**The EMBO Journal** Vol.17 No.8 pp.2156–2165, 1998

# Initial docking of ER-derived vesicles requires Uso1p and Ypt1p but is independent of SNARE proteins

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**ER-to-Golgi transport in yeast may be reproduced *in vitro* with washed membranes, purified proteins (COPII, Uso1p and LMA1) and energy. COPII coated vesicles that have budded from the ER are freely diffusible but then dock to Golgi membranes upon the addition of Uso1p. LMA1 and Sec18p are required for vesicle fusion after Uso1p function. Here, we report that the docking reaction is sensitive to excess levels of Sec19p (GDI), a treatment that removes the GTPase, Ypt1p. Once docked, however, vesicle fusion is no longer sensitive to GDI. *In vitro* binding experiments demonstrate that the amount of Uso1p associated with membranes is reduced when incubated with GDI and correlates with the level of membrane-bound Ypt1p, suggesting that this GTPase regulates Uso1p binding to membranes. To determine the influence of SNARE proteins on the vesicle docking step, thermosensitive mutations in Sed5p, Bet1p, Bos1p and Sly1p that prevent ER-to-Golgi transport *in vitro* at restrictive temperatures were employed. These mutations do not interfere with Uso1p-mediated docking, but block membrane fusion. We propose that an initial vesicle docking event of ER-derived vesicles, termed tethering, depends on Uso1p and Ypt1p but is independent of SNARE proteins.**

*Keywords*: COPII/SNARE proteins/Uso1p/vesicles/Ypt1p

## Introduction

Transport vesicle intermediates shuttle cargo between intracellular organelles through cycles of budding and membrane fusion. Vesicle budding from intracellular compartments is catalyzed by distinct sets of coat proteins that are shed prior to vesicle fusion (Schekman and Orci, 1996). For intracellular vesicle fusion, a conceptual framework is emerging whereby a set of general fusion factors, such as NSF/Sec18p and SNAP/Sec17p, act in concert with a related cast of compartment-specific factors, such as Rab/Ypt proteins and SNARE proteins (Rothman, 1994). The underlying mechanisms of intracellular membrane fusion appear to be conserved both within a species and from yeast to mammals, although a molecular description of this process is incomplete (for review, see Pfeffer, 1996). We are investigating this basic cell biological process through elucidation of vesicle fusion to the Golgi complex in the yeast *Saccharomyces cerevisiae*.

Several lines of experimental evidence indicate that vesicular transport from the ER to the Golgi in yeast is mediated by an ordered series of reactions: vesicle budding; vesicle docking; and membrane fusion (Kaiser and Schekman, 1990; Rexach and Schekman, 1991). The first reaction in this process, vesicle budding, may be reproduced *in vitro* with ER membranes and a set of soluble factors (Sar1p, Sec13p complex and Sec23p complex) that generate transport intermediates, termed COPII coated vesicles (Salama *et al*., 1993; Barlowe *et al*., 1994). The vesicle intermediates produced *in vitro* are distinct from the donor membranes in that they are enriched for cargo protein over resident ER protein. Furthermore, these intermediates are competent for fusion with the Golgi complex in a reaction requiring cytosol, ATP and GTP (Barlowe *et al*., 1994; Rexach *et al*., 1994; Lupashin *et al*., 1996). This assay was employed to isolate a minimal set of soluble proteins (Uso1p, Sec18p and LMA1) that replace the cytosol requirement for vesicle fusion. For overall ER-to-Golgi transport in yeast semi-intact cells, COPII proteins are required in addition to the isolated fusion factors, indicating that vesicle fusion is coupled to vesicle budding. Uso1p mediates vesicle docking and produces a dilution-resistant intermediate. Sec18p and LMA1 are not required for the docking phase, but are required for efficient fusion of vesicles (Barlowe, 1997).

These isolated fusion factors represent some, but not all, of the genetic requirements for fusion of ER-derived vesicles, established by analyses of secretion-defective cells (Kaiser *et al*., 1997). In this report, we use a reconstituted ER-to-Golgi transport assay to examine the roles of Sed5p, Sly1p, Bet1p, Bos1p and Ypt1p through selective inactivation of their functions. The integral membrane protein, Sed5p, is related to a family of target-SNARE (t-SNARE) proteins, and functions in transport from the ER to the Golgi complex *in vivo* (Hardwick and Pelham, 1992). Sly1p is a member of the Sec1 family of proteins, is peripherally associated with membranes and is required for ER-to-Golgi transport *in vivo* and *in vitro* (Dascher *et al*., 1991; Mizuta *et al*., 1994; Lupashin *et al*., 1996). Bet1p and Bos1p are essential ER-to-Golgi vesicle-SNAREs (v-SNAREs; Newman *et al*., 1990; Shim *et al*., 1991; Lian and Ferro-Novick, 1993), and *in vitro* experiments with purified proteins demonstrate that Bet1p increases the affinity of Bos1p binding to Sed5p (Stone *et al*., 1997). Ypt1p is essential for two steps in the yeast secretory pathway, ER-to-Golgi transport and transport between Golgi compartments (Segev *et al*., 1988). Ypt1p belongs to the Rab/Ypt family of GTPases which, in general, are thought to ensure the fidelity of vesicle targeting (Bourne *et al*., 1991; Rexach and Schekman, 1991; Segev, 1991). Immunoprecipitation experiments

from detergent lysates demonstrate Sed5p, Bet1p, Bos1p and Sly1p are in a stable SNARE complex when Sec18p is inactive (Sogaard *et al*., 1994). Ypt1p is proposed to regulate the assembly of this SNARE complex and a fraction of Ypt1p is also detected in association with Sed5p (Lian *et al*., 1994; Sogaard *et al*., 1994; Lupashin and Waters, 1997). After inactivation of Ypt1p, we find that Uso1p binding to membranes is prevented and ER-derived vesicles fail to dock. However, inactivation of Sed5p, Sly1p, Bet1p and Bos1p do not interfere with vesicle docking but prevent subsequent fusion. These results indicate that Uso1p mediated docking of ER-derived vesicles requires Ypt1p but does not rely directly on SNARE protein function.

