplest explanation for this multicopy suppression is that high levels of v-SNARE molecules can increase v-SNARE/t-SNARE complex assembly by virtue of mass action (see Fig. 8). Such enhanced assembly may be able to compensate, at least partially, for the loss of Uso1p function. This interpretation is supported by the finding that two of the v-SNAREs, Bet1p and Sec22p, can suppress a complete deletion of Uso1p. Overexpression of these v-SNAREs was also found to suppress the loss of Ypt1p (Dascher et al., 1991), albeit to a lesser degree (Dascher et al., 1991). The other two v-SNAREs, Bos1p and Ykt6p were able to suppress the temperature-sensitive alleles of *USO1* and *YPT1*, but were unable to suppress either the *uso1* null or the *ypt1* null. Although both of these proteins, along with Sec22p and Bet1p, are known to be members of the ER to Golgi SNARE complex (Søgaard et al., 1994), our results suggest that Bos1p and Ykt6p are functionally different from the other two v-SNAREs, Bet1p and Sec22p. Furthermore, since overexpression of Bet1p and Sec22p is able to suppress the *uso1* and *ypt1* null alleles, it appears that the v-SNAREs do not operate through Uso1p, but rather, that they function downstream of Uso1p. Consistent with this is the fact that overexpression of *USO1* is not able to suppress the temperature sensitivity of the v-SNARE mutants *sec22-3*, *bet1-1*, or *bos1-1* (data not shown).

In contrast to the v-SNAREs, overexpression of the t-SNARE Sed5p did not suppress a *uso1-1* mutation. This might be construed as conflicting with the idea that SNARE assembly can be driven by mass action. However, we believe this paradox can be resolved by considering the two activities of Sly1p. First, since *SLY1* is an essential gene (Dascher et al., 1991), and mutations in Sly1p disrupt ER to Golgi transport (Ossig et al., 1991), it must have a positive role in promoting this vesicular transport event.

*Table III. Summary of Multicopy Suppression Data*

| 2 μm plasmid | *uso1-1** | *ypt1-3** | *Δuso1*‡ |
|---|---|---|---|
| *YEp24* | − | − | − |
| *USO1* | +++ | + | +++ |
| *BET1* | ++ | ++ | ++ |
| *SEC22* | + | +++ | ++ |
| *BOS1* | ++ | ++ | − |
| *YKT6*§ | + | + | − |
| *SLY1-20*‖ | +++ | +++ | +++ |
| *YPT1* | +++ | +++ | + |

*Suppression of the temperature-sensitive phenotype.
‡Suppression of lethality.
§The *YKT6* gene in this plasmid is contained in the genomic fragment as it was isolated from the multicopy suppressor screen.
‖For suppression of the null allele, a CEN plasmid was used. For suppression of temperature sensitivity both CEN and 2 μm plasmids were tested; identical results were obtained.

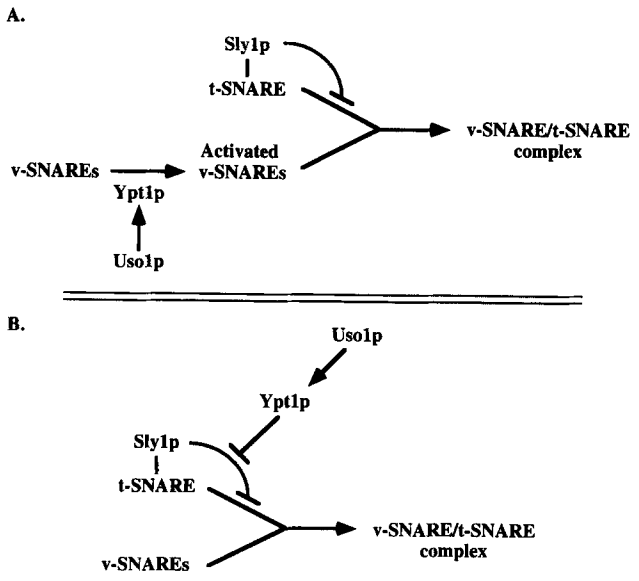


*Figure 8.* Potential sites of action for Uso1p and Ypt1p in assembly of the ER to Golgi SNARE complex. (*A*) Uso1p may facilitate the action of Ypt1p in v-SNARE activation. (*B*) Uso1p and Ypt1p may act by relieving the inhibitory effect that Sly1p imparts on v-SNARE/t-SNARE complex formation (see discussion for further explanation).

