

# Analysis of Sec22p in Endoplasmic Reticulum/Golgi Transport Reveals Cellular Redundancy in SNARE Protein Function

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Membrane-bound soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins form heteromeric complexes that are required for intracellular membrane fusion and are proposed to encode compartmental specificity. In yeast, the R-SNARE protein Sec22p acts in transport between the endoplasmic reticulum (ER) and Golgi compartments but is not essential for cell growth. Other SNARE proteins that function in association with Sec22p (i.e., Sed5p, Bos1p, and Bet1p) are essential, leading us to question how transport through the early secretory pathway is sustained in the absence of Sec22p. In wild-type strains, we show that Sec22p is directly required for fusion of ER-derived vesicles with Golgi acceptor membranes. In *sec22Δ* strains, Ykt6p, a related R-SNARE protein that operates in later stages of the secretory pathway, is up-regulated and functionally substitutes for Sec22p. In vivo combination of the *sec22Δ* mutation with a conditional *ykt6-1* allele results in lethality, consistent with a redundant mechanism. Our data indicate that the requirements for specific SNARE proteins in intracellular membrane fusion are less stringent than appreciated and suggest that combinatorial mechanisms using both upstream-targeting elements and SNARE proteins are required to maintain an essential level of compartmental organization.

## INTRODUCTION

In the eukaryotic secretory pathway, a multiplicity of proteins, lipids, and cofactors is required for organized transport. Proper organization is due in part to highly specific homotypic and heterotypic membrane fusion events that depend on a family of proteins termed soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors, or SNAREs (Sollner *et al.*, 1993). This family is typified by a conserved heptad repeat sequence or “SNARE motif” adjacent to a membrane-bound segment. Certain sets of SNARE proteins form stable complexes through assembly of their heptad repeat regions into a parallel four-helix coiled-coil structure (Hanson *et al.*, 1997; Katz *et al.*, 1998). A crystal structure of the neuronal SNARE complex consisting of synaptobrevin-II, syntaxin 1A, and SNAP-25B revealed a four-helix bundle held together by 16 layers of largely hydrophobic residues but with an ionic “zero layer” near the center of this bundle (Sutton *et al.*, 1998). The ionic layer seems to be a conserved feature of many different SNARE complexes and in most instances consists of one arginine

residue contributed by a synaptobrevin-like protein or R-SNARE and three glutamines residues contributed from three Q-SNARE helices (Fasshauer *et al.*, 1998).

The assembly of these four helix bundles with cognate sets of SNARE proteins contributed from opposing membranes has been proposed to catalyze bilayer fusion (Sollner *et al.*, 1993; Weber *et al.*, 1998) and to encode compartmental specificity (McNew *et al.*, 2000). However, other studies have suggested that SNARE proteins are not the sole determinants of intracellular fusion reactions and upstream targeting or tethering machines may work in concert with SNARE complexes to impart specificity (reviewed by Waters and Hughson, 2000). For example, a given SNARE protein can assemble into complexes with multiple SNARE partners (Fischer von Mollard *et al.*, 1997; Nichols and Pelham, 1998) and function in multiple membrane fusion reactions (Fischer von Mollard and Stevens, 1999). Moreover, studies with purified SNARE proteins demonstrate that stable complexes between some noncognate SNARE proteins form promiscuously (Fasshauer *et al.*, 1999; Yang *et al.*, 1999; Tsui and Banfield, 2000), although these associations may not reflect a capacity to fuse lipid bilayers. To test the role of SNARE proteins in specifying membrane fusion, a comprehensive study of SNARE proteins from *Saccharomyces cerevisiae* was undertaken to identify combinations that catalyze bilayer fusion when reconstituted with proteoliposomes bearing

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purified SNARE proteins. In large part, the compartmental specificity of intracellular membrane fusion was recapitulated with cognate SNARE proteins (McNew *et al.*, 2000).

In *S. cerevisiae*, genetic, biochemical, and morphological evidence indicates that the SNARE proteins Sed5p, Bet1p, Bos1p, and Sec22p mediate fusion of endoplasmic reticulum (ER)-derived transport vesicles with an early Golgi compartment (Kaiser *et al.*, 1990; Newman *et al.*, 1990; Dascher *et al.*, 1991; Hardwick and Pelham, 1992; Sogaard *et al.*, 1994). Indeed, membrane fusion reactions reconstituted with purified SNARE proteins in proteoliposomes demonstrated that of 11 SNARE proteins tested, only Bet1p sustained fusion activity when a ternary complex of Sed5p, Bos1p, and Sec22p was present on the opposing membrane (McNew *et al.*, 2000). Moreover, this set of SNARE proteins efficiently forms a stable quaternary complex upon mixing of purified components (Parlati *et al.*, 2000; Tsui *et al.*, 2001). However, the action of this SNARE complex in cellular membrane fusion has been enigmatic because the *SEC22* gene is dispensable for growth (Dascher *et al.*, 1991). In contrast, the genes encoding other members of this complex (*SED5*, *BET1*, and *BOS1*) are essential. These observations raised the possibility of redundancy in Sec22p function or that certain SNARE activities can somehow be bypassed.

In this report, we investigate the role of Sec22p in transport between the ER and Golgi by using a cell-free assay that reconstitutes this stage of transport in yeast (Baker *et al.* 1988; Barlowe, 1997). We show that in wild-type cells, Sec22p is required for transport to the Golgi but in its absence, Ykt6p, a protein with high sequence identity to Sec22p, is up-regulated and functionally substitutes for Sec22p. Given this apparent redundancy in SNARE protein function, our results suggest that additional specificity factors operate in concert with SNARE proteins to achieve an essential level of membrane organization.

## MATERIALS AND METHODS

### Strains and Plasmids

Yeast strains CBY740 (*MAT $\alpha$  his3 leu2 lys2 ura3*) and CBY773 (*MAT $\alpha$  his3 leu2 lys2 ura3 sec22 $\Delta$ ::KAN*) were purchased from Research Genetics (Huntsville, AL) and are isogenic to BY4742 (Winzeler *et al.*, 1999). Strain CBY1108 (*MAT $\alpha$  his3 leu2 lys2 ura3* with *pYKT6-2  $\mu$ m-URA3*) contains plasmid pSK60 (Sapperstein *et al.*, 1996) in CBY740. Wild-type strain FY834 (*MAT $\alpha$  his3 leu2 lys2 ura3 trp1*) has been described previously (Winston *et al.*, 1995), and CBY1236 (*MAT $\alpha$  his3 leu2 lys2 ura3 trp1 sec22 $\Delta$ ::KAN*) was constructed by backcrossing CBY773 with FY834 multiple times. The *ykt6-1* temperature-sensitive strain SARY166 (*MAT $\alpha$  his3 leu2 ura3 trp1 ykt6::LEU (CEN6, TRP1, ykt6-1)*) and isogenic wild-type SARY189 (*MAT $\alpha$  his3 leu2 ura3 trp1 ykt6 $\Delta$ ::LEU (2  $\mu$ , TRP1, YKT6)*) were from D. Banfield (Tsui and Banfield, 2000). The plasmid pGEX-2T-SEC22 was constructed by subcloning a 556-base pair polymerase chain reaction fragment carrying *SEC22* (1–180 aa) into the *Bam*HI-*Eco*RI sites of the pGEX-2T vector (Pharmacia, Peapack, NJ). Strain CBB1136 contains pGEX-2T-SEC22 in XL-1 Blue cells (Stratagene, La Jolla, CA). Yeast strains were grown in either rich medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) or selective medium (0.67% nitrogen base without amino acids, 2% dextrose) and required supplements. Bacterial strains were grown in LB medium (1% NaCl, 1% peptone, and 0.5% yeast extract) containing 100  $\mu$ g/ml ampicillin.

