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 Y pt1p 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 Y pt1p GTPase in this fusion event. When a transmembrane-anchored form of Y pt1p is used to restrict this GTPase to the acceptor compartment, vesicles depleted of Y pt1p 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), mediate a variety of intracellular membrane fusion reactions in eukaryotic cells (for review see Gott 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 kDa) and VAMP (vesicle-associated membrane protein, also known as synaptobrevin) that form a stable 7S complex (Benett 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.,...
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; M. ayer et al., 1996). A dictatorial 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 (Coorsen 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 (D. ascher 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 proteins may be crucial determinates in specifying membrane fusion.

In Saccharomyces cerevisiae, genetic and biochemical approaches have implicated the SNAREs Sec22p, Bet1p, Bos1p, Ykt6p, and Sed5p (Newman and Ferro-Novick, 1987; Newman et al., 1990; D. ascher et al., 1991; H. ardwick et al., 1992; Lian and Ferro-Novick, 1993; McNiew 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; B. Baker et al., 1988). A similar cast of proteins has been characterized in mammalian ER/Golgi transport and designated syntaxin 5, rsv1, rsec22, rbet1, membrin, and GOS28 (D. ascher et al., 1994; Nagahama et al., 1996; Subramaniam et al., 1996; H. ay et al., 1997, 1998; X u et al., 1997; Z. 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 (H. ay et al., 1998; R.owe 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 COP11-coated vesicles. The localization of Bet1p, Bos1p, Sed5p, and Ypt1p have been documented (Newman et al., 1992; H. ardwick 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 (MATa trpl-1 ade2-1 ura3-1 leu2-3,112 can1-100), CBY268 (MATa trpl-1 ade2-1 ura3-1 leu2-3,112 can1-100 sly1-1), CBY263 (MATa trpl-1 ade2-1 ura3-1 leu2-3,112 can1-100 sed5-1), RSY255 (MATa ura3-52 leu2-112), RSY944 (MATa ura3-52 lys2-801 bet1-1), RSY954 (MATa ura3-52 lys2-801 bos1-1), and CBY474 (MATa trpl-1 ade2-1 ura3-1 leu2-3,112 can1-100 ypt3-1) and they have been previously described (Cao et al., 1998). The ypt3-TM2p strain (ROH713-10A; MATa his3 leu2 ypt3Δ;HIS3 with a CENT-YPITTM2-EU2 plasmid) and isogenic wild-type strain (ROH713-10B; MATa his3 leu2) have been described previously (Ossig et al., 1995). The yeast strain containing a myc-tagged version of Sly1p, CBY73 (MATa ura3-52 lys2-801 ade2-101 trp1-163 his3Δ200 leu2-11 ypl1Δ;HIS3 with a CEN-LEU2-SLY1-YMYC 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). A nitobodies directed against α-1,6-mannose linkages (Barloue, 1997), Ypt1p (Rexach et al., 1994), Sec63p (Stirling et al., 1992), GPDase (Berninson et al., 1995), Sec12p (Powers and Barloue, 1998), Sec22p (Bednarek et al., 1995), Bet1p (Rexach et al., 1994), Bos1p (Sogaard et al., 1994), Sed5p (Cao et al., 1998), Ecp47p (Schroder et al., 1995), Sed23p (Hicke and Schekman, 1989), and the c-myc epitope (E van et al., 1985) have been described previously. Polyclonal antibodies prepared against Ytk6p were generated against a hexahistidine-tagged version of recombinant Ytk6p (MC Newman et al., 1997) as described (Ungergerman et al., 1999). For immunobots, 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 (A mersham).

