Asymmetric Requirements for a Rab GTPase and SNARE Proteins in Fusion of COPII Vesicles with Acceptor Membranes

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Abstract. Soluble NSF attachment protein receptor (SNARE) proteins are essential for membrane fusion in transport between the yeast ER and Golgi compartments. Subcellular fractionation experiments demonstrate that the ER/Golgi SNAREs Bos1p, Sec22p, Bet1p, Sed5p, and the Rab protein, Ypt1p, are distributed similarly but localize primarily with Golgi membranes. All of these SNARE proteins are efficiently packaged into COPII vesicles and suggest a dynamic cycling of SNARE machinery between ER and Golgi compartments. Ypt1p is not efficiently packaged into vesicles under these conditions. To determine in which membranes protein function is required, temperature-sensitive alleles of *BOS1*, *BET1*, *SED5*, *SLY1*, and *YPT1* that prevent ER/Golgi transport in vitro at restrictive temperatures were used to selectively inactivate these gene products on vesicles or on Golgi membranes. Vesicles bearing mutations in Bet1p or Bos1p inhibit fusion with wild-type acceptor membranes, but acceptor membranes containing these mutations are fully functional. In contrast, vesicles bearing mutations in Sed5p, Sly1p, or Ypt1p are functional, whereas acceptor membranes containing these mutations block fusion. Thus, this set of SNARE proteins is symmetrically distributed between vesicle and acceptor compartments, but they function asymmetrically such that Bet1p and Bos1p are required on vesicles and Sed5p activity is required on acceptor membranes. We propose the asymmetry in SNARE protein function is maintained by an asymmetric distribution and requirement for the Ypt1p GTPase in this fusion event. When a transmembrane-anchored form of Ypt1p is used to restrict this GTPase to the acceptor compartment, vesicles depleted of Ypt1p remain competent for fusion.

Key words: trafficking • Golgi apparatus • endoplasmic reticulum • secretion • membrane fusion

Introduction

A related family of integral membrane proteins, termed SNAREs (soluble NSF attachment protein receptors),[1] mediate a variety of intracellular membrane fusion reactions in eukaryotic cells (for review see Gotte and Fischer von Mollard, 1998). For example, SNARE proteins function in heterotypic fusion reactions including the fusion of synaptic vesicles with presynaptic membranes (Robinson and Martin, 1998) as well as homotypic fusion reactions that underlie organelle inheritance and dynamics (Nichols et al., 1997; Patel et al., 1998; Rabouille et al., 1998). Studies on the synaptic fusion machinery were the first to identify SNARE proteins, namely syntaxin 1, SNAP25 (synaptosome-associated protein of 25 kD) and VAMP (vesicle-associated membrane protein, also known as synaptobrevin) that form a stable 7S complex (Bennett et al., 1992). Based largely on sequence comparisons, intracellular SNAREs have been divided into two general categories, designated v-SNARE for vesicle-SNARE (related to synaptobrevin) and t-SNARE for target-SNARE (related to syntaxin 1 or SNAP25). However, these designations can be difficult in some instances because v- and t-SNAREs are likely to have evolved from a common ancestral gene and belong to the same superfamily (Weimbs et al., 1997, 1998). Both v- and t-SNARE proteins possess 50–60 amino acid stretches with the potential to form a coiled-coil structure that are typically near their COOH-terminal transmembrane domains. Indeed, structural analyses on neural SNARE proteins indicate that v- and t-SNAREs form parallel heterodimers (Lin and Scheller, 1997) and heterotetramers (Poirier et al., 1998; Sutton et al., 1998). The formation of this stable SNARE bundle has been proposed to create a fusion pore bridging opposed membranes (Sutton et al.,

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[1]*Abbreviations used in this paper:* gp-αF, glyco-pro-α factor; NSF, *N*-ethylmaleimide–sensitive factor; SNAP25, synaptosome-associated protein; SNARE, soluble NSF attachment protein receptor; t- or v-SNARE, target or vesicle SNARE, respectively.

1998; Weber et al., 1998). Although SNARE complexes are thought to be an essential feature of intracellular membrane fusion events, their precise function in bilayer fusion reactions remains obscure. It is generally accepted that the ATPase activity of NSF/Sec18p disrupts SNARE complexes (Wilson et al., 1992; Sollner et al., 1993b). However, different models have been proposed such that NSF drives bilayer fusion as SNARE proteins are disassembled (Sollner et al., 1993a) or, alternatively, that separation of SNARE complexes is a priming event that enables reassembly of SNARES in trans to promote bilayer fusion (Chamberlain et al., 1995; Mayer et al., 1996). Additional debate centers on the function of SNARE proteins in membrane fusion events; specifically, do these proteins form a fusion pore (Sutton et al., 1998; Weber et al., 1998) or are their associations part of a reaction series leading to bilayer fusion but do not constitute a fusion pore (Coorssen et al., 1998; Ungermann et al., 1998).

In addition to SNARE proteins, GTPases of the Rab/Ypt family appear to impart another layer of specificity to SNARE-dependent membrane fusion events through the concerted activity of distinct GTPases with specific sets of SNAREs. Rab/Ypt proteins appear to act before SNARE protein function (Dascher et al., 1991; Sogaard et al., 1994; Lupashin and Waters, 1997), and allow fusion partners to pair before engaging SNARE proteins (Cao et al., 1998). Because single SNARE proteins are capable of participating in multiple fusion events (Fischer von Mollard et al., 1997; Spang and Schekman, 1998), upstream regulation by Rab/Ypt members may be crucial determinates in specifying membrane fusion.

In *Saccharomyces cerevisiae*, genetic and biochemical approaches have implicated the SNAREs Sec22p, Bet1p, Bos1p, Ykt6, and Sed5p (Newman and Ferro-Novick, 1987; Newman et al., 1990; Dascher et al., 1991; Hardwick et al., 1992; Lian and Ferro-Novick, 1993; McNew et al., 1997) in transport between the ER and the Golgi complex. All of these SNAREs coprecipitate in a complex with Sed5p when isolated from a *sec18* mutant strain (Sogaard et al., 1994). In addition to these proteins, Sly1p, Sft1p, and p28 are found in this complex (Sogaard et al., 1994; Banfield et al., 1995). A similar cast of proteins has been characterized in mammalian ER/Golgi transport and designated syntaxin 5, rsly1, rsec22, rbet1, membrin, and GOS28 (Dascher et al., 1994; Nagahama et al., 1996; Subramaniam et al., 1996; Hay et al., 1997, 1998; Xu et al., 1997; Zhang et al., 1997). The subcellular distributions of Sed5p and its homologue syntaxin 5 have been investigated, and evidence indicates these proteins localize to Golgi membranes but cycle between the ER, intermediate compartment, and the Golgi complex (Hay et al., 1998; Rowe et al., 1998; Wooding and Pelham, 1998). A similar observation was documented for the neuronal plasmalemma t-SNARE syntaxin 1, which distributes between synaptic vesicles and the cell surface (Walch-Solimena et al., 1995). Thus, a strict distribution of some SNAREs to donor membranes and t-SNARES to acceptor membranes does not seem to be a general feature of these heterotypic fusion reactions. The question arises as to how directionality is imparted to transport processes if SNARE proteins are cycling between compartments such as the Golgi and ER.