## Results

### *GDI inhibits ER-to-Golgi transport, but does not affect vesicle budding*

Ypt1p, a small GTPase belonging to the Ypt/Rab family, is required for ER-to-Golgi transport *in vivo* (Segev *et al*., 1988) and *in vitro* (Baker *et al*., 1990). Yeast semi-intact cells prepared from a *ypt1-ts* strain or wild-type reactions inhibited by the addition of anti-Ypt1p Fabs accumulate ER-derived vesicles (Rexach and Schekman, 1991; Segev, 1991; Rexach *et al*., 1994), suggesting that Ypt1p is involved in docking ER-derived vesicles to the Golgi. We wanted to examine the role of Ypt1 in the Uso1p-dependent docking reaction using an ER-to-Golgi transport assay that depends on washed membranes and purified proteins (Barlowe, 1997). We first chose to inhibit Ypt1p activity by addition of GDI (Sec19p), since this inhibitor may be added at various stages of the transport reaction and allows us to establish a kinetic relationship of Ypt1p function with vesicle docking and membrane fusion. Excess levels of GDI extract GDP-bound forms of Rab/Ypt proteins from membranes and form soluble heterodimers *in vitro* (Araki *et al*., 1990; Sasaki *et al*., 1990). Elevated levels of GDI have been shown to extract a broad range of Rab/Ypt proteins and inhibit several intracellular transport reactions (Ullrich *et al*., 1993; Dirac-Svejstrup, 1994; Peter *et al*., 1994, Haas *et al*., 1995). Physiological levels of GDI in cells are thought to participate in the general regulation of the Rab/Ypt GTPase cycle and are essential for cell viability (Novick *et al*., 1980; Garrett *et al*., 1994).

First, we titrated the level of GDI required to inhibit our reconstituted ER-to-Golgi transport assay. In these experiments, washed semi-intact cells containing [$^{35}$S]gp-α-factor were incubated with COPII proteins, Uso1p, LMA1 and an ATP/GTP regeneration system. Additional Sec18p is not required in this assay because semi-intact cells contain ample membrane-bound Sec18p (Barlowe, 1997). Under these conditions, GDI concentrations >40 μg/ml inhibit transport (Figure 1A). Increasing the amount of purified GDI resulted in a dose-dependent inhibition of transport. To demonstrate that GDI inhibition of the reconstituted ER-to-Golgi transport assay was specific to later stages and not formation of ER-derived vesicles, we measured the rate of vesicle formation (Figure 1B) in the presence or absence of 200 μg/ml GDI. In this experiment, washed semi-intact cells containing [$^{35}$S]gp-α-factor were incubated with COPII proteins and an ATP/GTP regeneration system. Over time, ER-derived

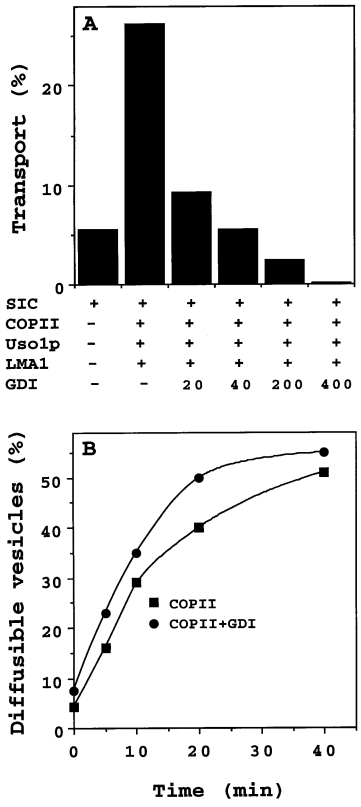


**Fig. 1.** GDI inhibits ER-to-Golgi transport, but not budding. (**A**) Transport in semi-intact cells (SIC) incubated with purified proteins (COPII, Uso1p and LMA1) in the presence or absence of increasing amounts of GDI (μg/ml) at 23°C for 1 h. The percent vesicle fusion represents the amount of [$^{35}$S]gp-α-factor that has been modified by the addition of Golgi-specific α-1,6-mannose residues. (**B**) Budding from semi-intact cells incubated with COPII in the presence (●) or absence (■) of GDI (200 μg/ml) at 23°C for various times. The percentage of diffusible vesicles represents the amount of [$^{35}$S]gp-α-factor released into a medium-speed supernatant fraction divided by the total amount of [$^{35}$S]gp-α-factor contained in the reaction.

vesicles containing [$^{35}$S]gp-α-factor were released as diffusible intermediates that remained in the supernatant fraction after centrifugation at 18 000 *g*. A concentration of GDI that inhibits transport does not reduce the rate or extent of budding driven by COPII. Experiments using crude cytosol to drive vesicle budding and transport produced a similar result (data not shown). Therefore, we conclude that GDI inhibits transport, but not vesicle budding.

### *GDI inhibits vesicle docking, not fusion*

Next, we studied the influence of GDI on the processes of vesicle docking and fusion. ER-derived vesicles dock to Golgi membranes and produce a dilution-resistant intermediate in the presence of Uso1p (Barlowe, 1997). To determine the effect of GDI on vesicle docking (Figure 2A), semi-intact cells were incubated with COPII while varying the order of Uso1p and GDI additions. Incubations with COPII and Uso1p, followed by addition of buffer at indicated time points, shows that the vesicles produced were efficiently docked under this condition (Figure 2A, open squares) whereas the COPII plus GDI condition produces vesicles that remained freely diffusible (Figure 2A, open circles). Reactions containing COPII

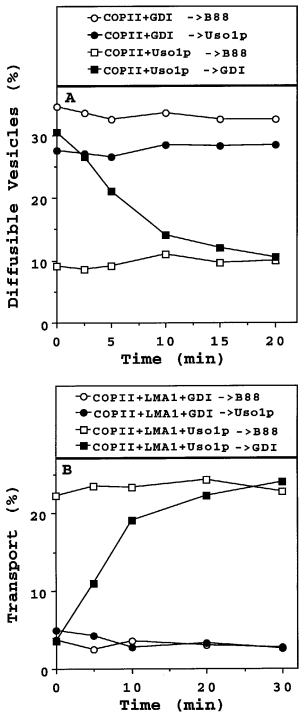


**Fig. 2.** GDI inhibits vesicle docking, not fusion. (**A**) Vesicle docking in semi-intact cells varying the order of Uso1p and GDI addition. Reactions were started with COPII and Uso1 or COPII and GDI. At indicated times, buffer (B88), Uso1p or GDI was added and incubations were continued for a total of 30 min. After stopping reactions on ice, the level of [$^{35}$S]gp-α-factor contained in freely diffusible vesicles was quantified. (**B**) Transport in semi-intact cells incubated with COPII, LMA1 and varying the order of Uso1p and GDI addition. Reactions were started with COPII, LMA1 and Uso1p or COPII, LMA1 and GDI. At indicated times, buffer (B88) or Uso1p or GDI was added and incubations were continued for a total of 90 min. The percentage transport was quantified after precipitation of outer-chain modified forms of [$^{35}$S]gp-α-factor.

plus GDI, followed by addition of Uso1p at indicated times, did not reduce the level of diffusible vesicles (Figure 2A, closed circles), and shows that GDI prevents docking. Finally, mixtures containing COPII plus Uso1p, followed by a time course of GDI addition, revealed a temporal sensitivity to GDI. Thus, GDI inhibits the docking reaction catalyzed by Uso1p but, once docked, GDI can not reverse this reaction.