### Antibodies and Immunoblotting

Antibodies directed against  $\alpha$ -1,6-mannose linkages Ypt1p, Sec61p, Bos1p, Bet1p, Sed5p, Sec23p, Ykt6p, and Sly1p have been described previously (Cao and Barlowe, 2000). Antibodies against Erv25p (Belden and Barlowe, 1996) and Erv41p (Otte *et al.*, 2001) were also used. Polyclonal antibodies were raised against a GST-Sec22p (NH<sub>2</sub>-terminal 1–180 aa) fusion protein expressed from plasmid pGEX-2T-SEC22. The fusion protein was purified according to the manufacturer's specifications (Pharmacia) and used to immunize rabbits by standard procedures. For affinity purification of anti-Sec22p antibodies, purified GST-Sec22p protein was coupled to Affi-Gel 10 as recommended by the manufacturer (Bio-Rad, Hercules, CA). Anti-Sec22p antibodies were bound and eluted from this matrix (Harlow and Lane, 1988) and then concentrated by centrifugation in a Centricon 30 microconcentrator (Amicon, Beverly, MA). Affinity-purified anti-Ykt6p antibodies were prepared as described previously (Ungermann *et al.*, 1999) and preimmune IgGs isolated on protein A-Sepharose (Harlow and Lane, 1988). Immunoblots were developed using the enhanced chemiluminescence method (Pharmacia). For densitometric analyses, films were scanned and plotted using NIH Image 1.52.

### Immunoprecipitations

Native immunoprecipitation of Bos1p from detergent-solubilized membranes was performed as follows. Wild-type and *sec22 $\Delta$*  strains were grown at a permissive temperature of 25°C. Semi-intact yeast cells were prepared (Baker *et al.*, 1988) and a 60- $\mu$ l aliquot was incubated in 180  $\mu$ l of lysis buffer (25 mM HEPES pH 7.0, 150 mM KOAc, 10 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) with 8 U of apyrase at 25 or 35°C for 10 min. Lysed cells were washed with 1 ml of lysis buffer and sedimented by centrifugation at 18,000  $\times$  g (14,000 rpm) for 3 min at 4°C. Pellets were resuspended with 200  $\mu$ l of lysis buffer (containing 13 U of apyrase), and an equal volume of lysis buffer containing 2% Triton X-100 was added to solubilize membranes on ice for 10 min. The detergent extract was centrifuged at 100,000  $\times$  g (50,000 rpm) at 4°C for 10 min in a TL-100 ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA). The supernatant fraction (380  $\mu$ l) was mixed with 100  $\mu$ l of buffer A (25 mM HEPES pH 7.0, 100 mM KOAc, and 0.1% Triton X-100) and 30  $\mu$ l of anti-Bos1p antibodies linked to protein A beads (50% solution) or 75  $\mu$ l of protein A beads (20%) was added. After a 2-h incubation at 4°C with gentle mixing, the beads were washed five times with cold buffer B (25 mM HEPES pH 7.0, 150 mM KOAc, and 0.1% Triton X-100) and bound proteins eluted by heating in 30  $\mu$ l of 2% SDS at 95°C for 1 min. The eluted proteins were diluted in SDS-PAGE sample buffer, resolved on 12.5% polyacrylamide gels, and transferred to nitrocellulose for immunoblot analysis.

### In Vitro Vesicle Budding, Tethering, and Transport Assays

Yeast semi-intact cells from either wild-type (CBY740) or *sec22 $\Delta$*  (CBY773) strains were prepared for in vitro tethering and transport assays as described previously (Barlowe, 1997; Cao *et al.*, 1998). For Sec22p immunodepletion experiments from COPII vesicles, budded vesicles were prepared and isolated from microsomes (Barlowe, 1997) with the following modification. Before the addition of COPII proteins, microsomes (0.1 ml of 4 mg/ml) containing [<sup>35</sup>S]gp- $\alpha$  factor were incubated in the presence or absence of affinity-purified anti-Sec22p antibodies (15  $\mu$ g/ml) on ice for 15 min. Depleted and wild-type budded vesicles were then isolated from density gradients and added to acceptor membranes (Barlowe, 1997). For in vitro assays, data points are the average of duplicate determinations and the error bars represent the range.

## RESULTS

### *Sec22p Is Required for Anterograde Transport to Golgi Complex In Vitro*

Genetic and biochemical experiments suggested a requirement for Sec22p in anterograde transport from the ER to the Golgi by forming a SNARE complex with Bos1p, Bet1p, and Sed5p (Kaiser and Schekman, 1990; Newman *et al.*, 1990; Sogaard *et al.*, 1994; Parlati *et al.*, 2000; Tsui *et al.*, 2001). Further studies showed that Sec22p also functions in retrograde transport from the Golgi to the ER and acts with the ER-localized SNARE protein Ufe1p (Lewis *et al.*, 1997). When examined in cell-free assays that reproduce anterograde (Cao and Barlowe, 2000) and retrograde transport (Spang and Schekman, 1998), the thermosensitive *sec22-3* allele inhibited retrograde and not anterograde transport at restrictive temperatures. These findings suggest that Sec22p does not act directly in anterograde transport although other explanations, such as allele specificity, are possible. Therefore, as an independent test of Sec22p function in anterograde transport, we prepared affinity-purified anti-Sec22p antibodies to neutralize Sec22p function in a reconstituted cell-free assay that measures transport to the Golgi. In this assay, washed semi-intact cell membranes containing [<sup>35</sup>S]glycoprotein- $\alpha$ -factor (gpaf) are incubated with purified transport factors (COPII, Uso1p, and LMA1) to drive transport of [<sup>35</sup>S]gpaf to the Golgi (Barlowe, 1997). On delivery to the Golgi complex, gpaf receives outer-chain  $\alpha$ 1,6-mannose residues that can be immunoprecipitated with  $\alpha$ 1,6-mannose-specific antiserum to quantify [<sup>35</sup>S]gpaf transport (Baker *et al.*, 1988). As seen in Figure 1A, reconstituted transport was sensitive to anti-Sec22p antibody, whereas preimmune IgGs at comparable concentrations do not inhibit transport. Cell-free transport can be divided into sub-reactions, each following movement of [<sup>35</sup>S]gpaf (Barlowe, 1997). Incubation of washed semi-intact cells with the purified COPII proteins generates freely diffusible vesicles containing gpaf that can be separated from larger membranes by differential centrifugation. Using these assays, we found that the inhibitory Sec22p antibodies specifically blocked the vesicle fusion stage of the reaction because COPII-dependent budding and Uso1p-dependent tethering were unaffected (Figure 1, B and C). The fact that anti-Sec22p antibodies had no effect on vesicle budding and tethering excluded the possibility that inhibition was due to aggregation of membranes. Taken together, these results indicated that Sec22p was directly required for anterograde transport of [<sup>35</sup>S]gpaf to the Golgi complex.