In this report, we first investigate the subcellular distribution of ER/Golgi SNARE proteins in yeast and measure their incorporation into COPII-coated vesicles. The localization of Bet1p, Bos1p, Sed5p, and Ypt1p have been documented (Newman et al., 1992; Hardwick and Pelham, 1992; Preuss et al., 1992; Lian and Ferro-Novick, 1993); however, we sought to directly compare the level of colocalization among these and other proteins involved in transport between the ER and the Golgi complex. We used a reconstituted ER/Golgi transport assay to determine the compartmental requirements for specific SNAREs in fusion of ER-derived vesicles with Golgi membranes. Our approach is through selective inactivation of protein function on isolated ER-derived vesicles or on isolated acceptor membranes. Although the SNARE molecules appear to be symmetrically distributed between vesicle and acceptor compartments, the functional requirements are asymmetric such that Bet1p and Bos1p are required on vesicles and Sed5p activity is required on acceptor membranes. A requirement for Sec22p activity in anterograde transport was not detected. We propose the asymmetry in SNARE protein function is maintained by an asymmetric distribution and requirement for Ypt1p in this fusion event.

Materials and Methods

General Materials and Techniques

Yeast strains used in this study are CBY267 *(MAT*α *trp1-1 ade2-1 ura3-1 leu2-3,112 can1-100*), CBY268 (*MAT*α *trp1-1 ade2-1 ura3-1 leu2-3,112 can1-100 sly1-ts*), CBY263 *(MAT*α *trp1-1 ade2-1 ura3-1 leu2-3,112 can1-100 sed5-1*), RSY255 (*MAT*α *ura3-52 leu2-3,112*), RSY944 (*MATa ura3-52 lys2-801 bet1-1*), RSY954 (*MATa leu2-3,112 lys2-801 bos1-1*), and CBY474 (*MAT*α *trp1-1 ade2-1 ura3-1 leu2-3,112 can1-100 ypt1-3*), and they have been previously described (Cao et al., 1998). The Ypt1-TM2p strain (ROH713-10A; *MAT*α *his3 leu2 ypt1*Δ*::HIS3* with a *CEN-YPT1TM2-LEU2* plasmid) and isogenic wild-type strain (ROH713-10B; *MAT*α *his3 leu2*) have been described previously (Ossig et al., 1995). The yeast strain containing a myc-tagged version of Sly1p, CBY73 (*MAT*α *ura3-52 lys2-801 ade2-101 trp1*Δ*63 his3-*Δ*200 leu2-*Δ*1 sly1*Δ*::HIS3* with a *CEN-LEU2-SLY1-MYC* plasmid) was constructed as described below. Strains were grown in rich medium (1% bactoyeast extract, 2% bactopeptone, and 2% glucose), and converted to semi-intact cells as described by Baker et al. (1988). Antibodies directed against α-1,6-mannose linkages (Barlowe, 1997), Ypt1p (Rexach et al., 1994), Sec61p (Stirling et al., 1992), GDPase (Berninsone et al., 1995), Sec12p (Powers and Barlowe, 1998), Sec22p (Bednarek et al., 1995), Bet1p (Rexach et al., 1994), Bos1p (Sogaard et al., 1994), Sed5p (Cao et al., 1998), Emp47p (Schroder et al., 1995), Sec23p (Hicke and Schekman, 1989), and the c-myc epitope (Evan et al., 1985) have been described previously. Polyclonal antibodies prepared against Ykt6p were generated against a hexahistidine-tagged version of recombinant Ykt6p (McNew et al., 1997) as described (Ungermann et al., 1999). For immunoblots, samples were resolved by SDS-PAGE (Lammeli, 1970), transferred to nitrocellulose (Towbin et al., 1979) and filter-bound secondary antibodies were detected by peroxidase-catalyzed chemiluminescence (Amersham).

Plasmid Construction

The plasmid YEP51 containing the *SLY1* gene (Dascher et al., 1991) was a gift from H.D. Schmitt (Max Plank Institute, Gottingen). The restriction sites BamHI and EcoRV were used to subclone the 3′ end of *SLY1* (nucleotides 2,550–3,557) into the BamHI and EcoRV sites of pBluescript SK+ (Stratagene) to construct pXC1. The restriction sites SpeI were used to subclone the 5′ end of *SLY1* (nucleotides 728–2,834) into the SpeI sites of pXC1 to produce pXC2 (pBS-*SLY1*). The peptide sequence EEQKLISEEDLHHHHHH (c-myc epitope and hexahistide tag) was fused to the COOH terminus of Sly1p as follows. Two complementary oligonucleotides A25635 (5′-GAGGAGCAGAA ATTAATCAG CGAAGAGGAC CTCCTCAGGA AGAGGCATCA CCATCACCAT CACTAAGATA TCTGCA-3′) and A25636 (5′-GATATCTTAG TGATGGTGAT GGT-

GATGCCTCT TCCTGAGGAG GTCCTCTTCG CTGATTAATT TCTGCTCCTC TGCA-3′) were annealed to produce a DNA fragment flanked by ends compatible with sites generated by an NsiI digest. The annealed oligonucleotides were inserted into the NsiI site of pXC2 (a unique restriction site directly before the stop codon of *SLY1*) to produce pXC4 (pBS-SLY1-MYC-6HIS). This construct was sequenced to confirm faithful insertion of the fusion sequences. Finally, the restriction sites XhoI and XbaI of pXC4 were used to subclone *SLY1-MYC-6HIS* into the XhoI and XbaI sites of pRS315 (Sikorski and Hieter, 1989) to produce pXC5r (*pRS315-SLY1-MYC-6HIS*).

Strain Construction

The *SLY1* locus was targeted for disruption with the *HIS3* gene (Baudin et al. 1993). PCR was used to amplify a *HIS3* disruption fragment flanked by sequences immediately before and after the *SLY1* open reading frame, using the primers C35571 (5′-ATATATATAT ATTAGTCTAT CGTCATTGGG GCTAGATGCC AATTAGCGCG CCTCGTTCAG AATG-3′) and C33626 (5′-GTCATTGCCA GTTGCTAAGT ATCTTTGACC AAAAATCACA ACATCGGCCT CCTCTAGTAC ACTC-3′). The *HIS3* homologous regions are underlined. The resulting product has the *HIS3* gene flanked by 45 bp of the *SLY1* gene directly before the ATG start and directly after the stop codon. Strain YPH501 (Sikorski and Hieter, 1989) was transformed with this PCR product, and histidine prototrophs were screened by colony PCR using the primer SLY15 (5′-CCGTTTCCCT CTTCGCG-3′; 142 bp upstream of the *SLY1* start codon) and an internal *HIS3* primer (5′-GGCTCATCCAAAGGCGC-3′). Several colonies were identified that contained a *HIS3* disruption of *SLY1*. One strain, designated CBY69, was heterozygous at the *SLY1* locus and used in further studies. CBY69 was transformed with pXC5r (*pRS315-SLY1-MYC-6HIS*) and grown under conditions to induce sporulation. Viable haploid disruptants of *SLY1* containing pXC5r were identified, and exhibited growth properties that were indistinguishable form wild-type strains. One strain (CBY73) was used in the following studies.

Subcellular Fractionation

Membrane organelles prepared from cell lysates were resolved on 22–60% sucrose density gradients (Antebi and Fink, 1992) with minor modifications as described by Powers and Barlowe (1998). Gradient fractions were collected and diluted 1:1 with SDS-PAGE sample buffer and immunoblotted for Sec61p (ER marker), Emp47p (Golgi marker), Bet1p, Bos1p, Sec22p, Sed5p, Sly1p, Ypt1p, and Ykt6p. Relative levels of specific proteins in each fraction were quantified by densitometric scanning of immunoblots. GDPase activity (Golgi marker) was determined as described (Yanagisawa et al., 1990) using CDP to subtract nonspecific phosphatase activity. Sucrose concentrations of individual fractions were determined by measuring the refractive index with an Abbe refractometer (American Optical).