Because GDI prevents vesicle docking, the effects of this inhibitor on transport should exhibit similar kinetics (Figure 2B). In this experiment, LMA1 was included to drive vesicle fusion, and GDI was added at indicated time points. Again, addition of GDI at early time points inhibited fusion due to a failure of vesicles to dock. At ~10 min, vesicle docking had neared completion (Figure 2A, squares), and indeed, transport was largely insensitive to GDI after this time. This result suggests that membrane fusion is not sensitive to GDI. However, the conditions of this experiment would not prevent fusion should it occur instantly after docking, since LMA1 is present at the start of reactions. To address this possibility, we formed docked intermediates with COPII and Uso1p for 30 min, then added GDI and LMA1 simultaneously, to test the effect of GDI on LMA1-catalyzed fusion. As seen in Figure 3, addition of LMA1 at the start of the reaction or after 30 min with GDI produced efficient

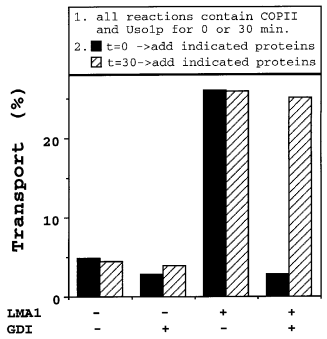


**Fig. 3.** GDI does not inhibit vesicle fusion when added after vesicle docking. Semi-intact cells were incubated with COPII and Uso1p for 0 or 30 min, then GDI, LMA1 or GDI and LMA1 was added and incubations continued for a total of 90 min. The percentage transport was determined as in Figure 1.

fusion (compare hatched bars, columns 3 and 4). In summary, once ER-derived vesicles have docked, GDI cannot reverse the docked species, nor can it interfere with the membrane fusion reaction even if added concomitantly with LMA1.

### *GDI extracts Ypt1p and prevents Uso1p binding*

Based on the above observations, we speculate that Ypt1p regulates Uso1p membrane association in docking ER-derived vesicles to the Golgi complex. If Ypt1p comprises a binding site for Uso1p, then removal of this GTPase should reduce the amount of Uso1p associated with membranes. Uso1p behaves as a peripheral membrane protein in cell fractionation studies (Seog *et al*., 1994). Most of the Uso1p partitions to a 100 000 *g* supernatant fluid, but a significant fraction of this species sediments with the 100 000 *g* pellet. To determine the influence of GDI on Uso1p membrane association, we employed a medium speed supernatant fraction containing cytosol and Golgi membranes that is active for vesicle docking and fusion. Incubation of this extract with GDI reveals a dose-dependent extraction of Ypt1p and a corresponding decrease in Uso1p from the 100 000 *g* pellet fraction (Figure 4A). The GDI treatment does not affect the integrity of Golgi membranes as evidenced by equal recovery of the Golgi localized integral membrane protein Emp47p (Schroder *et al*., 1995). Furthermore, GDI does not act as a general chaotropic agent because the level of peripherally associated Sly1p is not affected by this treatment.

GDI extraction is specific for the GDP forms of Rab/Ypt proteins and is not effective if the GTPase is bound to a non-hydrolyzable analog of GTP, such as GTPγS (Araki *et al*., 1990; Garrett *et al*., 1993). Pre-incubation of extracts with GTPγS (0.4 mM) reduced the level of extractable Ypt1p and mirrored the level of extractable Uso1p. In similar experiments, we have examined Uso1p binding to mutant *ypt1-3* membranes, and found that reduced levels of both Ypt1p and Uso1p bound after incubation at permissive and restrictive temperatures (data not shown). Therefore, we conclude that the membrane association of Uso1p correlates strictly with the level of membrane-bound Ypt1p. Uso1p could bind directly to Ypt1p or, alternatively, Ypt1p could regulate an Uso1p receptor complex.

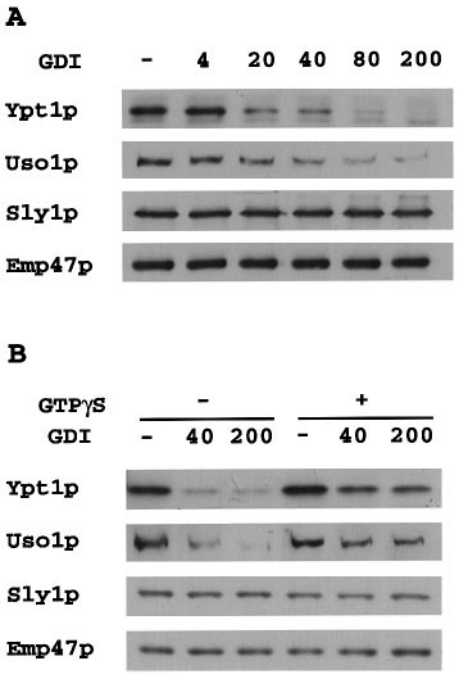


**Fig. 4.** GDI treatment of membranes. (**A**) A medium speed supernatant fraction prepared from a yeast lysate of strain CBY324 was incubated with increasing amounts of GDI (μg/ml) at 20°C for 30 min. Membranes were isolated by centrifugation at 100 000 *g* and the resulting pellets washed with Buffer 88 and spun again. The final pellet was resuspended with SDS–PAGE sample buffer and resolved on 12.5 or 7% polyacrylamide gels. Immunoblot analyses using the ECL detection method document the content of individual proteins contained in the membrane pellet. (**B**) The experiment is similar to (A), except lysates were pre-incubated with GTPγS (0.4 mM) prior to the addition of GDI.

### *SNARE molecules are not required for Uso1p docking*

Uso1p and Ypt1p represent some, but not all, of the requirements for fusion of ER-derived vesicles. Genetic and biochemical approaches have implicated Sec22p, Bet1p, Bos1p, Sly1p, Sed5p and Ykt6, in this step of ER-to-Golgi transport (Newman *et al*., 1990, 1992; Dascher *et al*., 1991; Hardwick *et al*., 1992; Sogaard *et al*., 1994). Several of these proteins are found in a stable SNARE complex when isolated from a *sec18-1* strain (Sogaard *et al*., 1994). Interestingly, Uso1p and Ypt1p are not found in a stable association with the isolated SNARE complex (Sapperstein *et al*., 1995; Lupashin *et al*., 1997). We speculate that the SNARE proteins are not required for docking of ER-derived vesicles since Sec18p was not required for formation of an Uso1p dilution-resistant intermediate (Barlowe, 1997). However, there are alternative explanations for this result depending on the model for Sec18p function that is favored. The SNARE hypothesis proposes that pairing of v-SNARE with t-SNARE docks vesicles in a Sec18p/NSF-independent process, followed by recruitment of Sec18p/NSF to form the 20S complex. ATP hydrolysis by Sec18p/NSF dissociates SNARE proteins and permits membrane fusion (Sollner *et al*., 1993; Sogaard, 1994). Therefore, Sec18p-independent docking is consistent with the SNARE hypothesis as proposed by Rothman and colleagues (Sollner *et al*., 1993). However, recent studies suggest Sec18p is indeed required for ATP-catalyzed separation of SNARE proteins, but that this