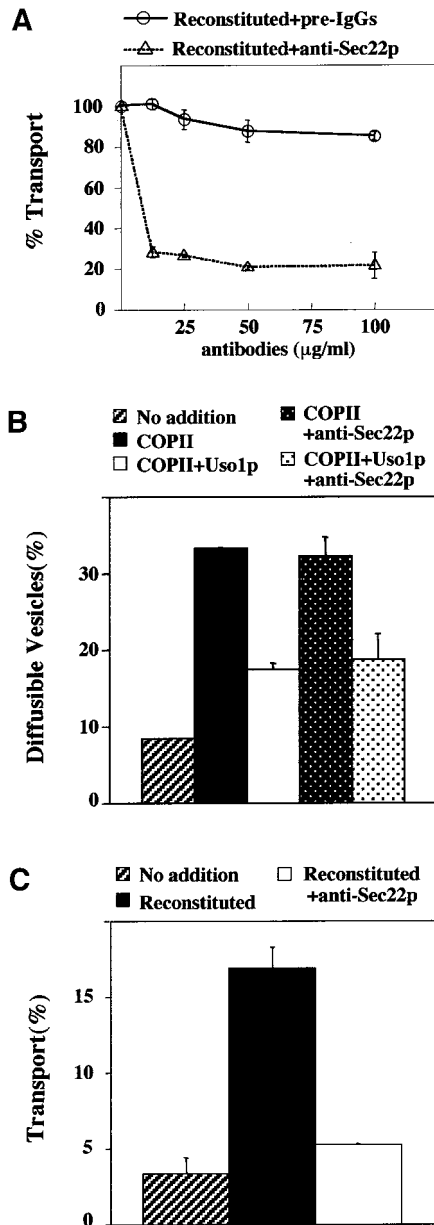
Unlike the other ER/Golgi SNAREs required for anterograde transport (i.e., *BET1*, *BOS1*, and *SED5*), the *SEC22* gene is not essential although the *sec22 $\Delta$*  allele results in slowed growth and temperature sensitivity (Dascher *et al.*, 1991). Given our in vitro findings, these observations raise an interesting paradox. If Sec22p is required for an essential transport step, how do cells survive in its absence? We speculated that the Sec22p-dependent step was somehow bypassed or that a redundant activity substituted for Sec22p function. To address these possibilities, we first investigated anterograde transport in *sec22 $\Delta$*  cells by using the reconstituted cell-free assay. As seen in Figure 2A, ~40% of the [<sup>35</sup>S]gpaf was budded into diffusible vesicles in a wild-type strain when COPII proteins were added. Addition of Uso1p-

tethered COPII vesicles to acceptor membranes resulted in an ~50% reduction of diffusible vesicles (Figure 2A, open bars). Efficient fusion of tethered vesicles required the addition of LMA1 and yielded ~18% transport of [<sup>35</sup>S]gpaf to the Golgi complex in wild-type membranes (Figure 2B). In *sec22 $\Delta$*  semi-intact cell membranes, reconstituted budding and transport efficiencies were significantly reduced, whereas Uso1p-dependent tethering remained ~50% efficient (Figure 2). It is not entirely clear why budding and fusion are compromised in *sec22 $\Delta$*  cells, although it is known that this deletion causes activation of the unfolded protein response (Belden and Barlowe, 2001). Regardless, these results indicated that a COPII- and Uso1p-dependent transport pathway was operational in the complete absence of Sec22p. Therefore, we consider it unlikely that the normal anterograde transport pathway was bypassed in *sec22 $\Delta$*  cells.

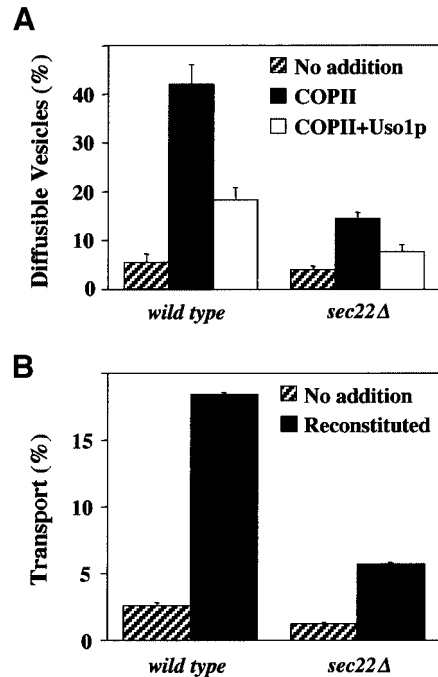
### *Ykt6p Assembles into Specific ER/Golgi SNARE Complex in sec22 $\Delta$ Strains*

We next investigated whether a redundant activity was substituting for Sec22p function. Of the 21 predicted SNARE proteins in *S. cerevisiae*, Ykt6p shares the highest degree of amino acid identity (28%) with Sec22p. Furthermore, the core sequences of Ykt6p and Sec22p share an even greater degree of amino acid identity (40%) with the zero layer arginine residue present in both proteins. Thus, substitution of Sec22p with Ykt6p in a tetrameric SNARE complex consisting of Sed5p, Bet1p, Bos1p, and Ykt6p would preserve a 3Q:1R ratio. Ykt6p has been reported to act in multiple trafficking steps in yeast, including retrograde transport to the *cis*-Golgi (McNew *et al.*, 1997), homotypic vacuole fusion (Ungermann *et al.*, 1999), and anterograde transport from the Golgi complex to the vacuole (Dilcher *et al.*, 2001). Although Ykt6p has not been directly implicated in anterograde transport from the ER to the Golgi complex in yeast, the temperature-sensitive *sec22-1* allele is suppressed by multicopy *YKT6* (Banfield *et al.*, 1995). To investigate the possibility that Ykt6p functionally replaced Sec22p, we first examined the expression level of the ER/Golgi SNAREs in whole cell membranes (Figure 3). Sed5p, Sly1p, and Bos1p (our unpublished data) expression levels were unchanged in the *sec22 $\Delta$*  strain, whereas Ykt6p expression was increased 3.4-fold. Furthermore, Ykt6p remained membrane bound in the *sec22 $\Delta$*  strain, indicating efficient posttranslational prenylation of the overexpressed protein. Other proteins involved in budding (Sec23p) and tethering (Ypt1p) of ER-derived transport vesicles were not elevated in the *sec22 $\Delta$*  strain. Interestingly, a 1.6-fold increase in the ER-translocon protein Sec61p was observed in the *sec22 $\Delta$*  strain and was probably due to activation of the unfolded protein response (UPR) caused by this deletion (Travers *et al.*, 2000; Belden and Barlowe, 2001). However, it should be noted that the expression level of Ykt6p was not induced simply by activating the UPR with dithiothreitol (our unpublished data), a result that is in accord with microarray analysis of UPR-induced messages (Travers *et al.*, 2000). In summary, deletion of *SEC22* increases the expression level of Ykt6p, the yeast SNARE protein that shares highest identity to Sec22p.