**Plasmid Construction**

The plasmid Y ES1 containing the SL Y1 gene (D. ascher 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 SL Y1 (nucleotides 2,350–3,557) 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 SL Y1 (nucleotides 728–2,834) into the SpeI sites of pXCl to produce pXC2 (pBS-SLY1). The peptide sequence EEQKLISEEDLHLLLLHHH (c-myc epitope and hexahistide tag) was fused to the COOH terminus of Sly1p as follows. Two complementary oligonucleotides A25635 (5′-GAGGAGCATCA CCATACACCAT CACTAGATA TCGCCA-3′) and A25636 (5′-GATATCTTAG TGATGGTGAT GGTATTCGTT TCTGCA-3′) were used.
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 open reading frame, using the primers C35571 (5'-ATATAATAT ATTTGCTAT CGT- CTTGGG GCTAGATGCC AATTAGCGCG CCTCGTTCAG AATG-3') and C36326 (5'-GATGCCTCT TCCTGAGGAG GTCCTCTTCG CTGATTAATT CCGTTTCCCT CTTCGCG-3'). Several colonies were identified that contained a HIS3 disruption of SLY1. One strain, designated CBY69, was heterozygous at the HIS3 locus and in further studies. CBY69 was transformed with pXC5 (Cao and Barlowe, 1998) and histidine prototrophic cells were prepared (Baker et al., 1988) from wild-type, semi-intact cells by incubation with purified COPII proteins (Sar1p, Sec23p complex, and Sec13p complex) at 20°C for 2 min. Solubilized membranes were diluted 20-fold with IP buffer, followed by the addition of anti-aα-1,6-mannose-specific serum and protein A-Sepharose (Pharmacia Biotech). Outdoor chain-modified forms of [35S]gp-a-factor (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 [35S]gp-a-factor divided by the total amount of protease-protected ConA-precipitable [35S]gp-a-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 SNAP-25 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 compartmentalized 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 t- and v-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 t-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 and Sed5p (H ardwick et al., 1992) have been documented and published 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 (A ntegi 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, Sed6p 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 yeast strains, and very similar fractionation profiles were observed, such that these SNA RE proteins distributed between the 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-
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 (CBY 73) 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 through 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 1. Subcellular distribution of ER/Golgi proteins by sucrose gradient. Lysed spheroplasts made from CBY 409 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 Ca2+-dependent GDPase activity and serves as a marker for Golgi membranes.

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.
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.

**Table I. Efficiency of Protein Packaging into COPII Vesicles**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Percent incorporated (+ Reconst.)</th>
<th>Percent incorporated (+ Reconst.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sec61p</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Sec12p</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>GDPase</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Ypt1p</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Ykt6</td>
<td>0.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Sec22p</td>
<td>0.6</td>
<td>9.9</td>
</tr>
<tr>
<td>Bos1p</td>
<td>0.4</td>
<td>10.3</td>
</tr>
<tr>
<td>Bet1p</td>
<td>0.4</td>
<td>19.4</td>
</tr>
<tr>
<td>Sed5p</td>
<td>0.6</td>
<td>8.8</td>
</tr>
<tr>
<td>Sly1p</td>
<td>0.8</td>
<td>4.9</td>
</tr>
</tbody>
</table>

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.

**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 sly1-1 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 [35S]gp-αF. COPII produces freely diffusible vesicle intermediates containing [35S]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 [35S]gp-αF that had acquired the Golgi-specific outer chain α-1,6-mannose modification. An example is shown in Fig. 3, where incubation of wild-type vesicles containing [35S]gp-αF with the membranes from the bet1-1 mutant strainresulted in fusion and outer chain modification of [35S]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 [35S]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 [35S]gp-αF to the wild-type acceptor at both temperatures, whereas acceptor membranes from the sed5-1 strain were not functional at a
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 (Ascher et al., 1991; Sogaard et al., 1994). Our analyses of Sly1p on sucrose gradients and on COP II 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-
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 generation system alone (M em. + V es., open bars) or an ATP regeneration system with Uso1p and LMA1 (M em. + Ves. + Fusion Factors, black bars). The percent fusion represents the amount of the outer chain-modified forms of $[^{35}S]$gp-αF.

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. Ypt1p-TM2p behaves as an integral membrane protein and expression complements a ypt1Δ 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 should allow us to test if transfer of Ypt1p 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 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.

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 Receptor proteins (COPII, Uso1p, and LMA1) after 60 min at 23°C. Samples were processed as described previously (Cao et al., 1998).
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 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 (Nicholls and Pelham, 1998; Gotta 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. A nontrivial 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 sec22A 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 sec22A 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 stoichiometry 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 SNARE P25 contributes two (Poirier et al., 1998; Sutton et al., 1998). Previous reports have noted that Bet1p shares a high degree of sequence identity with SNARE P25 (Weimbs et al., 1997), leading to the prediction that the role of Bet1p may be comparable to SNAP25 (Nichols et al., 1992; Haas et al., 1995). For homotypic fusion of vacuoles, 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 COPII 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; Exach et al., 1994). This is thought to arise from the fusion of ER-derived vesicles with a cis-Golgi-like compartment. Alternatively, COPII 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 COPII in anterograde transport of gp-αF to the α-1,6-mannosyl transferase-containing compartment has not been established, whereas COPI and COPII 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. COP II 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 COP II 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 COPII 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.

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