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sly1(258) | R | G | V | **L** | I | I | L | **D** | **R** | N | I | **D** | F |
| rSly1(261) | R | P | L | **L** | V | L | V | **D** | **R** | N | I | **D** | L |
| Unc18(227) | R | S | Q | **L** | L | I | L | **D** | **R** | G | F | **D** | P |
| cUnc18(225) | R | S | Q | **L** | I | I | I | **D** | **R** | G | Y | **D** | A |
| n-Sec1(227) | R | S | Q | **L** | L | I | L | **D** | **R** | G | F | **D** | P |
| scSec1(244) | R | S | I | **L** | I | I | T | **D** | **R** | T | L | **D** | P |
| Vps45(226) | T | P | V | **L** | L | I | L | **D** | **R** | N | T | **D** | P |
| mVps45(212) | P | P | L | **L** | L | I | L | **D** | **R** | C | D | **D** | A |
| Rop(238) | R | S | Q | **L** | L | I | L | **D** | **R** | G | F | **D** | C |

**Fig. 5.** *sly1-ts* mutation alters an invariant arginine residue. Amino acid sequence of Sly1p surrounding the temperature-sensitive mutation and comparison with selected homologues. Sly1p contains five domains that share high sequence identity (25–47%) with a family of related proteins. In the *sly1-ts* strain, arginine 266 (highlighted) is converted to a lysine. The amino acid numbers in parentheses correspond to the first residue shown, and the abbreviations are as follows: Sly1, Sly1p from *S.cerevisiae* (Z48784); rSly1, rat Sly1p (U57687); Unc18, *Caenorhabditis elegans* unc-18 (S66176); cUnc18, *Caenorhabditis briggsae* unc-18 homologue (D63505); n-Sec1, neuronal Sec1p homologue from rat (A53455); scSec1, *S.cerevisiae* Sec1p (P30619); Vps45, *S.cerevisiae* Vps45p (U07972); mVps45, mouse Vps45p homologue (U66865); Rop, Ras2 opposite protein from *Drosophila melanogaster* (S33578).

reaction activates SNARE proteins and precedes docking (Mayer *et al*., 1996; Ungermann *et al*., 1998). Regardless of the precise temporal mode of Sec18p action, both models postulate that SNARE proteins dock donor and acceptor membranes. We pursued a series of experiments to determine if Sed5p (t-SNARE) and the Sed5p associated protein, Sly1p, participate in formation of Uso1p docked vesicles.

The *sed5-1* allele was isolated through an *in vitro* mutagenesis approach and is due to a single point mutation (R255G) that results in temperature-sensitive (ts) growth. At 25°C, *sed5-1* strains grow and transport secretory proteins whereas growth is inhibited at 37°C and ER-forms of secretory proteins accumulate (Banfield *et al*., 1995). The *sly1-ts* allele was identified in a screen for ribosome-synthesis mutants, and it was subsequently determined that several mutations blocking the secretory process, in general, inhibit expression of ribosomal subunits (Mizuta and Warner, 1994). Again, *sly1-ts* strains grow at 25°C but when shifted to 37°C, growth is inhibited and ER-forms of secretory proteins accumulate (Mizuta and Warner, 1994). We recovered the *sly1-ts* allele for sequence analysis and determined that temperature sensitivity is due to a single point mutation (R266K) in Sly1p that alters an invariant amino acid residue found in the Sec1p family of proteins (Figure 5). It remains to be determined whether mutation of this residue in other family members produces temperature-sensitive phenotypes. Examination of the *sly1-ts* strain indicates that the expression level and subcellular fractionation behavior of the Sly1p-R266K protein is indistinguishable from the wild-type species (data not shown). Both the *sed5-1* and *sly1-ts* alleles are recessive mutations.

We prepared an isogenic set of strains (wild-type, *ypt1-3*, *sed5-1* and *sly1-ts*) for comparison of vesicle docking and fusion in our reconstituted assay. All strains were grown at 23°C before preparation of semi-intact cells. Addition of purified COPII and fusion factors drove ER-to-Golgi transport in these strains when the reaction temperature was 23°C (Figure 6). However, membranes prepared from the *sed5-1* and *sly1-ts* strains display temperature sensitivity in transport, with a marked inhibition observed at 29°C (Figure 6). For the *ypt1-3* strain, transport was

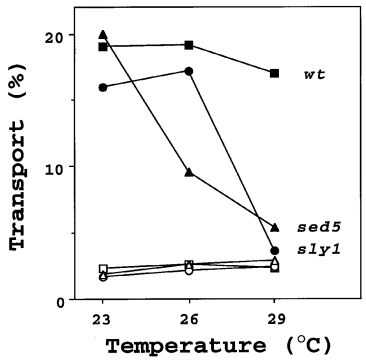


**Fig. 6.** Temperature-sensitive mutations in *SLY1* or *SED5* inhibit ER-to-Golgi transport at an elevated temperature. Semi-intact cells from wild-type (squares), *sed5-1* (triangles) or *sly1-ts* (circles) were incubated alone (open symbols) or with COPII, Uso1p and LMA1 (closed symbols) for 90 min at 23, 26 or 29°C (restrictive temperature). The percentage transport was quantified after precipitation of outer-chain modified forms of [$^{35}$S]gp-α-factor.