Previous reports suggested that Ykt6p functions on Golgi membranes and later compartments of the secretory pathway, including the vacuole (McNew *et al.*, 1997; Ungermann



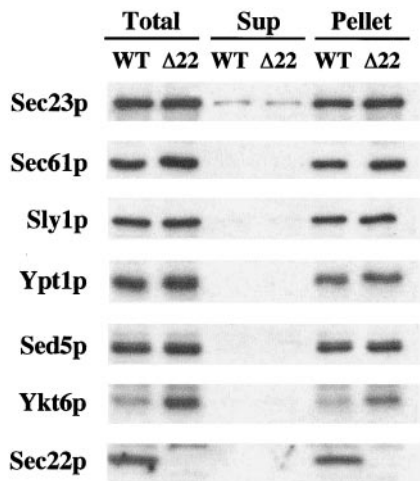
**Figure 1.** Anti-Sec22p antibodies inhibit transport to the Golgi complex in vitro. (A) Wild-type (CBY740) semi-intact cells containing [<sup>35</sup>S]gp- $\alpha$ -factor in the ER were incubated with COPII proteins, Uso1p, LMA1, and an ATP regeneration system. After 80 min at 23°C, the amount of Golgi-modified [<sup>35</sup>S]gp- $\alpha$ -factor was measured to determine transport efficiency. Varying amounts of affinity-purified anti-Sec22p antibodies or preimmune IgGs were added into cell-free transport reactions. In this experiment, background transport (semi-intact cells with an ATP regeneration system) was 1.3% and reconstituted transport in the absence of antibodies was 12.1%. (B) Semi-intact cells prepared as in A were incubated with COPII or COPII plus Uso1p to measure budding and tethering in the presence or absence of anti-Sec22p (15  $\mu$ g/ml). After 30 min at 23°C, freely diffusible vesicles containing [<sup>35</sup>S]gp- $\alpha$ -factor were separated from semi-intact membranes by centrifugation at 18,000  $\times$  g. (C) A parallel transport reaction for the experiment shown in B to demonstrate effective inhibition of fusion with anti-Sec22p.



**Figure 2.** Influence of *sec22 $\Delta$*  on steps in ER-Golgi transport. Washed semi-intact cells containing [<sup>35</sup>S]gp- $\alpha$ -factor were prepared from wild-type (CBY740) and *sec22 $\Delta$*  (CBY773) strains. (A) Vesicle budding and tethering in reactions that contained an ATP regeneration system alone (no addition, hatched bars), plus COPII (solid bars) or plus COPII and Uso1p (open bars). (B) Transport assays contained an ATP regeneration system alone (no addition, hatched bars) or were supplemented with COPII, Uso1p, and LMA1 (reconstituted, solid bars).

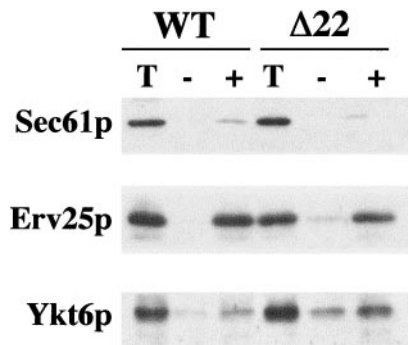
*et al.*, 1999; Cao and Barlowe, 2000; Dilcher *et al.*, 2001). If Ykt6p was substituting for Sec22p function in transport between the ER and Golgi, we expected that a fraction of overexpressed Ykt6p would be found on ER-derived transport vesicles. To explore this possibility, we compared COPII vesicles isolated from wild-type and *sec22 $\Delta$*  strains. Vesicle budding was reconstituted from ER membranes by incubating purified COPII proteins with washed membranes (Figure 4). Membranes lacking Sec22p produced COPII-coated vesicles, albeit less efficiently than wild-type, as evidenced by budding of the vesicle marker protein Erv25p (Belden and Barlowe, 1996). This result was also in accord with decreased [<sup>35</sup>S]gpaf budding observed in Figure 2. Although budding was less efficient in *sec22 $\Delta$*  membranes, COPII vesicles from these membranes contained an elevated level of Ykt6p compared with other vesicle marker proteins. We conclude that overexpressed Ykt6p was contained on COPII vesicles and therefore in a location to participate in this stage of transport.

We next tested whether the overexpressed Ykt6p detected in *sec22 $\Delta$*  strains was associated with other SNARE proteins that operate in transport between the ER and Golgi complex. Previous reports indicated that some Ykt6p coimmunoprecipitated with Sed5p from detergent-solubilized membranes (Sogaard *et al.*, 1994; McNew *et al.*, 1997). We immunoprecipitated Bos1p from wild-type and *sec22 $\Delta$* -solubilized