In Vitro Budding and Transport Assays

Microsomes were isolated from the CBY73 strain and incubated in the presence or absence of proteins required for reconstitution of vesicle formation as described in Barlowe et al. (1994). A 30-μl aliquot of the total reaction and 300 μl of a supernatant fluid containing vesicles released from budding reactions were centrifuged at 100,000 *g* in a TLA100.3 rotor (Beckman Instruments) to collect membranes. This resulting membrane pellet was solubilized in 30 μl of SDS-PAGE sample buffer, and 10 μl was resolved on 12.5% polyacrylamide gels and immunoblotted for various proteins as indicated in the figure legends.

For in vitro fusion assays, ER-derived vesicles and acceptor membranes were isolated from indicated strains as follows. Semi-intact yeast cells were prepared (Baker et al., 1988) from wild-type, *bet1-1*, *bos1-1*, *sed5-1*, *sly1-ts*, and *ypt1-3* strains that were grown at 24°C. To prepare ER-derived vesicles that contained [^{35}S]glyco-pro-α factor (gp-αF), semi-intact cells were thawed quickly and washed three times with buffer 88 (25 mM Hepes, pH 7.0, 150 mM potassium acetate, 250 mM sorbitol, and 5 mM magnesium acetate) to remove cytosol. Each wash was followed by centrifugation at 15,000 *g* (12,000 rpm) in an Eppendorf refrigerated centrifuge (model 5417). gp-αF was posttranslationally translocated into the ER of semi-intact cells at 10°C for 10 min in the presence of an ATP regeneration system (Baker et al., 1988). Vesicles were synthesized from semi-intact cells by incubation with purified COPII proteins (Sar1p, Sec23p complex, and Sec13p complex) at 20°C for 10 min as previously described (Barlowe, 1997). After budding, the reactions were chilled on ice and centrifuged for 5 min at 20,000 *g* (14,000 rpm in an Eppendorf refrigerated centrifuge). The supernatant fluid, containing budded vesicles, was collected and used in subsequent fusion assays.

To prepare acceptor membranes, semi-intact cells were thawed and washed three times as described above. Fusion reactions were performed by incubating isolated vesicles (containing ~5,000 cpm of [^{35}S]gp-αF) with washed semi-intact cells in the presence of Uso1p and LMA1 at 23 or 29°C (Barlowe, 1997). Under these conditions, washed semi-intact cells provide the acceptor membrane for vesicle fusion. After 90-min incubations, transport reactions were stopped by the addition of SDS to a final concentration of 1% and heated to 95°C for 2 min. Solubilized membranes were diluted 20-fold with IP buffer, followed by the addition of anti–α-1,6-mannose-specific serum and protein A–Sepharose (Pharmacia Biotech). Outer chain–modified forms of [^{35}S]gp-αF (reflecting delivery to a Golgi compartment) were precipitated at room temperature for 2 h and processed as described (Baker et al., 1988). The percent transport is the amount of outer chain–modified [^{35}S]gp-αF divided by the total amount of protease-protected ConA-precipitable [^{35}S]gp-α-factor contained in isolated vesicles. For each figure, multiple data sets were obtained, and a representative experiment plotting the mean and range of duplicate samples is shown.

Results

Subcellular Distribution of SNAREs between the ER and Golgi Membranes

One model for vesicle-mediated transport suggests that t- and v-SNAREs reside on separate compartments (Sollner et al., 1993a; Rothman and Wieland, 1996), although there is experimental evidence indicating these species are not strictly compartment localized in heterotypic systems (Walch-Solimena et al., 1995; Rowe et al., 1998). Beyond these cellular localization studies, little has been done to distinguish in which vesicle or a membrane compartment distinct v- and t-SNARE proteins are functionally required. Here, we address these questions in a simplified system of membrane fusion between ER-derived vesicles and Golgi membranes. First, we examined the subcellular distribution of the SNARE proteins involved in this fusion event. The fractionation behavior of Bet1p, Bos1p (Shim et al., 1991; Newman et al., 1992; Lian and Ferro-Novick, 1993), and Sed5p (Hardwick et al., 1992) have been documented by various methods; however, we sought to directly compare the level of colocalization among these and other proteins involved in transport the between ER and Golgi. To determine their steady state distribution, a standard procedure was employed (Antebi and Fink, 1992) to resolve ER and Golgi compartments by velocity sedimentation on sucrose density gradients (Fig. 1). The resolution of Golgi membranes and the ER by this method was confirmed through the analysis of Golgi-localized markers, GDPase and Emp47, which peaked in fraction 7 and the ER marker, Sec61p, which peaked in fraction 13. Immunoblot analysis of these same gradient fractions with antibodies specific for Bos1p, Bet1p, Sec22p, Sed5p, Sly1p, Ypt1p, and Ykt6p are shown in Fig. 1 (A–H). Notably, Bos1p, Bet1p, and Sed5p displayed similar distribution patterns with major peaks that were coincident with Golgi and ER markers. This method was performed multiple times with different wild-type yeast strains, and very similar fractionation profiles were observed, such that these SNARE proteins distributed between ER and Golgi compartments with a majority (~60–80%) localizing with Golgi membranes. Sly1p is peripherally associated with membranes, in part, through binding to Sed5p, and we observed signifi-

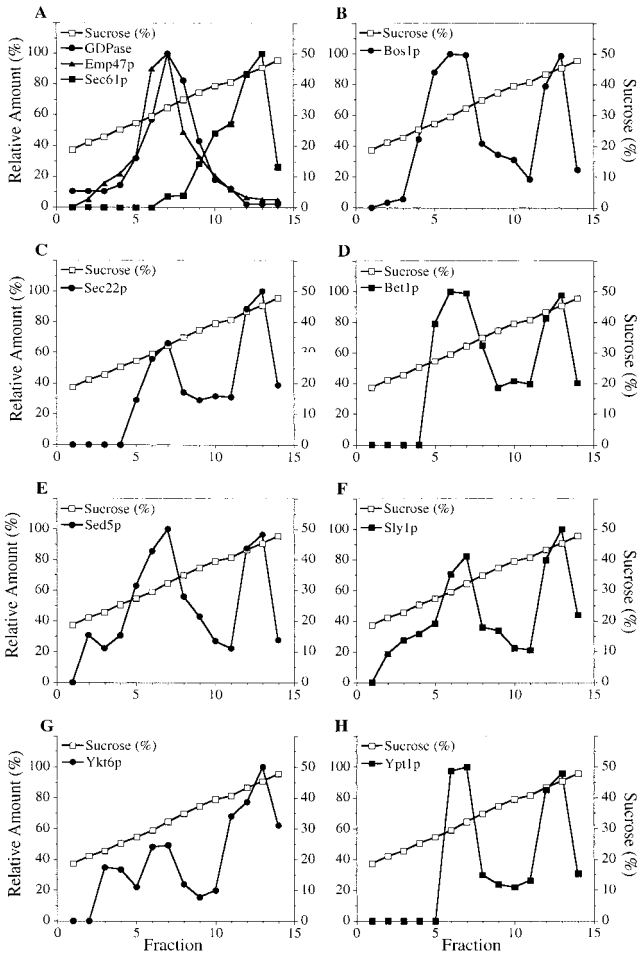

Figure 1. Subcellular distribution of ER/Golgi proteins by sucrose gradient. Lysed spheroplasts made from CBY409 cells were loaded on a 20–60% sucrose gradient. The gradients were centrifuged at 35,000 rpm in an SW40 rotor for 2.5 h at 4°C. Fractions from the gradient were resolved on SDS-PAGE and immunoblotted for Sec61p (ER marker), Emp47p (Golgi marker), Sec22p, Bet1p, Bos1p (v-SNARE), Sed5p (t-SNARE), and the t-SNARE–associated protein, Sly1p. The relative abundance contained in each fraction was determined by densitometry using NIH image. The GDPase assay measures Ca^{2+}-dependent GDPase activity and serves as a marker for Golgi membranes.