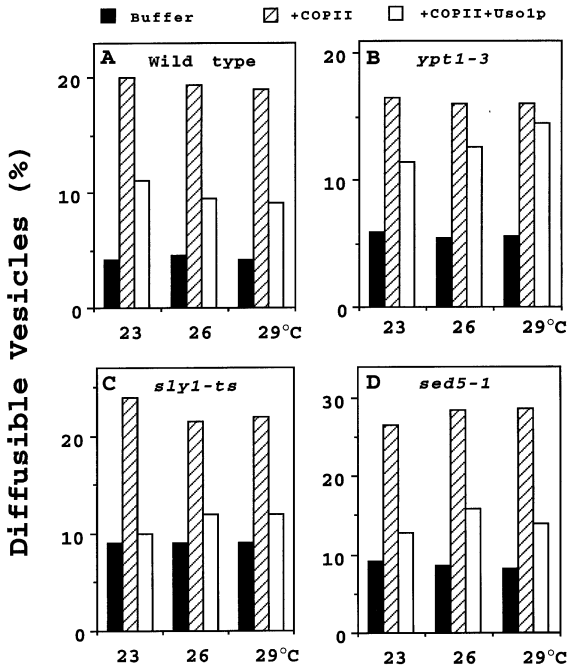


**Fig. 7.** Ypt1p, but not Sly1p and Sed5p, is required for vesicle docking. (**A**) Wild-type semi-intact cells (SIC) were incubated alone (dark bars), with COPII (hatched bars) or COPII plus Uso1p (open bars) at 23, 26 and 29°C for 30 min. Freely diffusible vesicles containing [$^{35}$S]gp-α-factor were separated from semi-intact cells by centrifugation at 18 000 *g* for 3 min and quantified by Con A precipitation. (**B**) Docking in semi-intact cells prepared from *ypt1-3*. (**C**) Docking in semi-intact cells prepared from *sly1-ts*. (**D**) Docking in semi-intact cells prepared from a *sed5-1* strain, as in (A).

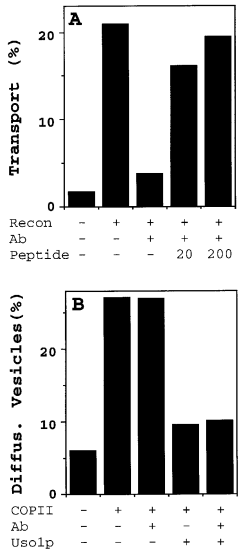


**Fig. 8.** Sed5p antibodies block vesicle fusion but not docking. (**A**) ER-to-Golgi transport in wild-type semi-intact cells that were incubated with COPII proteins, Uso1p and LMA1 (Recon), 250 μg/ml anti-Sed5p immune IgG (Ab) or competitor Sed5p peptide (20 or 200 μg/ml) as indicated. The percent transport was quantified after precipitation of outer-chain modified forms of [$^{35}$S]gp-α-factor. (**B**) Levels of freely diffusible vesicles in wild-type semi-intact cells that were incubated with COPII proteins, 250 μg/ml anti-Sed5p immune IgG (Ab) or Uso1p as indicated. Freely diffusible vesicles containing [$^{35}$S]gp-α-factor were separated from semi-intact cells by centrifugation at 18 000 *g* for 3 min and quantified by Con A precipitation.

reduced even at permissive temperatures, but completely blocked at 29°C (data not shown). This reduced level of transport at 23°C is probably due to lower amounts of mutant Ypt1p in these cells (Sogaard *et al.*, 1994). These results indicate that loss-of-function mutations in Ypt1p, Sly1p and Sed5p prevent delivery of [$^{35}$S]gp-α-factor to the Golgi complex. To distinguish between possible defects in vesicle docking and fusion, we measured the level of freely diffusible ER-derived vesicles in the presence and absence of Uso1p (Figure 7). Docking in the *ypt1-3* strain was inhibited at 29°C and reduced compared with a wild-type strain at 23°C (Figure 7A and B). Vesicle docking achieved through Uso1p function was efficient in *sly1-ts* and *sed5-1* strains at permissive and restrictive temperatures (Figure 7C and D). Furthermore, docking remains equally sensitive to GDI treatment in the mutant strains at 29°C as was observed in wild-type reactions at 23°C (data not shown). In summary, these results suggest docking of ER-derived vesicles does not directly require Sed5p and Sly1p, but subsequent fusion depends on their activity.

As an independent approach to determine the role of Sed5p in vesicle docking and fusion, we used neutralizing antibodies specific for Sed5p to inhibit function. Antibodies were raised against a synthetic peptide comprising the N-terminal 28 amino acid residues of Sed5p and tested for inhibition of *in vitro* transport. A titration of anti-Sed5p (from total immune IgG) showed potent inhibition of reconstituted transport that was maximal at 0.25 mg/ml (data not shown). Furthermore, this inhibition could be alleviated by incubation of immune IgG with the synthetic peptide used to illicit the anti-Sed5p immune response (Figure 8A). Antibody inhibition could be reversed if competitor peptide was added at $t_0$ or after a 20 min incubation under standard assay conditions (data not shown). The reversibility of the anti-Sed5p block demonstrates that this inhibition is specific and does not damage Sed5p or proteins associated with this t-SNARE. When the docking assay was performed in the presence of inhibitory concentrations of anti-Sed5p IgG (Figure 8B), Uso1p reduced the level of freely diffusible vesicles as efficiently as in untreated reactions. The addition of anti-Sed5p IgG does not affect the vesicle budding reaction (Figure 8B, third column), excluding the possibility that the reduction in freely diffusible vesicles was due to

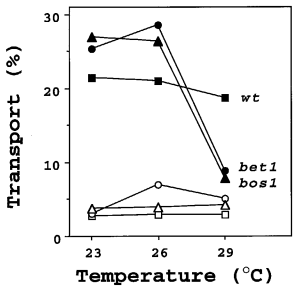


**Fig. 9.** Temperature-sensitive mutations in *BET1* or *BOS1* inhibit ER-to-Golgi transport at an elevated temperature. Semi-intact cells from the wild-type strain RSY255 (squares), *bet1-1* (circles) or *bos1-1* (triangles) were incubated alone (open symbols) or with COPII, Uso1p and LMA1 (closed symbols) for 90 min at 23, 26 or 29 (restrictive temperature). The percentage transport was quantified after precipitation of outer-chain modified forms of [$^{35}$S]gp-α-factor.

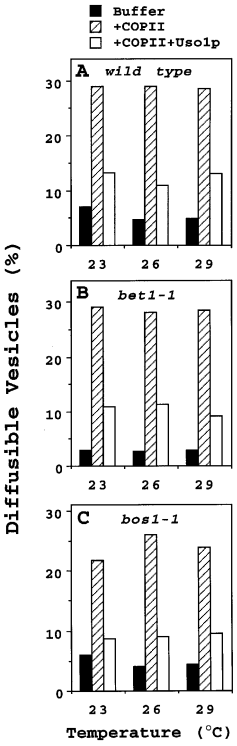


**Fig. 10.** Bet1p and Bos1p are not required for vesicle docking. Wild-type (**A**), *bet1-1* (**B**) or *bos1-1* (**C**) semi-intact cells were incubated alone (dark bars), with COPII (hatched bars) or COPII plus Uso1p (open bars) at 23, 26 and 29 for 30 min. Freely diffusible vesicles containing [$^{35}$S]gp-α-factor were separated from semi-intact cells by centrifugation at 18 000 *g* for 3 min and quantified by Con A precipitation.

inhibition of the budding step. In summary, antibody inhibition of Sed5p function confirms results obtained using the *sed5-1* allele that this t-SNARE is required for membrane fusion but not for vesicle docking.