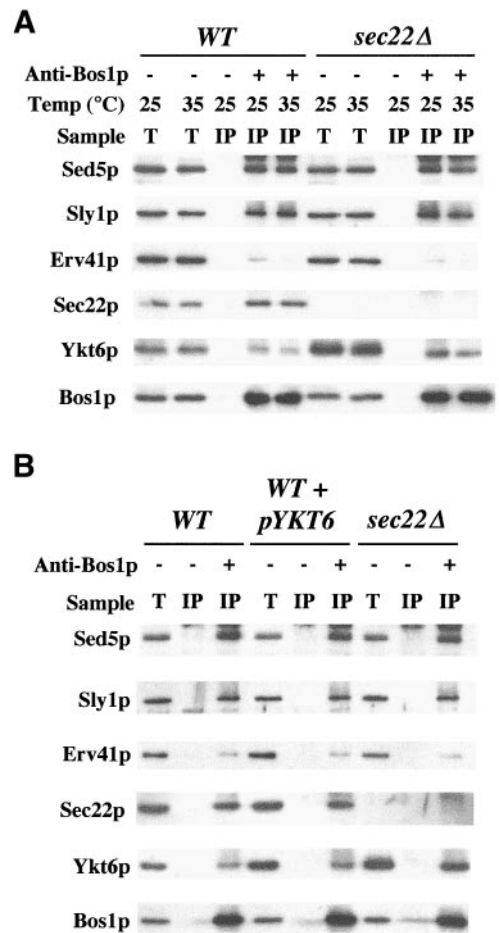


**Figure 3.** Deletion of *SEC22* increases Ykt6p expression level. Immunoblot to monitor expression levels and subcellular distributions of proteins in WT (CBY740) and *sec22Δ* (CBY773) cells. Washed semi-intact cells were incubated in buffer (20 mM HEPES pH 7.0, 150 mM KOAc, and 5 mM MgOAc) and then fractionated by centrifugation at 100,000 × *g* for 10 min to generate supernatant (Sup) and pellet fractions. The total lysate and aliquots of each fraction were resolved by SDS-PAGE, followed by immunoblot for Sec23p, Sec61p, Sly1p, Ypt1p, Sed5p, Ykt6p, and Sec22p.

membranes and monitored the amount of Ykt6p and other proteins that coprecipitated (Figure 5A). Membranes were preincubated for a brief period at 25 or 35°C to monitor the relative stability of these associations. After preincubation at 25°C, equivalent amounts of Sed5p, Sly1p, and Bet1p (our unpublished data) coimmunoprecipitated with Bos1p in wild-type and *sec22Δ* strains; however, the amount of Ykt6p associated with Bos1p immunoprecipitates was increased 2.7-fold in *sec22Δ* membranes. Erv41p, an integral membrane protein that localizes to ER/Golgi membranes (Otte *et al.*, 2001), was not efficiently immunoprecipitated and served



**Figure 4.** Packaging of Ykt6p into COPII-coated vesicles. COPII budding reactions with semi-intact cells from WT (CBY740) and *sec22Δ* (CBY773) strains. One-tenth of a total reaction (T), budded vesicles isolated after incubation with COPII proteins (+), or a mock reaction without COPII proteins (-). Samples were resolved by SDS-PAGE, followed by immunoblot for Sec61p (ER resident protein), Erv25p (vesicle protein), and Ykt6p.



**Figure 5.** Incorporation of Ykt6p into ER-Golgi SNARE complexes. Solubilized proteins were bound to anti-Bos1p coupled to protein A beads (+) or beads alone (-) as described in MATERIAL AND METHODS. (A) Semi-intact cells from WT (CBY740) and *sec22Δ* (CBY773) strains were preincubated at 25 or 35°C and then placed on ice before solubilization with Triton X-100. Total solubilized extracts (T) and immunoprecipitates (IP) were resolved on SDS-PAGE followed by immunoblot for indicated proteins. (B) Semi-intact cells from WT (CBY740), WT+pYKT6-2 μm (CBY1108), and *sec22Δ* (CBY773) strains were preincubated at 25°C, solubilized, and immunoprecipitated with anti-Bos1p-coupled protein A beads as described above.

as a negative control for these experiments. If membranes were preincubated at 35°C before Bos1p immunoprecipitation, the level of Sed5p and Sly1p was unchanged; however, the amount of bound Ykt6p was decreased 1.5-fold in *sec22Δ* membranes compared with wild type. Comparable levels of Bos1p were recovered from both membrane preparations at 25 or 35°C. To summarize, more Ykt6p was associated with Bos1p in strains lacking Sec22p and this association was thermosensitive. The observed instability of this Bos1p complex in *sec22Δ* membranes may underlie the temperature sensitivity exhibited by *sec22Δ* strains.

The association of Ykt6p with Bos1p was also examined in a wild-type strain that overproduced Ykt6p (Figure 5B). We were concerned that association of Ykt6p with Bos1p was a

nonspecific consequence of Ykt6p overexpression in the *sec22Δ* strain. However, if Sec22p and Ykt6p competed for a specific association with a Sed5p-Bet1p-Bos1p SNARE complex, one would expect that *YKT6* overexpression in a wild-type strain would yield less Ykt6p in association with Bos1p than in a *sec22Δ* strain. Indeed, a threefold overproduction of Ykt6p in the presence of normal levels of Sec22p resulted in a modest increase in Ykt6p that was coimmunoprecipitated with Bos1p. This level was significantly less than the amount of Ykt6p associated with Bos1p in *sec22Δ* membranes (Figure 5B, compare Ykt6p in lanes 3, 6 and 9). These observations indicate that in the absence of Sec22p, Ykt6p protein levels are increased and Ykt6p assembles into a specific SNARE complex with Bos1p.

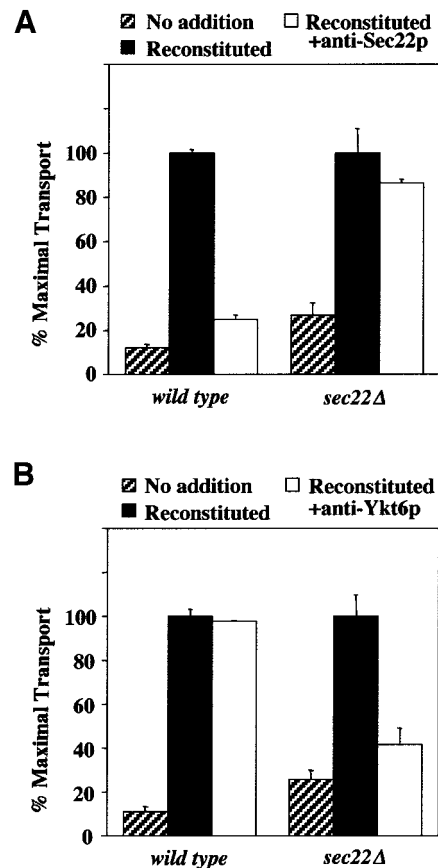
### Ykt6p Functionally Substitutes for Sec22p

If Ykt6p functionally substitutes for Sec22p in *sec22Δ* strains, we hypothesized that strains lacking Sec22p would be sensitive to inhibitors of Ykt6p activity in the ER/Golgi cell-free transport assay. To test this idea, we used the reconstituted transport assay described in Figure 2 and selectively neutralized Sec22p or Ykt6p activity with affinity-purified antibodies directed against these proteins. Addition of anti-Sec22p antibodies to wild-type reactions inhibited transport by 90%, whereas addition of an identical dose of antibodies to *sec22Δ* reactions reduced transport by 10% (Figure 6A). In contrast, wild-type transport reactions were insensitive to anti-Ykt6p antibodies but *sec22Δ* membranes were strongly inhibited by this addition (Figure 6B). Moreover, anti-Ykt6p antibodies did not inhibit COPII vesicle budding in *sec22Δ* membranes (our unpublished observation), indicating a specific block in the fusion stage. These findings demonstrate that Ykt6p is functionally required for anterograde transport to the Golgi complex in *sec22Δ* strains.