cant overlap between Sly1p and Sed5p. Ypt1p followed a similar distribution with a majority localized to Golgi membrane fractions, however, some Ypt1p migrated with ER membranes. A fraction of Ykt6p colocalized with ER and Golgi markers, but the overall pattern was distinct from other ER/Golgi SNARE, proteins and may reflect a role for this protein in fusion events later in the secretory pathway (Ungermann et al., 1999). In summary, the steady state distributions of ER/Golgi SNAREs (Bet1p, Bos1p, Sec22p, and Sed5p) are similar and do not appear to be restricted to single membrane compartments. These observations on Sed5p are consistent with data suggesting the t-SNARE Sed5p/Syn5 cycles between the ER and Golgi compartments (Rowe et al., 1998; Wooding and Pelham, 1998).

Incorporation of SNAREs and SNARE Regulatory Proteins into COPII-coated Vesicles

The primary route of protein transport from the ER is thought to be via COPII-coated transport vesicles (Schekman and Orci, 1996). Methods have been established to isolate in vitro synthesized COPII vesicles (Barlowe et al., 1994; Rexach et al., 1994), and we sought to determine the efficiency with which distinct SNARE and SNARE regulatory proteins are packaged into isolated COPII vesicles from microsomes. For these studies, microsomes were prepared from a yeast strain (CBY73) containing an epitope-tagged version of Sly1p (see Materials and Methods). Incubation of microsomes with purified Sar1p, Sec23p complex, and Sec13p complex, in the presence of ATP and GTP, produced COPII vesicles that were separated from larger ER membranes by differential centrifugation. The COPII vesicles contained in the supernatant fraction were collected by centrifugation at 100,000 *g*. Fig. 2 shows the content of specific proteins contained on these isolated vesicles. First, resident ER proteins such as Sec61p and Sec12p were not packaged into COPII-coated vesicles as has been previously observed (Barlowe et al., 1994; Rexach et al., 1994), indicating a faithful reproduction of sorting during in vitro budding, and that the integrity of ER membranes was preserved though this procedure. Second, GDPase (a Golgi resident) was not efficiently packaged into COPII-coated vesicles, suggesting a selectivity of the COPII coat for ER membranes even though isolated microsomes contain a significant amount of Golgi membranes. Finally, the ER to Golgi SNARE proteins monitored (Bos1p, Bet1p, Sec22p, and Sed5p) and the t-SNARE–associated protein (Sly1p) were specifically packaged into COPII-coated vesicles. Subsequent repetitions of this experiment produced qualitatively similar results, and the efficiency of their incorporation into COPII-

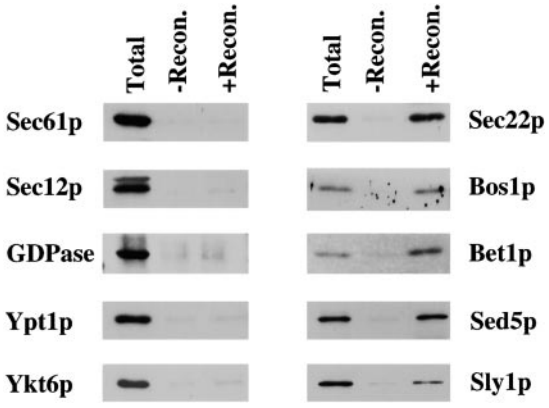

Figure 2. SNARE proteins are incorporated into ER-derived vesicles. COPII-coated vesicles were synthesized from ER membranes and collected by centrifugation. Lanes labeled "Total" are membranes from one-tenth of a total reaction (containing both vesicles and ER). Lanes labeled "+Recon" are vesicles produced under conditions of reconstituted vesicle formation by addition of COPII proteins. Lanes labeled "−Recon" are those produced in the absence of COPII proteins. Samples were resolved on 12.5% SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies specific for indicated proteins.

Table I. Efficiency of Protein Packaging into COPII Vesicles

Protein	Percent incorporated (−Recon.)	Percent incorporated (+Recon.)
Sec61p	0.3	0.4
Sec12p	0.2	0.2
GDPase	0.6	0.9
Ypt1p	0.8	1.0
Ykt6	0.7	1.1
Sec22p	0.6	9.9
Bos1p	0.4	10.3
Bet1p	0.4	19.4
Sed5p	0.6	8.8
Sly1p	0.8	4.9

Values represent the percentage of each protein in vesicles compared to total microsomes and were determined by densitometric scanning of blots shown in Fig. 2.

coated vesicles from the experiment shown in Fig. 2 is listed in Table I. Interestingly, the percentage of Bet1p incorporated into the vesicles was reproducibly twofold higher than the other SNAREs monitored in these experiments (Sec22p, Bos1p, Bet1p, and Sed5p). In addition, the level of the GTPase Ypt1p packaged into the COPII vesicles was quite low, and suggested this protein was not a constituent of these transport intermediates, an observation that is consistent with previous experiments (Barlowe et al., 1994; Rexach et al., 1994). A similar result was obtained in analysis of Ykt6p, as this protein was not efficiently packaged into COPII-coated vesicles. Together with the subcellular fractionation results, these observations are consistent with the notion that specific sets of SNARE proteins cycle between the ER and Golgi instead of a hypothesis that v-SNAREs are enriched on transport vesicles and t-SNAREs localize to acceptor membranes (Rothman and Wieland, 1996). In contrast, the small GTPase Ypt1p was not efficiently packaged into COPII vesicles, even though the subcellular distribution of Ypt1p is similar to ER/Golgi SNARE proteins.

Localized Requirements for SNARE Protein Function

The above experiments indicate that ER/Golgi SNAREs are similarly distributed between these compartments, and are efficiently packaged into ER-derived vesicles. However, these results do not indicate in which compartments their activities are required, although there is ample evidence indicating that Bet1p, Bos1p, and Sed5p function in the fusion of ER-derived vesicles with Golgi membranes (Newman et al., 1992; Lian and Ferro-Novick, 1993; Cao et al., 1998). To test the functional requirements for these activities on vesicles and acceptor membranes, we used *bet1-1*, *bos1-1*, *sed5-1*, and *sly1-ts* mutant strains that allow for selective inactivation of specific proteins on vesicles or acceptor membranes. Our previous experiments have established that the mutated versions of Bet1p, Bos1p, Sed5p, and Sly1p cause thermosensitive blocks in cell-free vesicle fusion assays (Cao et al., 1998). Here, we employ these same mutations to inactivate SNARE proteins on vesicles or acceptor membranes and monitor fusion efficiency.