Our results demonstrate a clear role for Sed5p and Sly1p in overall transport, yet these proteins are not required for the initial vesicle docking stage. However, recent evidence indicates that there is not a strict separation of t-SNAREs to target compartments and v-SNAREs to donor vesicle compartments (Walch-Solimena *et al*., 1995; C.Barlowe and X.Cao, unpublished observation), and we explored the possibility that v-SNARE molecules such as Bet1p and Bos1p provide necessary SNARE contacts during Uso1p-mediated docking. We used temperature-sensitive *bet1-1* and *bos1-1* mutations in our reconstituted docking and transport assays. As with the *sed5-1* and *sly1-ts* alleles, these are recessive loss-of-function mutations *in vivo* (Wuestehube *et al*., 1996). As seen in Figure 9, temperature profiles of these mutant strains in reconstituted transport show efficient transport at 23°C, whereas a marked inhibition was observed in both mutant strains at 29°C compared with their wild-type counterpart strain RSY255). Finally, docking in the *bet1-1* and *bos1-1* semi-intact cells was comparable with wild-type when Uso1p was added to the reactions (Figure 10). Therefore, we conclude that in addition to the t-SNARE protein Sed5p, the v-SNARES Bet1p and Bos1p are not required for initial docking of ER-derived vesicles to the Golgi complex. In contrast, two independent approaches (GDI extraction or mutation of Ypt1p) demonstrate that Ypt1p function is required for this docking reaction.

## Discussion

In this report, we demonstrate that initial docking of ER-derived vesicles depends on Uso1p and Ypt1p, but does not require the SNARE proteins Sed5p, Bet1p or Bos1p. We will refer to this Uso1p-dependent step as 'tethering' to distinguish it from docking reactions mediated by SNARE proteins (Sollner *et al*., 1993). This Uso1p-tethered vesicle is a functional intermediate that is no longer sensitive to GDI, but requires the action of Sed5p, Bet1p, Bos1p, Sly1p, Sec18p and LMA1 for fusion. A model, based on these results and a series of genetic experiments, is proposed as follows. First, uncoated ER-derived vesicles bind Uso1p and are tethered to Golgi membranes in a Ypt1p-dependent process. Next, the tethering event transmits a signal to the SNARE machinery, possibly through activation of Ypt1p. Once this signal is received, SNARE proteins are paired and vesicles fuse through the action of Sec18p and LMA1. This model is consistent with a general hypothesis for intracellular transport where distinct vesicles find their target site more efficiently via GTPase regulated 'Velcro' factors (Pfeffer, 1996). In the case of ER-to-Golgi transport, Uso1p probably represents the Velcro molecule that leads to pairing of v- and t-SNARE proteins. Aspects of this model are now considered in the context of reported genetic and *in vitro* observations.

The participation of Uso1p and Ypt1p in a distinct biochemical reaction prior to Sed5p, Sly1p, Bet1p and Bos1p function is compatible with genetic analyses in yeast. Overexpression of v-SNARE proteins (Sec22, Bet1p or Bos1p) suppress loss-of-function *ypt1* and *uso1* mutations. Furthermore, a gain-of-function *SLY1* allele (*SLY1-20*) obviates the need for Uso1p and Ypt1p (Dascher *et al*., 1991; Sapperstein *et al*., 1996). These results have led to the proposal that an increase in active SNARE proteins bypasses the need for Uso1p and Ypt1p function by mass action (Pfeffer, 1996; Sapperstein *et al*., 1996). This report now correlates these genetic relationships with the biochemically distinguishable steps of vesicle tethering and SNARE protein-dependent fusion. However, there is genetic and biochemical evidence suggesting that proteins involved in vesicle tethering communicate with downstream SNARE proteins. For example, a complete deletion

of *USO1* can be weakly suppressed by *YPT1* overexpression, but the converse does not hold true (Sapperstein *et al*., 1996). Additionally, Ypt1p has been shown to associate transiently with Sed5p (Lupashin and Waters, 1997). Based on these observations, we speculate that the tethering event transmits information to SNARE proteins, making them competent for activation and thereby ensuring correct SNARE assembly only after vesicle tethering. A primary suspect to relay this signal is the small GTPase, Ypt1p. Our results indicate that, once a vesicle has tethered, fusion is no longer sensitive to GDI, possibly due to conversion of Ypt1p to the GTP-bound form. This event may be transduced to the Sed5p–Sly1p complex. In the absence of Uso1p-mediated tethering *in vivo*, overproduction of Ypt1p could yield a significant level of GTP-bound Ypt1p and send a false signal that activates downstream SNARE proteins. We suggest activation of Ypt1p to the GTP-bound form is important for activation of SNARE proteins; however, vesicle fusion may not depend on GTP hydrolysis by Ypt1p. This feature is consistent with *in vivo* results with an activated form of Ypt1p (Richardson *et al*., 1998) and *in vitro* analyses with an XTP-dependent form of Rab5 protein (Rybin *et al*., 1996), indicating GTP hydrolysis by Ypt/Rab proteins is not directly required for membrane fusion.

Surprisingly, ER-derived vesicles remain tethered in the absence of essential SNARE protein function under our conditions. Previous *in vitro* analyses indicate Sec18p action is required for vesicle docking when intermediates of ER-to-Golgi transport were analyzed on sucrose gradients (Rexach and Schekman, 1991). In contrast, we propose Sec18p activity is not required for tethering although the behavior of Uso1p-tethered vesicles on sucrose gradients has not been examined to determine if this intermediate withstands such a treatment. It remains possible that a second stage of docking depends on SNARE proteins and produces a more firmly bound vesicle. We do not think the temperature-sensitive alleles used in these experiments are semi-functional, such that mutant SNARE proteins dock vesicles in a non-productive manner. *In vivo*, such an effect may produce dominant phenotypes where mutant SNARES would engage vesicles but not fuse, and then block access of subsequent ER-derived vesicles. All of the mutations used in these experiments are recessive, loss-of-function mutations. Although we cannot exclude semi-functional activities *in vitro*, the similar behavior displayed by all of the SNARE alleles tested argues against this possibility. Furthermore, as an independent line of evidence, we have used antibody inhibition specifically to prevent Sed5p function. An identical conclusion is reached, based on these inhibition experiments, that Sed5p is not required for Uso1p tethering.