### Efficient Transport Requires Sec22p Activity on ER-derived Vesicles or Acceptor Membranes

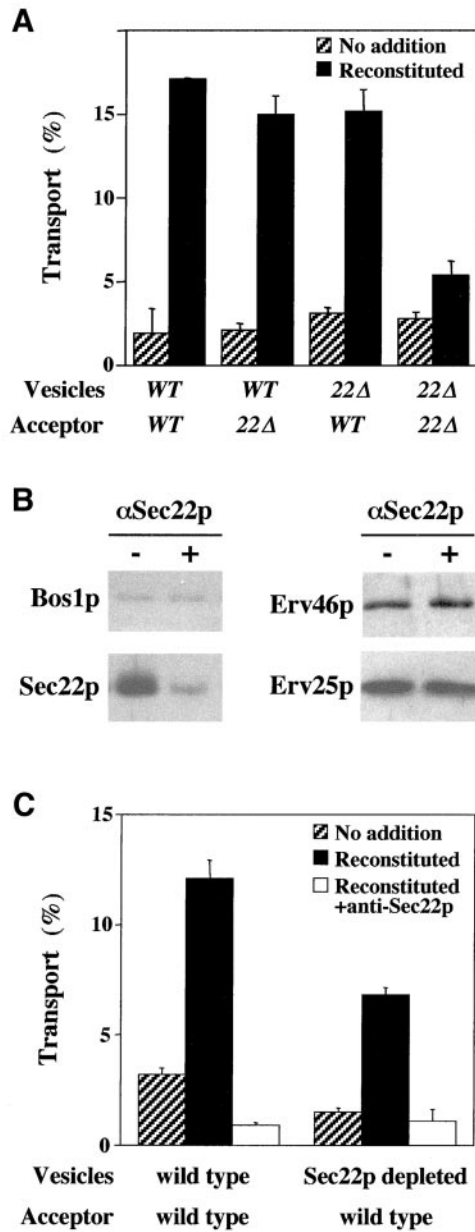
Having established that Sec22p was directly required for anterograde transport, we next investigated whether this activity localized to vesicle or acceptor membranes. Our previous experiments indicated that Bet1p and Bos1p were functionally required on vesicles, whereas Sed5p acted on the acceptor membrane fraction (Cao and Barlowe, 2000). To determine sites of action, we isolated COPII vesicles containing [<sup>35</sup>S]gpaf from wild-type or *sec22Δ* membranes and added equal amounts of each to wild-type or *sec22Δ* acceptor membranes. Vesicles or acceptor membranes lacking Sec22p fused efficiently when mixed with their wild-type counterpart (Figure 7A). Only when Sec22p was absent from both vesicles and acceptor membranes was transport significantly reduced. This level corresponded to that observed when overall transport was reconstituted in *sec22Δ* membranes (Figure 2B). Presumably, this transport level is sustained by Ykt6p substitution. These results indicate that either the vesicle or the acceptor membrane fraction can provide Sec22p activity.

Because the *sec22Δ* mutation reduced the efficiency of vesicle budding and fusion assays, we sought a second line of experimentation to confirm the localized requirements for Sec22p by using wild-type membranes. Previous reports have shown that antibodies directed against specific ER-



**Figure 6.** Selective inhibition of ER-Golgi transport by anti-Ykt6p antibodies. Cell free transport assays in wild-type (CBY740) and *sec22Δ* (CBY773) semi-intact cells. (A) Assays contained an ATP regeneration system alone (no addition, hatched bars), plus reconstitution proteins (solid bars) or reconstitution proteins and anti-Sec22p. (B) As in A except anti-Ykt6p (75  $\mu$ g/ml) antibodies were used instead of anti-Sec22p antibodies. In these experiments, maximal transport of [<sup>35</sup>S]gp- $\alpha$  factor was ~15% for the wild-type and ~5% for the *sec22Δ* semi-intact cells.

vesicle proteins can inhibit their incorporation into these vesicles when added during vesicle-budding reactions (Rowe *et al.*, 1998; Allan *et al.*, 2000). Presumably, antibody-bound proteins are not recognized by the COPII-budding machinery and therefore are not efficiently packaged into transport vesicles. Therefore, we sought to deplete Sec22p from ER-derived vesicles by adding affinity-purified anti-Sec22p antibodies to a COPII-budding reaction. As seen in Figure 7B, inclusion of anti-Sec22p inhibited Sec22p packaging into COPII-synthesized vesicles but did not inhibit overall vesicle budding because other vesicle proteins, including Bos1p, Sed5p (our unpublished data), Erv25p, and Erv46p, were packaged efficiently in the presence of this antibody. We then purified wild-type and Sec22p-depleted vesicles on density gradients and measured their capacity to fuse with wild-type acceptor membranes (Figure 7C). No anti-Sec22p antibodies were detected on vesicles after gradient-purification (our unpublished observation); therefore, any influence on fusion efficiency can be attributed to depletion and not



**Figure 7.** Sec22p acts on vesicle or acceptor membranes. (A) COPII vesicles containing [<sup>35</sup>S]gp- $\alpha$ -factor were synthesized from wild-type (WT) or *sec22Δ* (22Δ) membranes and mixed with wild-type or *sec22Δ* semi-intact cell acceptor membranes in transport assays. No addition indicates ATP regeneration system alone, and reconstituted indicates addition of Uso1 and LMA1. (B) Immunoblot of floated ER-derived vesicles that had been depleted of Sec22p by addition of anti-Sec22p during COPII budding. (C) Incubation of wild-type or Sec22p-depleted vesicles with wild-type acceptor membranes in transport assays. Addition of anti-Sec22p antibody inhibited fusion efficiency in wild-type and depleted vesicles.

carryover of antibody. In these experiments, we observed that fusion of purified wild-type vesicles remained sensitive to anti-Sec22p antibodies, indicating a direct role for Sec22p

in this fusion stage of anterograde transport. We also found that Sec22p depletion from vesicles reduced their fusion efficiency (~2-fold) but some fusion activity remained. This residual vesicle fusion activity relied on Sec22p because addition of anti-Sec22p antibody inhibited this fusion signal to near background levels. Therefore, a >90% reduction in Sec22p from transport vesicles caused only a 50% reduction in fusion efficiency. Together with the *sec22Δ* experiments, these results suggest that optimal fusion efficiency requires Sec22p on vesicles and acceptor membranes, but fusion can proceed if activity is present on either membrane. These findings are similar to those reported for Nyv1p-dependent fusion of vacuoles (Nichols *et al.*, 1997). In this situation, deletion of Nyv1p (an R-SNARE) from one vacuole reduced but did not block fusion, whereas deletion from both vacuoles blocked membrane fusion.