Transport between the ER and Golgi may be reproduced with washed semi-intact cells incubated with purified COPII proteins, Uso1p, and LMA1, and is monitored by the processing of [^{35}S]gp-αF. COPII produces freely diffusible vesicle intermediates containing [^{35}S]gp-αF that then tether to Golgi membranes in the presence of Uso1p. Fusion of tethered vesicles requires the activities of Sec18p and LMA1 (Barlowe, 1997). With this refined transport assay, we can isolate freely diffusible vesicles from membranes incubated with COPII and, in a second stage, incubate vesicles with Golgi membranes from washed semi-intact cells in the presence of fusion factors. The percentage of vesicles that fuse with acceptor membranes was quantified by determining the amount of [^{35}S]gp-αF that had acquired the Golgi-specific outer chain α-1,6-mannose modification. An example is shown in Fig. 3 (A), where incubation of wild-type vesicles containing [^{35}S]gp-αF with the membranes from the *bet1-1* mutant strain resulted in fusion and outer chain modification of [^{35}S]gp-αF at permissive or nonpermissive temperatures. However, incubation of vesicles from the *bet1-1* mutant strain with wild-type acceptor membranes at a restrictive temperature–blocked vesicle fusion, indicating that the activity of Bet1p is required on ER-derived vesicles. Similar experiments were performed using components from the *bos1-1* and *sed5-1* mutant strains to determine the compartmental requirements for Bos1p and Sed5p. As shown in Fig. 3 (B), vesicles carrying mutant Bos1p failed to deliver [^{35}S]gp-αF to wild-type Golgi membranes at 29°C. This result is consistent with previous studies that demonstrated a requirement for Bos1p on ER-derived vesicles when neutralizing anti-Bos1p antibodies were used (Lian and Ferro-Novick, 1993). In contrast, Fig. 4 shows that vesicles carrying the thermosensitive version of Sed5p were fully functional for delivery of [^{35}S]gp-αF to the wild-type acceptor at both temperatures, whereas acceptor membranes from the *sed5-1* strain were not functional at a

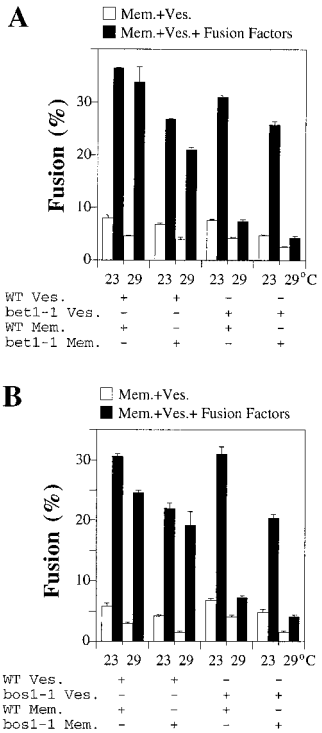

Figure 3. The v-SNAREs Bet1p and Bos1p are functionally required on ER-derived vesicles. COPII vesicles, which were synthesized in vitro from wild-type or mutant ER membranes at 20°C, were incubated in a second stage with wild-type or mutant Golgi membranes at 23 or 29°C. (A) Vesicle fusion with membrane components from a *bet1-1* strain or (B) a *bos1-1* strain. Reactions contained an ATP regeneration system alone (Mem. + Ves., open bars) or an ATP regeneration system with Uso1p and LMA1 (Mem. + Ves. + Fusion Factors, black bars). The percent fusion was quantified after precipitation of the outer chain–modified forms of [^{35}S]gp-αF.

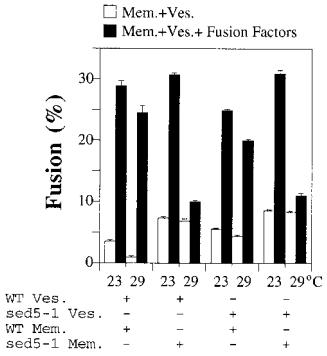

Figure 4. The t-SNARE Sed5p is functionally required on Golgi membranes. COPII vesicles synthesized in vitro from wild-type or *sed5-1* ER membranes at 20°C were incubated in a second stage with wild-type or *sed5-1* Golgi membranes at 23 or 29°C. Reactions contained an ATP regeneration system alone (Mem. + Ves., open bars) or an ATP regeneration system with Uso1p and LMA1 (Mem. + Ves. + Fusion Factors, black bars). The percent fusion represents the amount of the outer chain–modified forms of [^{35}S]gp-αF.

restrictive temperature. These results indicate that ER/Golgi SNAREs display spatially distinct requirements in the fusion of ER-derived vesicles with acceptor membranes. Bet1p and Bos1p functioned on vesicles, whereas Sed5p was required on acceptor membranes.

From the experiments shown in Figs. 3 and 4, we hypothesized that vesicles prepared from the *sed5-1* strain would fuse with acceptor Golgi membranes prepared from the *bet1-1* strain even at restrictive temperatures. In other words, simultaneous inactivation of a v-SNARE on the acceptor and a t-SNARE on the vesicle should not inhibit fusion. Indeed, as shown in Fig. 5, vesicles from the *sed5-1* strain and Golgi membranes from the *bet1-1* strain fused at both 23 and 29°C. As controls for this experiment, other combinations of these mutant components showed clear temperature sensitivity. A similar result was observed when the experiment was performed with *sed5-1* vesicles and *bos1-1* acceptor membranes (not shown), although the magnitude of the fusion signal was lower than for the *bet1-1* experiment. Taken together, these results indicated a requirement for Bet1p and Bos1p on vesicles and the t-SNARE Sed5p on the acceptor compartment.

Asymmetric Requirements for SNARE Regulatory Proteins

At this point, we have examined the localized requirements for SNARE proteins in ER/Golgi transport. Although SNARE proteins are central components, additional proteins that appear to regulate SNARE protein function are essential for membrane fusion, and we next investigated the requirements for two of these regulators, Ypt1p and Sly1p (Lian et al., 1994; Lupashin and Waters, 1997). Sly1p is an essential 84-kD protein that binds to Sed5p (Dascher et al., 1991; Sogaard et al., 1994). Our analyses of Sly1p on sucrose gradients and on COPII vesicles showed that Sly1p distributes between ER and Golgi similarly to SNAREs, but was incorporated into vesicles less efficiently than Sed5p or other ER/Golgi SNAREs. We have previously shown that the *sly1-ts* mutation produces a temperature-dependent block in our in vitro transport assay (Cao et al., 1998). As shown in Fig. 6, we found that the requirement for Sly1p resided specifically on acceptor membranes, whereas the ER-derived vesicles prepared from this strain were fully functional. Therefore, the distribution and spatial requirement for Sly1p were similar to Sed5p and may reflect a linked function for these molecules.

Previous studies have shown that isolated vesicles accumulated in a *ypt1* temperature-sensitive mutant were functional for fusion with wild-type Golgi membranes (Rexach et al., 1994), suggesting functional Ypt1p was not required on vesicles. This published observation employed a rich cytosol, which contains some soluble Ypt1p, to drive the second stage fusion reaction. Therefore, we reevaluated the requirements for Ypt1p in our cell-free assay under conditions where soluble Ypt1p or Gdi1p was not provided. We have previously shown that the *ypt1-3* mutation displays some temperature sensitivity in vitro when the reaction was driven with purified transport factors; however, even at permissive temperatures, the transport efficiency was low in reactions that employ the *ypt1-3* mutation (Cao et al., 1998). As seen in Fig. 7, Golgi membranes from *ypt1-3* cells did not function with wild-type vesicles, whereas vesicles isolated from the *ypt1-3* strain were fully active for fusion with wild-type acceptor membranes. Under conditions where *ypt1-3* acceptor membranes were used, we detected only modest amounts of transport at permissive temperatures. These results suggest that Ypt1p function was restricted to Golgi membranes as was observed for Sed5p and Sly1p and places this set of mole-

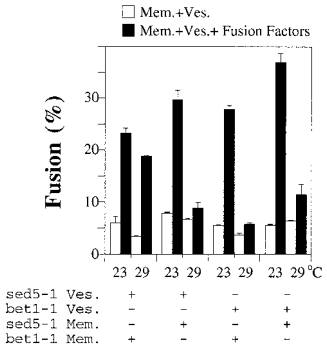

Figure 5. The v-SNARE Bet1p is required on ER-derived vesicles, and the t-SNARE Sed5p is required on Golgi membranes. Vesicles synthesized from a *bet1-1* strain and Golgi membranes from a *sed5-1* strain or vice versa were incubated in a second stage at 23 or 29°C. Reactions contained an ATP regeneration system alone (Mem. + Ves., open bars) or an ATP regeneration system with Uso1p and LMA1 (Mem. + Ves. + Fusion Factors, black bars). The percent fusion represents the amount of the outer chain–modified forms of [^{35}S]gp-αF.