At present, we can not conclude with certainty whether Uso1p binds directly to Ypt1p, or whether Ypt1p is in association with an Uso1p receptor complex. We are continuing to investigate proteins that bind to Uso1p and the orientation of Uso1p with respect to the docked vesicle and Golgi compartment. Uso1p exists as a parallel homodimer in solution with two globular heads and a long C-terminal coiled-coil domain (Seog *et al*., 1994; Yamakawa *et al*., 1996). Two general models for Uso1p action can be envisaged. First, Uso1p may act asymmetrically in vesicle docking such that the tail portion binds to one membrane surface while the globular domain binds to the other. Secondly, Uso1p may tether vesicles through a symmetrical arrangement whereby the head or tail domain binds to both membrane surfaces. Within these two general modes of action, the Uso1p dimer may represent the functional unit, or higher order multimers may form. Since Ypt1p regulates Uso1p binding, a symmetrical model would imply Ypt1p function on both compartments and, at present, there is only evidence to indicate Ypt1p function on the Golgi compartment. Vesicles synthesized from a mutant *ypt1* strain are fully functional for fusion with wild-type Golgi membranes (Rexach *et al*., 1994). Furthermore, we find that treatment of ER-derived vesicles with GDI prior to isolation on density gradients does not reduce their docking and fusion efficiency (C.Barlowe, unpublished observation). Therefore, we favor some form of the asymmetrical model for the docking process of ER-derived vesicles with the Golgi.

How does Sly1p participate in this process? Sly1p is related to a large family of proteins, including Sec1p, and associates with the t-SNARE molecule Sed5p (Sogaard *et al*., 1994). These proteins have been described as a combination of positive and negative regulators (Pevsner *et al*., 1994; Dascher and Balch, 1996; Lupashin and Waters, 1997) that protect the t-SNAREs (Pfeffer, 1996) until an appropriate v-SNARE is presented. The *sly1-ts* allele employed in our experiments is a recessive, loss-of-function mutation and if Sly1p were simply a negative regulator, a loss-of-function mutation could allow tethered vesicles to fuse. However, Sly1p function appears to be required for vesicle fusion in this cell-free reaction after vesicle tethering. Our results are most consistent with a model proposed by Lupahsin and Waters (1997), and we consider Sly1p to be a component of the t-SNARE, such that a conformational change in the Sly1p–Sed5p complex leads to productive interactions with vesicle SNAREs. The nucleotide-bound state of Ypt1p may influence this conformational change.

We speculate that a tethering event precedes SNARE assembly in vesicular transport processes beyond the ER-to-Golgi step. Putative 'Velcro' factors have been identified for endosome–endosome fusion (Stenmark *et al*., 1995) and for fusion of secretory vesicles with the plasma membrane during exocytosis (Terbush and Novick, 1995). For synaptic vesicle exocytosis, a tethering activity has also been proposed (Sudhof, 1995) based on the following evidence. Treatment of nerve terminals with botulinum or tetanus toxin cleaves the cognate v- and t-SNAREs (syntaxin and VAMP) and prevents neurotransmitter release. Interestingly, the cleavage event is not accompanied by release of docked vesicles from the pre-synaptic membrane. Instead, vesicles remain firmly attached (Hunt *et al*., 1994). This may be explained if there are docking proteins that keep synaptic vesicles tethered to the pre-synaptic membrane in the absence of SNARE protein interactions. Similarly, we find that an essential t-SNARE molecule (Sed5p) is not required for stable docking of ER-derived vesicles to the Golgi complex. Using this refined *in vitro* system, we continue to explore the molecular detail of this Uso1p-tethered intermediate.

**Table I.** Yeast strains

| Strain | Genotype | Source |
|---|---|---|
| sed5-1 | *MATα his3-Δ200 leu2-3-112 ura3-52 trp1Δ901 lys2-810 suc2Δ9 sed5-1* | D.Banfield |
| J1003.1d | *MATα trp1-1 ade2-1 ura3-1 leu2-3,112 can1-100* | J.Warner |
| 312 | *MATα trp1-1 ade2-1 ura3-1 leu2-3,112 can1-100 sly1-ts* | J.Warner |
| RSY255 | *MATα ura3-52 leu2-3,–112* | R.Schekman |
| RSY944 | *MATa bet1-1 lys2-801 ura3-52* | R.Schekman |
| RSY954 | *MATa bos1-1 lys2-801 leu2-3,–112* | R.Schekman |
| RSY976 | *MATa ura3-52 ypt1-3* | R.Schekman |
| CBY324 | *MATα his3Δ200 leu2Δ1 lys2Δ202 ura3–52 trp1Δ63 uso1-1 pUSO1-Myc* | this laboratory |
| CBY263 | *MATα trp1-1 ade2-1 ura3-1 leu2-3,112 can1-100 sed5-1* | this study |
| CBY474 | *MATα trp1-1 ade2-1 ura3-1 leu2-3,112 can1-100 ypt1-3* | this study |

## Materials and methods

### General materials and techniques

Yeast strains used in this study are listed in Table I and were grown in rich medium (1% Bacto-yeast extract, 2% Bacto-peptone and 2% dextrose) or selective medium (0.67% nitrogen base without amino acids, 2% dextrose) and required supplements. The *sed5-1* and *ypt1-3* strains were back-crossed multiple times through the W303 derivative J1003.1D (Mizuta and Warner, 1994) to generate an isogenic set of strains for these studies. Rabbit antiserum specific for α-1,6-mannose linkages was prepared by intravenous injection of heated RSY919 cells as described previously (Ballou, 1970). For the preparation of Sed5p specific antibodies, a synthetic peptide that contains the N-terminal 28 residues of Sed5p with an added cysteine at position 29 was used as the antigen. This peptide was coupled to BSA (20:1 molar ratio) using the cross-linker disuccinimidyl suberate, and linked conjugates were isolated on a gel filtration column equilibrated in phosphate buffered saline. The peptide–BSA conjugate (~0.5 mg) was mixed with Freund's complete adjuvant and injected into rabbits, followed by monthly boosts with ~0.2 mg of conjugate in Freund's incomplete adjuvant. Serum isolated from immunized rabbits cross-reacted with a 46 kDa species on immunoblots, and this reactivity was specifically competed by incubation of synthetic peptide with antiserum prior to immunoblots. IgG was purified from serum using a protein A affinity matrix as described (Harlow and Lane, 1988). Antibodies directed against Ypt1p (Rexach *et al*., 1994), Emp47p (Schroder *et al*., 1995) and c-Myc (Evan *et al*., 1985) have been described. Anti-Sly1p serum was a generous gift of H.Dieter Schmitt (Gottingen, Germany). For immunoblots, proteins were resolved by 12.5% SDS–PAGE (Laemmli, 1970), transferred to nitrocellulose filters (Towbin *et al*., 1979) and filter-bound primary antibodies were detected by peroxidase-catalyzed chemiluminescence (ECL method, Amersham).