### Genetic Experiments Reveal a Synthetic Lethal Relationship between *sec22Δ* and *ykt6-1*

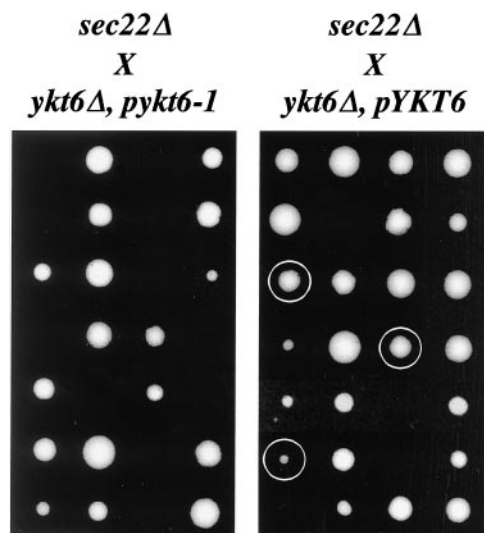
If Ykt6p substitutes for Sec22p in vivo, we hypothesized that a crippled version of Ykt6p may not fulfill this requirement. *YKT6* is an essential gene (McNew *et al.*, 1997); however, a previous report described a temperature-sensitive *ykt6-1* allele that inhibits intra-Golgi and/or post-Golgi transport when incubated at restrictive temperatures (Tsui and Banfield, 2000). We tested the genetic relationship between the *sec22Δ* and *ykt6-1* alleles by crossing strain CBY1236 (*sec22Δ*) with SARY166 (*ykt6Δ*, *pykt6-1*) and SARY189 (*ykt6Δ*, *pYKT6*). After sporulation and tetrad dissection of the *sec22Δ* X *ykt6Δ*, *pykt6-1* cross, no haploid strains containing the *sec22Δ*, *ykt6Δ* and *pykt6-1* alleles were recovered (Figure 8A). In similar analyses of *sec22Δ* X *ykt6Δ*, *pYKT6* tetrads, several spores containing the *sec22Δ*, *ykt6Δ* and *pYKT6* alleles were recovered (Figure 8B). Based on these results, we conclude that *sec22Δ* strains cannot survive if *YKT6* function is compromised. These in vivo results corroborate our in vitro findings indicating that wild-type Ykt6p can substitute for Sec22p in fusion of ER-derived transport vesicles.

## DISCUSSION

### SNAREs in ER/Golgi Transport

In this report, we investigated the mechanisms by which yeast cells lacking the Sec22p R-SNARE protein can maintain anterograde transport between the ER and Golgi complex. Our experiments demonstrated that Ykt6p, the R-SNARE most related to Sec22p, was up-regulated and formed a specific SNARE complex with Bos1p and Sed5p when Sec22p was absent. Under this condition, Ykt6p was also efficiently packaged into ER-derived transport vesicles and was required for fusion of these vesicles with acceptor Golgi membranes. Although Ykt6p can substitute for Sec22p activity, replacement was not optimal because cell growth rates are reduced (Dascher *et al.*, 1991) and in vitro transport efficiency was decreased (Figure 2). When Ykt6p function was further compromised in a *sec22Δ* background, cell viability was lost. Based on these findings, we conclude that Ykt6p provides a redundant activity for Sec22p.

Our findings answer a long-standing question concerning the viability of *sec22Δ* strains and their resulting phenotypes (Semenza *et al.*, 1990; Dascher *et al.*, 1991). A role for Sec22p



**Figure 8.** Synthetic lethal interaction between *ykt6-1* and *sec22Δ*. (A) A diploid strain generated from the cross of CBY1236 (*sec22Δ*) and SARY166 (*ykt6Δ, pykt6-1*) was sporulated, dissected, and incubated on YPD plates at room temperature for 5 d. Seven (from a total of 14) representative tetrads are shown. No strains containing *sec22Δ ykt6Δ* and *pykt6-1* were recovered. (B) As in A, except CBY1236 (*sec22Δ*) was crossed with SARY189 (*ykt6Δ, pYKT6*) and sporulated. Seven (from a total of 14) representative tetrads are shown. Circled colonies indicate strains in which the *sec22Δ ykt6Δ* and *pYKT6* alleles cosegregated. Some inviability results from a failure to segregate the *pYKT6* plasmid into *ykt6Δ* spores.

in retrograde transport from the Golgi complex to the ER had been suggested (Semenza *et al.*, 1990) and demonstrated (Spang and Schekman, 1998), but a direct requirement for Sec22p activity in anterograde transport to the Golgi has not been reported (Cao *et al.*, 1998; Spang and Schekman, 1998). Perhaps the conditional *sec22-3* allele used in these experiments selectively inhibits retrograde and not anterograde transport. Regardless, studies now indicate direct requirements for Sed5p, Bet1p, Bos1p, and Sec22p in anterograde traffic to the Golgi complex (Lian and Ferro-Novick, 1993; Cao and Barlowe, 2000). We propose that a SNARE complex formed from Sed5p, Bet1p, Bos1p, and Sec22p catalyzes membrane fusion in accord with studies showing formation of a stable quaternary complex between these proteins (Parlati *et al.*, 2000; Tsui *et al.*, 2001) and a capacity for this subset of SNAREs to fuse proteoliposomes (McNew *et al.*, 2000). In vitro data suggest that Bos1p and Bet1p act on ER-derived vesicles, whereas Sed5p acts on acceptor membranes (Lian and Ferro-Novick, 1993; Cao and Barlowe, 2000). In this report, we show that Sec22p acts on either vesicles or acceptor membranes. These findings contrast the minimal fusion assay where only a combination of Sed5p, Bos1p, and Sec22p in one bilayer fused with partner liposomes containing Bet1p (Parlati *et al.*, 2000). It remains to be determined how SNARE regulatory proteins such as Sly1p may influence these topological requirements. Last, we hypothesize that the Sed5p-Bet1p-Bos1p-Sec22p complex acts in fusion of ER-derived membranes with Golgi acceptor membranes that house outer-chain oligosaccharide modification activities;

however, these SNAREs may also act in homotypic fusion of ER-derived vesicles in a step that precedes heterotypic fusion (Rowe *et al.*, 1998).