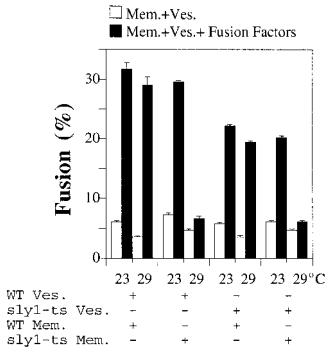

Figure 6. Sly1p function is required on Golgi membranes. COPII vesicles synthesized in vitro from wild-type or *sly1-ts* ER membranes at 20°C were incubated in a second stage with wild-type or *sly1-ts* Golgi membranes at 23 or 29°C. Reactions contained an ATP regeneration system alone (Mem. + Ves., open bars) or an ATP regeneration system with Uso1p and LMA1 (Mem. + Ves. + Fusion Factors, black bars). The percent fusion represents the amount of outer chain–modified forms of [^{35}S]gp-αF.

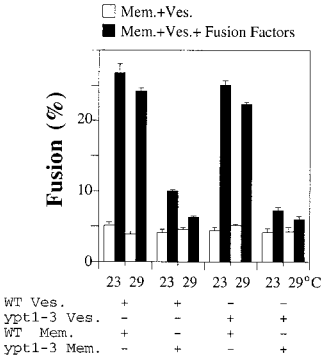

Figure 7. The small GTPase Ypt1p is functionally required on Golgi membranes. COPII vesicles, which were synthesized in vitro from wild-type or *ypt1-3* membranes at 20°C, were incubated in a second stage with wild-type or *ypt1-3* Golgi membranes at 23 or 29°C. Reactions contained an ATP regeneration system alone (Mem. + Ves., open bars) or an ATP regeneration system with Uso1p and LMA1 (Mem. + Ves. + Fusion Factors, black bars). The percent fusion represents the amount of the outer chain–modified forms of [^{35}S]gp-αF.

cules in a distinct group from Bet1p and Bos1p. However, we cannot exclude the possibility that wild-type acceptor membranes transferred membrane-bound Ypt1p to incoming vesicles, although we consider this unlikely because the addition of varying amounts of Gdi1p does not stimulate this reaction (data not shown).

A Requirement for Ypt1p Can Be Fulfilled When This GTPase Is Restricted to Acceptor Membranes

As an independent test for localized Ypt1p activity on acceptor membranes, we used a membrane-anchored form of this protein (Ypt1-TM2p), which has been previously described in the literature (Ossig et al., 1995). We reasoned that if vesicles do not require Ypt1p bound to their surface for function, then acceptor membranes containing Ypt1-TM2p should support vesicle fusion when transfer is prevented by anchoring. The membrane-anchored form used in our experiments fuses the transmembrane domain of Sec22p to the COOH terminus of Ypt1p in a manner that replaces the CAAX sequence of this GTPase. Ypt1-TM2p behaves as an integral membrane protein and expression complements a *ypt1*Δ strain. However, the Ypt1p-TM2p must be overproduced (approximately twofold) for complementation and, even when overexpressed, the growth of the Ypt1-TM2p strain was slightly slower (20%) than a wild-type rate. Regardless, transport of secretory proteins and Golgi function appeared normal in the Ypt1-TM2p strain where membrane detachment is prevented (Ossig et al., 1995).

We first characterized the properties of the Ypt1-TM2 strain in our reconstituted transport assay and observed that vesicles were synthesized in a COPII-dependent manner, ER-derived vesicles were tethered in an Uso1p-dependent process, and fusion required Sec18p and LMA1p (Table II). However, the efficiency of vesicle formation and fusion were reduced in comparison to a wild-type strain, yet vesicle tethering remained efficient in the Ypt1p-TM2 strain. These results indicate the Ypt1-TM2p membranes are not optimal for ER/Golgi transport in our reconstituted assays, but that [^{35}S]gp-αF was transported in a conventional manner and, importantly, anchoring Ypt1p yields active acceptor membranes. Therefore, acceptor membranes, which were prepared from this strain

Table II. Budding, Tethering and Fusion with Wild-type and Ypt1p-TM2 Membranes

	Diffusible vesicles (%)			Transport (%)	
Membranes	NA	COPII	COPII/Uso1p	NA	Recon.
WT	4.5 ± 0.4	36.2 ± 0.4	25.9 ± 0.2	4.6 ± 0.2	22.9 ± 0.5
TM2	4.7 ± 0.2	22.6 ± 0.6	14.7 ± 2.0	4.5 ± 0.9	12.9 ± 1.0

The percentages of freely diffusible [^{35}S]gp-α Factor–containing vesicles were determined in the presence of COPII (budding) and COPII + Uso1p (tethering) after 30 min at 20°C. In separate reactions, the percentage of transport was quantified by precipitation of the outer-chain modified forms of [^{35}S]gp-α Factor in the presence of Recon. proteins (COPII, Uso1p and LMA1) after 60 min at 23°C. Samples were processed as described previously (Cao et al., 1998).

should allow us to test if transfer of Ytp1p from donor membranes to vesicles is a requirement for their fusion.

The yeast GDP dissociation inhibitor, Gdi1p, is an inhibitor of Ypt and Rab GTPases that binds and extracts the GDP-bound form of these proteins from intracellular membranes (Garrett et al., 1994). We tested if Ypt1-TM2p was sensitive to extraction by Gdi1p for two purposes. First, although the transmembrane form of Ypt1p behaves as an integral membrane protein, we wanted to directly demonstrate that the anchored form of Ypt1p was not solubilized by Gdi1p to exclude the possibility of transfer from acceptor membranes to vesicles in our reaction. Second, vesicles generated from wild-type or the *ypt1-3* strain could contain trace amounts of Ypt1p that may be sufficient for vesicle fusion. We reasoned that if Ypt1-TM2p was insensitive to Gdi1p extraction, addition of excess Gdi1p to vesicles should selectively inhibit the activity of lipid-anchored forms of Ypt1p and not membrane-anchored Ypt1p. As seen in Fig. 8, wild-type membranes, which contain the lipid-anchored form of Ypt1p, are sensitive to Gdi1p, and Ypt1p was extracted upon addition of excess Gdi1p, whereas Ypt1p-TM2p was not extractable. In this experiment, Sec23p, a peripherally associated membrane protein (Hicke and Schekman, 1989), and Sec61p, an integral membrane protein (Stirling et al., 1992), served as controls to demonstrate effective separation of cytosol (supernatant) and membrane (pellet) fractions. The Ypt1-TM2p fusion migrates as a larger species