### Recovery of the sly1-ts allele

The *sly1-ts* mutation was isolated by the gap-repair method (Rothstein, 1991). Plasmid pXC3 contains the complementing 2.5 kb *Spe*I–*Eco*RV fragment from pYep51-*SLY1* (Dascher *et al*., 1991) in pRS315 (Sikorski and Heiter, 1989). Gapped pXC3 (lacking the 1 kb *Pst*I–*Nsi*I fragment) was used to transform strain 312 (*sly1-ts*) to leucine prototrophy and several temperature-sensitive transformants were obtained. Plasmid DNA was isolated from one of these transformants and amplified in *Escherichia coli*. The recovered plasmid (pXC3-*sly1*) contained the full-length gene but could not complement a *sly1-ts* strain for growth at 37°C. Furthermore, a *sly1* null strain harboring pXC3-*sly1* displayed a temperature-sensitive growth phenotype as observed in the original *sly1-ts* strain. Sequencing of pXC3-*sly1* revealed a G to A mutation near the *Pst*I site that changes amino acid 266 from an arginine residue to a lysine residue.

### Protein purification

Yeast GDI (Sec19p) was expressed in *E.coli* and purified as described previously (Garrett *et al*., 1994). The peak fractions were dialyzed against Buffer 88 [20 mM HEPES (pH 7.0), 150 mM KOAc, 5 mM MgOAc] containing 1 mM DTT and 1 mM PMSF. Aliquots were frozen in liquid nitrogen and stored at –70°C. The COPII proteins (Barlowe *et al*., 1994) and Uso1p (Barlowe, 1997) were prepared as described. Purified LMA1 (Xu *et al*., 1997) was a generous gift of Zuoyu Xu (this department).

### Assays for docking and fusion of ER-derived vesicles

Yeast semi-intact cells were prepared from log-phase cultures of strains grown at 23°C and stored frozen at –70°C (Baker *et al*., 1988). Prior to assays, a tube of cells was quickly thawed and washed three times in Buffer 88 to remove cytosol, each wash was followed by centrifugation at 15 000 *g* (in 1.5 ml tubes, 12 000 r.p.m., Eppendorf model 5417 refrigerated centrifuge). Then, a 0.4 ml translocation reaction containing washed semi-intact cells, [$^{35}$S]pre-pro-α-factor and an ATP regenerating system was performed at 10°C for 10 min (Baker *et al*., 1988). After translocation of [$^{35}$S]pre-pro-α-factor into ER membranes, semi-intact cells were chilled on ice and washed three times in Buffer 88 as described above.

For docking assays, semi-intact cells were incubated with COPII proteins and various additions in reactions that contained an ATP-regenerating system and 0.1 mM GTP. The standard condition was 23°C for 30 min in 25 μl or as indicated in the figure legends. At the end of the incubations, tubes were placed on ice for 5 min and then centrifuged at 18 000 *g* (13 000 r.p.m. in Eppendorf model 5417 refrigerated centrifuge) for 3 min. The amount of freely diffusible ER-derived vesicles was quantified by measuring protease-protected Con A–Sepharose-precipitable [$^{35}$S]gp-α-factor contained in the 18 000 *g* supernatant fraction as follows. A portion (10 μl) of the supernatant fraction was treated with trypsin (0.5 mg/ml) for 10 min on ice followed by trypsin inhibitor (1 mg/ml) for 10 min on ice. Membranes were then solubilized in 1% SDS at 95°C for 2 min, diluted 20-fold in IP buffer (25 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100), and [$^{35}$S]gp-α-factor was precipitated with Con A–Sepharose at room temperature for 2 h (Baker *et al*., 1988). A total reaction (no centrifugation after incubation) was processed in an identical manner. The percentage of freely diffusible vesicles represents the amount of [$^{35}$S]gp-α-factor contained in the 18 000 *g* supernatant fraction divided by the total amount of [$^{35}$S]gp-α-factor. Data points represent the average of duplicate determinations, where each duplicate set varied by less than 10%.

For transport assays, a parallel reaction with docking was performed and incubated at indicated temperatures. The standard reaction was 23°C for 60 min in 25 μl or as indicated in the figure legends. Transport reactions were stopped by addition of SDS to a final concentration of 1% and heated at 95°C for 2 min. Solubilized membranes were diluted 20-fold with IP buffer followed by the addition of anti-α1,6-mannose-specific serum and protein A Sepharose (Pharmacia Biotech). Outer-chain modified forms of [$^{35}$S]gp-α-factor (reflecting delivery to the Golgi) were precipitated at room temperature for 2 h and processed as described previously (Baker *et al*., 1988). The percentage transport is the amount of outer-chain modified [$^{35}$S]gp-α-factor divided by the total amount of protease-protected Con A-precipitable [$^{35}$S]gp-α-factor. For temperature-sensitive experiments, both docking and transport reactions were performed at 23, 26 and 29°C. Data points represent the average of duplicate determinations, where each duplicate set varied by less than 10%.

### Membrane binding assay for Ypt1p and Uso1p

Strain CBY324, expressing a c-myc tagged version of Uso1p, was grown to mid-log phase, harvested and lysed in liquid nitrogen as described (Barlowe, 1997). The lysate was centrifuged at 25 000 *g* (14 000 r.p.m. in a Sorvall SS34 rotor) for 15 min and aliquots of the supernatant fraction were frozen for storage at –70°C. For binding assays, 20 μl of extracts (~10 mg/ml protein) were incubated in the presence or absence

of GDI with an ATP regenerating system and 0.2 mM GTP in 25 µl reactions at 20°C for 30 min. After chilling on ice, samples were diluted with 0.2 ml of Buffer 88 and yeast membranes were collected by centrifugation at 100 000 *g* (60 K in a TLA100 rotor, Beckman Instruments). The resulting membrane pellet was washed once with Buffer 88, and solubilized in 30 µl of SDS sample buffer. The amounts of Ypt1p, Sly1p, Uso1p and Emp47p bound to membranes were visualized by immunoblotting. To determine the effect of guanine nucleotide on membrane binding, GTPγS was exchanged onto GTPases through an incubation with 7 mM EDTA in the presence or absence of 0.4 mM GTPγS at 20°C for 5 min, followed by a second incubation at 20°C in the presence of additional MgOAc (final concentration 12 mM) (Garrett *et al*., 1993). The effects of GDI on Ypt1p and Uso1p after GTPγS loading were determined as described above.

## Acknowledgements

We thank David Banfield for providing the *sed5-1* strain, Hans Dieter Schmitt for the anti-Sly1p serum, John Warner for the *sly1-ts* strain and Zuoyu Xu for purified LMA1. Christian Ungermann and Gerry Waters are thanked for critical comments on this manuscript. This work was supported by grants from the National Institute of General Medial Sciences and the Pew Scholars Program in the Biomedical Sciences.

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*Received November 3, 1997; revised January 9, 1998; accepted February 23, 1998*