Secondly, Sly1p appears to act as a negative regulator of ER to Golgi transport because a dominant gain of function allele, *SLY1-20*, can abrogate the requirement for both Ypt1p (Ossig et al., 1991), and Uso1p in vesicle docking. A plausible model (Dascher et al., 1991; Søgaard et al., 1994) to explain these observations is that Sly1p, which is found in association with the t-SNARE Sed5p (Søgaard et al., 1994), impacts on Sed5p activity in a regulated fashion. Thus, the association of Sly1p with Sed5p may prevent illicit interactions of Sed5p with v-SNAREs until Sly1p is converted to another conformation, perhaps one similar to that of Sly1-20p, that permits Sed5p to interact with the v-SNAREs. Similarly, n-sec1 (the neuronal homologue of Sly1p) appears to regulate the ability of syntaxin (the neuronal homologue of Sed5p) to interact with VAMP (the neuronal homologue of the v-SNAREs) (Pevsner et al., 1994). In support of this model is our finding that overexpression of wild-type Sly1p (the inhibitory conformation) was unable to suppress the *uso1-1* mutation, while expression of even moderate levels of Sly1-20p (the stimulatory conformation) can completely restore ER to Golgi transport in the *uso1-1* mutant. Therefore, it appears that the lack of v-SNARE/t-SNARE complex assembly in *uso1-1* cells can be compensated for by either increasing the concentration of any of the v-SNAREs, or by increasing the effective concentration of the "active" t-SNARE.

Interestingly, all of the *uso1-1* suppressors (with the exception of *YKT6*) had been previously described as having some genetic interaction with *YPT1* (Dascher et al., 1991). In this study we have shown that all of the *uso1-1* suppressors can suppress the *ypt1-3* temperature-sensitive phenotype and that *uso1-1* and *ypt1-3* mutations display a synthetic lethal interaction (Fig. 6). These data, together with our observation that Uso1p, like Ypt1p (Lian et al., 1994; Søgaard et al., 1994), is required for assembly of the ER to Golgi v-SNARE/t-SNARE complex, suggest that Uso1p and Ypt1p function in the same process. We have also observed that overexpression of Uso1p can suppress reduced levels of Ypt1p, but it cannot compensate for the complete loss of Ypt1p. In contrast, the overexpression of *YPT1* is capable of weakly suppressing the complete loss of Uso1p. Thus, it appears that Uso1p requires some minimal amount of Ypt1p to impact on SNARE assembly, but that Ypt1p, when present at high levels, can function in the complete absence of Uso1p. Therefore, Uso1p most likely functions upstream of Ypt1p. An alternative possibility is that Uso1p functions in conjunction with Ypt1p, and that the high levels of Ypt1p, even in the absence of Uso1p, impart sufficient function to allow slow growth.

What is the nature of the interaction between Uso1p and Ypt1p? Since Ypt1p is a member of the rab family of small GTP-binding proteins, its function should be regulated by other proteins. These would include a GTPase activating protein (GAP), a GTP dissociation inhibitor (GDI), and a GDP dissociation stimulator (GDS) (Nuoffer and Balch, 1994). Uso1p is unlikely to be the GDI for Ypt1p since it is known that Sec19p can function as a Ypt1p GDI (Garrett et al., 1994). Potential GAP or GDS activities of Uso1p however remain to be examined. Another possibility is that Uso1p impacts on Ypt1p indirectly, perhaps through an as of yet undiscovered factor.

Since Uso1p functions in ER to Golgi transport, and its mammalian homolog p115 functions in both intra-Golgi transport and transcytosis, we, and others, had proposed that p115 is a general transport factor (Barroso et al., 1995; Sapperstein et al., 1995). By analogy, if Uso1p is a general transport factor, one would predict that overexpression of ER to Golgi SNAREs might facilitate movement of proteins from the ER to the Golgi, but that later intra-Golgi transport steps would still be defective. Surprisingly, we have not observed a defect in intra-Golgi transport when the *uso1-1* secretory defect was suppressed by overexpression of the ER to Golgi v-SNAREs (see Fig. 2). Therefore, either Uso1p is specifically required for the ER to Golgi transport step, or it is required for multiple steps with the ER to Golgi step being the most sensitive to perturbations in Uso1p function.

Fig. 8 presents two models for how Ypt1p and Uso1p could function to regulate v-SNARE/t-SNARE complex assembly. The first model (Fig. 8 *A*), based on the proposal of Lian et al. (Lian et al., 1994), and consistent with the experiments presented here, posits that Uso1p and Ypt1p activate the v-SNAREs (perhaps by facilitating their association) rendering them competent for interaction with the t-SNARE. Alternatively (Fig. 8 *B*), Uso1p and Ypt1p may impact on Sly1p, perhaps to relieve an inhibitory effect of Sly1p on SNARE complex assembly. In this regard, it is noteworthy that *SLY1-20*, the dominant gain of function allele of *SLY1*, is the best suppressor of *ypt1-3* and *uso1-1* temperature sensitivity, and of suppression of the *uso1-1* transport defect. The models depicted in Fig. 8 are, however, not mutually exclusive: Uso1p and Ypt1p could impact on both v-SNARE activation and the relief of t-SNARE inhibition, perhaps in concert, to promote assembly of the v-SNARE/t-SNARE complex. In addition, Uso1p could also function as a docking checkpoint by monitoring whether the correct v-SNAREs are

present on the vesicle, or whether the v-SNAREs have been sufficiently activated.

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