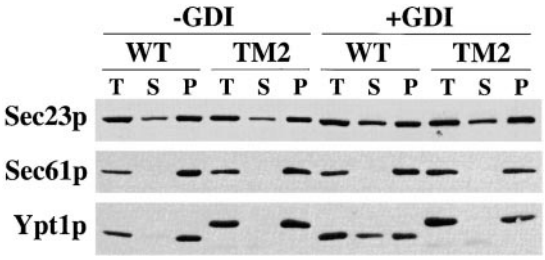

Figure 8. Gdi1p extracts Ypt1p but not Ypt1-TM2p. Wild-type (WT) or Ypt1-TM2p (TM2) semi-intact cells were incubated with Gdi1p (50 μg/ml) for 20 min at 25°C. Total reactions (T) were centrifuged at 100,000 *g* to generate the membrane pellet (P) and supernatant (S) fractions. Fractions were resolved on 12.5% polyacrylamide gels, and immunoblot analyses were performed with specific antibodies to measure the content of Sec23p (a peripheral membrane protein control), Sec61p (control for integral membrane protein), and the lipid-anchored or membrane-anchored form of Ypt1p.

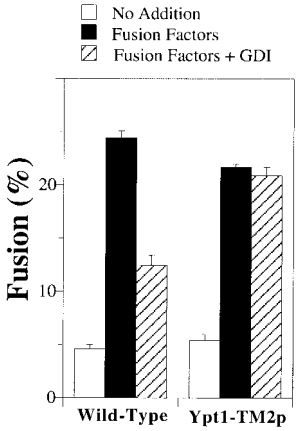

Figure 9. Ypt1-TM2p functions on Golgi membranes. COPII vesicles synthesized in vitro from *ypt1-3* membranes were incubated in a second stage with wild-type or Ypt1-TM2p Golgi membranes. Reactions contained an ATP regeneration system alone (No addition, open bars) or supplemented with Uso1p and LMA1 (Fusion Factors, black bars) or Uso1p and LMA1 and 50 μg/ml Gdi1p (Fusion Factors + GDI, hatched bars). The percent fusion represents the amount of outer chain–modified forms of [^{35}S]gp-αF.

because of the addition of 26 amino acid residues from the transmembrane domain of Sec22p.

To determine if vesicles could fuse with acceptor membranes when Ypt1p transfer was prevented by membrane anchoring, we prepared vesicles from the *ypt1-3* strain and acceptor membranes from wild-type or Ypt1-TM2p strains. As seen in Fig. 9, vesicles incubated with wild-type acceptor membranes fused at an efficient level, and this fusion was sensitive to Gdi1p. When vesicles were incubated with acceptor membranes that contained Ypt1-TM2p, vesicles fused at a somewhat lower efficiency but, importantly, this reaction was insensitive to Gdi1p. This experiment indicates that ER-derived vesicles that were depleted of Ypt1p by *ypt1-3* mutation and treated with Gdi1p still fused with the acceptor when Ypt1-TM2 was restricted to acceptor membranes. Based on these results, we propose that the requirement for Ypt1p in the fusion of ER-derived vesicles with acceptor membranes resides in the Golgi compartment.

Discussion

Questions concerning the regulation and function of distinct SNARE proteins in intracellular fusion reactions have become increasingly complex (Nichols and Pelham, 1998; Gotte and Fischer von Mollard, 1998). For example, fusion events involving ER and Golgi membranes presumably include fusion of ER-derived vesicles with Golgi membranes (Rexach and Schekman, 1991; Cao et al., 1998), fusion of Golgi-derived retrograde vesicles with the ER (Lewis and Pelham, 1996; Spang and Schekman, 1998), as well as homotypic membrane fusions between ER (Latterich et al., 1995; Patel et al., 1998), between Golgi (Rabouille et al., 1998), and between ER-derived vesicles (Rowe et al., 1998). In all of these examples, SNARE proteins have been implicated and, in some instances, distinct fusion reactions appear to employ an identical SNARE (Patel et al., 1998; Spang and Schekman, 1998). How does each of these fusion events determine which components will be used when, or is there promiscuity such that any given set can operate? To answer these questions, we believe it will be important to determine precisely which sets of proteins are capable of mediating specific membrane fusion events, and on which compartments they are functionally required.

In this manuscript, we report that ER/Golgi SNARE proteins display similar distributions, but are asymmetrically required in fusion between ER-derived vesicles and Golgi membranes. Using thermosensitive versions of SNAREs and SNARE regulatory proteins, we observe that Bet1p and Bos1p are functionally required on ER-derived vesicles, whereas the t-SNARE (Sed5p) and t-SNARE–associated proteins (Sly1p) are specifically required on the Golgi acceptor compartment. A recently discovered multispanning membrane protein, termed Got1p, also functions specifically on this acceptor compartment and appears to facilitate Sed5p activity (Conchon et al., 1999). These distinct spatial requirements suggest that heterotypic fusion events depend on compartment-specific cues to regulate SNARE protein function. An important component of this regulation appears to be the small GTPase Ypt1p. Because Ypt1p is asymmetrically enriched and required on Golgi membranes with respect to vesicles in our reaction, we propose that Ypt/Rab proteins play an important role in compartment-specific regulation. One manner in which this could be accomplished is that upon the binding of ER-derived vesicles to Golgi membranes, a process which requires Ypt1p (Cao et al., 1998), this GTPase is activated and signals the Sed5p/Sly1p complex, enabling Golgi SNAREs to engage vesicle SNAREs.

We have not detected a requirement for Sec22p in anterograde fusion of ER-derived vesicles with Golgi membranes (Cao, X., and C. Barlowe, unpublished observation), as might be predicted from in vivo studies (Kaiser and Schekman, 1990). It is possible that the temperature-sensitive *sec22-3* allele remains functional in our in vitro assay, even at restrictive temperatures, although this seems unlikely because this same allele exhibits sensitivity in an in vitro assay that measures COPI-dependent retrograde transport from the Golgi to the ER (Spang and Schekman, 1998). Because strains containing a *sec22*Δ allele are viable, this SNARE is apparently not essential for anterograde or retrograde transport (Ossig et al., 1991), however, in vitro fusion assays with membrane components prepared from this deletion strain may allow for a more rigorous test of Sec22p function. In addition to Sec22p, the ER-localized t-SNARE, Ufe1p, appears to operate specifically in retrograde transport to the ER and not in anterograde transport (Lewis and Pelham, 1996; Lewis et al., 1997; Spang and Schekman, 1998). This is in contrast to Bet1p, which appears to operate in both anterograde and retrograde fusion events (Cao et al., 1998; Spang and Schekman, 1998).