If Sec22p is required for retrograde transport from the Golgi to ER (Spang and Schekman, 1998), does Ykt6p also substitute in the retrograde pathway when Sec22p is absent? We speculate that Ykt6p satisfies this requirement as well. Other characterized proteins that operate in retrograde traffic to the ER are essential (Lewis *et al.*, 1997; Spang and Schekman, 1998; Reilly *et al.*, 2001); therefore, it seems unlikely that a parallel pathway operates in the absence of Sec22p. Rather, Ykt6p may substitute for Sec22p yielding a SNARE complex consisting of Ufe1p, Bos1p, Bet1p, and Ykt6p that catalyzes retrograde fusion in *sec22Δ* strains. Given that Ufe1p is largely ER localized and Sed5p is Golgi localized, it is not at all clear how anterograde and retrograde vesicles are distinct with respect to their SNARE machinery. Perhaps upstream-tethering components that probably include Uso1p, Ypt1p, and TRAPP (Sacher *et al.*, 2001) for anterograde movement, and Sec20p, Tip20, and Dsl1p (Reilly *et al.*, 2001) for retrograde transport, could decipher features on these distinct carrier vesicles.

Substantial progress has been made in characterizing SNARE proteins that mediate transport through the early secretory pathway in mammalian cells. The mammalian homologs of Sed5p (syntaxin 5), Bet1p (rbet), Bos1p (membrin), Sec22p (Sec22b), and Ykt6p (Ykt6) have been functionally implicated in transport between the ER and Golgi complex (Rowe *et al.*, 1998; Zhang *et al.*, 1999; Allan *et al.*, 2000; Xu *et al.*, 2000; Zhang and Hong, 2001). Interestingly, antibodies against mammalian Ykt6p inhibited a late stage of ER-Golgi transport of vesicular stomatitis virus-G protein protein in vitro (Zhang and Hong, 2001), in contrast to our observation in yeast. However, it may be difficult to draw direct parallels between yeast and mammals because there seem to be multiple isoforms of ER/Golgi SNARE proteins that localize to distinct compartments in mammalian cells, and it seems that the organization of the early secretory pathway across species is distinct (Glick, 2000; Zhang and Hong, 2001).

### Specificity of SNAREs

Other studies in yeast have suggested cellular redundancy in SNARE protein functions through genetic experiments (Protopopov *et al.*, 1993; Darsow *et al.*, 1997; Nichols *et al.*, 1997; Dilcher *et al.*, 2001; Tsui *et al.*, 2001) although in these instances the data could be explained by substitution or by activation of parallel processes. Indeed, the situation is complicated because a single SNARE protein can operate in multiple trafficking pathways (Fischer von Mollard and Stevens, 1999), and transport between some membranes can use multiple routes (Lewis *et al.*, 2000; Harsay and Schekman, 2002). Importantly, the findings in this report demonstrate that a single SNARE protein that normally operates in other trafficking steps can be conscripted to act in another. This apparent flexibility in SNARE protein requirements seems inconsistent with a role in specifying fusion partners (McNew *et al.*, 2000).

Biochemical studies indicate significant promiscuity in SNARE complex assembly when purified cognate and non-cognate SNARE proteins are mixed in solution (Fasshauer *et al.*, 1999; Yang *et al.*, 1999; Tsui and Banfield, 2000). In



contrast, reconstituted liposome fusion assays suggest that cognate SNARE complexes are largely required to drive bilayer fusion. For example, proteoliposomes containing a Sed5p-Bos1p-Sec22p complex fused specifically with partner liposomes containing Bet1p but not with 10 other SNARE proteins tested (McNew *et al.*, 2000). Interestingly, Ykt6p was not able to substitute in this assay when modified with a lipid anchor. When a transmembrane domain was fused to Ykt6p, this integral membrane species promoted fusion with a plasma membrane SNARE complex consisting of Sso1p-Sec9p. The transmembrane-anchored form of Ykt6p was apparently not tested in combinations with Sed5p, Bos1p, and Bet1p. It seems probable that Ykt6p would at least partially substitute for Sec22p in the reconstituted fusion assay although additional SNARE regulatory factors may be required to recapitulate this reaction.

Ykt6p may be well suited for promiscuous behavior because it is a lipid-anchored protein that is partially soluble (McNew *et al.*, 1997) and displays a broad intracellular distribution (Cao and Barlowe, 2000). In fact, Ykt6p substitution for other related R-SNAREs could explain the nonlethal phenotypes associated with strains lacking Snc1p/Snc2p (Protopopov *et al.*, 1993) or Nyv1p (Nichols *et al.*, 1997). The Snc1p/Snc2p R-SNAREs operate in fusion at the cell surface, and when both are deleted, fusion of exocytic vesicles is reduced but cells remain viable. The other Q-SNAREs that operate in this stage of transport, Sso1p/Sso2p and Sec9p, are essential. Therefore these properties are reminiscent of the ER/Golgi situation because the R-SNARE (Sec22p) is nonessential and the other Q-SNAREs (Sed5p, Bos1p, and Bet1p) are essential. In homotypic vacuole fusion, the R-SNARE proteins Nyv1p and/or Ykt6p are thought to act with the Q-SNAREs Vam3p, Vam7p, and Vti1p (Nichols *et al.*, 1997; Ungermann *et al.*, 1999; McNew *et al.*, 2000). The phenotypes associated with *nyv1Δ* are mild compared with the vacuolar fragmentation patterns displayed by deletion of the associated Q-SNAREs (Nichols *et al.*, 1997). Again, Ykt6p may substitute for Nyv1p in this fusion pathway, a proposal that is supported by *in vitro* studies showing Ykt6p competes with Nyv1p for binding to a ternary SNARE complex consisting of Vam3p, Vam7p, and Vti1p (McNew *et al.*, 2000).

Is the situation with Sec22p in yeast an isolated case or could SNARE substitution be more widespread in nature? Given that deletion of the R-SNARE synaptobrevin in flies (Deitcher *et al.*, 1998), worms (Nonet *et al.*, 1998), and mice (Schoch *et al.*, 2001) does not block fusion, redundancy seems a probable explanation. Closer examination of the synaptobrevin knockout mice by electrophysiology reveals that spontaneous synaptic vesicle fusion was decreased 10-fold in the neural synapse (Schoch *et al.*, 2001). Indeed, one explanation given for the reduced fusion efficiency was that a noncognate SNARE that did not normally function in synaptic vesicle fusion could partially substitute for synaptobrevin in the mutant neurons (Scales *et al.*, 2001; Schoch *et al.*, 2001). Given our current findings, it may be informative to test whether the mammalian version of Ykt6p is expressed and can substitute for synaptobrevin at the neural synapse.

If there are SNARE proteins that can operate in many steps, and substitute for one another, how is compartmental

organization maintained? Perhaps some inappropriate fusion can be tolerated although within the limits of detection these events seem minor. Alternatively, membrane fusion reactions could be highly selective. The collective data on SNARE proteins now suggest they provide some selectivity but are unlikely to be the sole determinants of specificity. Previous studies with Rab GTPase chimeras indicated that they too are unlikely to provide a needed level of specificity (Brennwald and Novick, 1993). Therefore, a more likely explanation is that combinatorial mechanisms that use upstream targeting elements and SNARE proteins are required to maintain compartmental identity.

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