Several lines of evidence indicate ER/Golgi SNARE proteins exist in heteromeric complexes under specific conditions in yeast (Lian et al., 1994; Sogaard et al., 1994; Lewis et al., 1997; Lupashin and Waters, 1997) and mammalian cells (Hay et al., 1998; Rowe et al., 1998). However, in most instances, these studies were performed with whole cell extracts, therefore, it is difficult to determine in which membrane compartment(s) these complexes exist. Experiments with purified proteins indicate that Bos1p and Sec22p can bind directly to Sed5p (Sacher et al., 1997), and Bet1p appears to increase the affinity of Bos1p for Sed5p (Stone et al., 1997). We have detected some Sed5p, Bet1p, and Bos1p in a complex on ER-derived vesicles through a cross-linking immunoprecipitation approach (Cao, X., and

C. Barlowe, unpublished observation), although it will require further studies to determine the stochiometry and composition of SNARE complexes contained on COPII vesicles. Structural studies on neural SNARE proteins indicate the formation of a stable core complex composed of four parallel coiled-coil domains, such that syntaxin and synaptobrevin each contribute a single coiled-coil to this structure, whereas SNAP25 contributes two (Poirier et al., 1998; Sutton et al., 1998). Previous reports have noted that Bet1p shares a high degree of sequence identity with SNAP25 (Weimbs et al., 1997), leading to the prediction that the role of Bet1p may be comparable to SNAP25 (Stone et al., 1997; Weimbs et al., 1997). Because Bet1p contains a single α-helical domain, it may contribute two molecules in fulfilling a putative SNAP25 role (Weimbs et al., 1998). We have observed that the percentage of Bet1p packaged into COPII vesicles from starting microsomes is twice that of Bos1p, Sed5p, and Sec22p (Fig. 2 and Table I). This may reflect the stochiometry of ER/Golgi SNARE complexes that are packaged into COPII vesicles. Alternatively, Bet1p could simply posses a higher affinity for subunits of the COPII coat, resulting in an increased packaging efficiency. However, based on the collective results, we speculate that a SNARE complex consisting of Sed5p, Bos1p, and two molecules of Bet1p functions in fusion of anterograde vesicles, whereas a complex of Ufe1p, Sec22p, and two molecules of Bet1p would be required for fusion of retrograde vesicles with the ER.

Our results indicate some parallels with homotypic membrane fusion reactions, notably the fusion of vacuoles. These reactions employ a similar cast of characters (SNAREs, Rab proteins, Sec18p, etc.) and, in some respects, appear to operate by similar mechanisms such that membranes first bind in a reaction that requires a Ypt protein for tethering before engaging SNARE protein machinery (Ungermann et al., 1998). A distinct difference in these reactions, however, is a symmetric requirement for Ypt7p, which is the counterpart of Ypt1p (Wichmann et al., 1992; Haas et al., 1995). For homotypic fusion of vacuoles, Ypt7p is required on both compartments. Perhaps this indicates a symmetric nature to the tethering reactions in homotypic membrane pairing. The vacuolar reaction can be altered to generate a pseudoheterotypic condition by deleting a v-SNARE from one vacuole and a t-SNARE from another. Under this condition, fusion proceeds at a lowered efficiency, but proceeds nonetheless (Nichols et al., 1997). Interestingly, the requirement for Sec18p now becomes asymmetric, such that membranes containing the t-SNARE molecule (Vam3p) depend on Sec18p for fusion but a Δ*vam3* vacuole does not. This observation suggests that ER-derived vesicles may not require Sec18p for fusion, whereas the Golgi acceptor would. Sec18p is clearly required for the fusion of ER-derived vesicles with the Golgi (Rexach and Schekman, 1991; Barlowe, 1997), although we currently are not able to distinguish if this requirement is refined to acceptor membranes, vesicles, or both.

Initial models for SNARE protein function suggested that t-SNARE proteins remain largely associated with target membranes, and v-SNARE proteins would be found on both vesicles and target membranes (Sollner et al., 1993a). However, a strict separation of v- and t-SNARE proteins does not appear to be a general feature of membrane fusion reactions (Walch-Solimena et al., 1995). With respect to Sed5p and Syn5, studies in yeast (Wooding and Pelham, 1998) and mammalian cells (Rowe et al., 1998) indicate that this protein is rapidly cycling between early compartments of the secretory pathway. In spite of Sed5p cycling through the ER, we find that this t-SNARE is functionally required on acceptor membranes and not vesicles. Our findings are not entirely consistent with those reported on the mammalian Syn5 protein, where function was restricted to ER-derived vesicles. In these experiments, treatment of ER-derived vesicles with neutralizing anti-Syn5 antibodies prevented the formation of vesicular-tubular pre-Golgi intermediates, but these same antibodies did not affect the competency of Golgi membranes to act as an acceptor (Rowe et al., 1998). These disparate observations are not easily explained, but may be related to the use of different inhibitors in these experimental approaches or may reflect differences in organization of the early secretory pathway in *S. cerevisiae* and mammals. A recent morphometric study on the organization of the yeast secretory pathway indicates many parallels between *S. cerevisiae* and mammals (Morin-Ganet et al., 1999). In both, ER-derived vesicles appear to fuse with or form vesicular tubular clusters that recruit COPI components, and then fuse with or mature into cis-Golgi networks (Bannykh et al., 1996; Presley et al., 1997; Scales et al., 1997; Bonfanti et al., 1998; Morin-Ganet et al., 1999). Precisely where specific outer-chain carbohydrate modifications occur in this scheme remains to be determined. The yeast ER/Golgi transport assay measures a heterotypic membrane fusion reaction that results in a mixture of ER-derived vesicles containing secretory protein (gp-αF) with a compartment that contains α-1,6-mannosyl transferase activity (Baker et al., 1988; Rexach et al., 1994). This is thought to arise from the fusion of ER-derived vesicles with a cis-Golgi-like compartment. Alternatively, COPI vesicles containing α-1,6-mannosyl transferase could deliver this activity to a vesicular-tubular cluster of COPII vesicles (Pelham, 1998; Lin et al., 1999). A direct requirement for COPI in anterograde transport of gp-αF to the α-1,6-mannosyl transferase–containing compartment has not been established, whereas COPI and COPI assembly proteins are required for in vitro retrograde transport of an HDEL-tagged protein from the Golgi to the ER (Spang and Schekman, 1998; Poon et al., 1999). Therefore, we speculate that our in vitro assay measures the fusion of COPII vesicles with a compartment that contains α-1,6-mannosyl transferase and not direct fusion with COPI vesicles derived from the Golgi. We cannot exclude the possibility that ER-derived vesicles fuse homotypically because our in vitro assay would not detect this event. However, if homotypic fusion of ER-derived vesicles is catalyzed by Sed5p, this event does not appear to be a requirement for ER/Golgi transport in our assay. In mammalian cells, such a homotypic fusion process may be required for further progress through the early secretory pathway, and could explain the different Sed5p/Syn5 requirements observed in these assays.

If Sed5p is not functionally required on ER-derived vesicles, why does this protein actively cycle between the ER and Golgi compartments? Sed5p may be incorporated into

transport vesicles as a consequence of complex formations with Bet1p, Bos1p, and Sec22p. COPII proteins have been demonstrated to bind directly to the soluble domains of Bet1p and Bos1p (Springer and Schekman, 1998) and Sed5p (Peng et al., 1999). Presumably COPI binds specific ER/Golgi SNARE proteins for retrograde transport back to the ER; therefore, Sed5p may be incorporated into these vesicle carriers either by association with specific SNAREs or direct interactions with COPI subunits. In other words, Sed5p may cycle because it is in complex with proteins that must cycle. Alternatively, Sed5p cycling between ER and Golgi may be functionally important for homotypic fusion of ER-derived vesicles (Rowe et al., 1998) or for other fusion events that are currently uncharacterized. In any event, a biochemical dissection of the mechanisms underlying this spatial regulation should provide important insights into SNARE-dependent membrane fusion.

We thank Hans Dieter Schmitt and Dieter Gallwitz for providing antibodies and strains used in these studies.

This work was supported by grants from the National Institute of General Medical Sciences (GM52549) and the Pew Scholars Program in the Biomedical Sciences.

Submitted: 23 August 1999
Revised: 22 February 2000
Accepted: 24 February 2000

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