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The Synaptobrevin-related Domains of Bos1p and Sec22p Bind to the Syntaxin-like Region of Sed5p*

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SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors) are cytoplasmically oriented membrane proteins that reside on vesicular carriers (v-SNARE) and target organelles (t-SNARE). The pairing of a stage-specific v-SNARE with its cognate t-SNARE may mediate the specificity of membrane traffic. In the yeast *Saccharomyces cerevisiae* transport between the endoplasmic reticulum and Golgi complex employs two v-SNAREs, Bos1p and Sec22p, each containing a domain that is related to the neuronal v-SNARE synaptobrevin. Sed5p, which is homologous to syntaxin, is the t-SNARE that functions at this stage of the secretory pathway. Here we report that regions of Bos1p and Sec22p, which are homologous to synaptobrevin, bind to the syntaxin-like domain of Sed5p. Furthermore, we demonstrate that efficient v-SNARE/t-SNARE interactions require the participation of both v-SNAREs, indicating that, unlike post-Golgi membrane traffic, the active form of the endoplasmic reticulum to Golgi v-SNARE is a heteromeric complex.

The secretory pathway is composed of several distinct membrane-bound compartments, each with a unique set of proteins. The maintenance of these organelles is governed by the proper targeting of vesicular carriers that move to and from these compartments. For example, to maintain its integrity, the *cis*-Golgi complex must receive anterograde transport vesicles that originate from the endoplasmic reticulum (ER)[1] or retrograde vesicles that bud from later Golgi compartments. How does a vesicular carrier find its acceptor membrane? The SNARE hypothesis (1) states that each vesicle contains a specific membrane protein (v-SNARE) which recognizes a unique receptor that resides on the target organelle (t-SNARE). Pairing of a v-SNARE with its cognate t-SNARE ensures that a vesicle will dock and fuse with its appropriate target membrane.

SNAREs have distinct features. Besides their vesicular localization, v-SNAREs are cytoplasmically oriented transmembrane proteins that are homologous to the synaptic vesicle protein synaptobrevin. t-SNAREs, on the other hand, are homologous to one of two neuronal presynaptic membrane proteins, syntaxin or SNAP-25. The yeast gene products *BOS1* (2) and *SEC22* (3), which are constituents of the ER to Golgi transport vesicles, fit the criteria of v-SNAREs. Their putative receptor is the t-SNARE Sed5p, a type II transmembrane Golgi protein that is related to syntaxin (4). Interestingly, Bet1p, which contains a domain that is related to SNAP-25, resides primarily on the ER instead of the target membrane (5, 6).

A yeast ER to Golgi SNARE complex accumulates in *sec18* mutant cells that are blocked in membrane fusion (7). Included in this complex are the v-SNAREs Bos1p and Sec22p, the t-SNARE Sed5p, small amounts of Bet1p, and six other proteins (6, 7). *In vitro* binding studies have demonstrated that the essential v-SNARE Bos1p binds directly to Sed5p, and Bet1p potentiates this interaction (6). The role of the nonessential v-SNARE, Sec22p, is discussed in this report.

Although Bos1p and Sec22p colocalize to the ER and carrier vesicles, they only form a complex on ER-derived transport vesicles where Bos1p functions (8, 9). Here we performed *in vitro* binding studies to demonstrate directly that the pairing of these proteins modulates the activity of the v-SNARE. Furthermore, deletion analysis revealed that a domain of Sed5p which is highly homologous to syntaxin binds to the synaptobrevin-like regions of Bos1p and Sec22p.

EXPERIMENTAL PROCEDURES

Yeast Strains and Genetic Techniques—Yeast strains used in this study were: SFNY26-6A (*MATα, his4–619*), ANY112 (*MAT***a**, *bet1–1*, *ura3–52*), SFNY411 (*MATα, sec22–3*, *ura3–52*, *ade2–801*, *leu2-Δ98*), SFNY412 (*MAT***a**, *bos1–1*, *ura3–52*, *leu2–3, 112*), SFNY357 (*MATα, ura3–52*, *leu2–3, 112*, *SEC22::URA3*), SFNY358 (*MAT***a**, *ura3–52*, *leu2–3, 112*), SFNY82 (*Mat***a**/*α*, *leu2–3*, *112/leu2–3*, *112*, *ura3–52/ura3–52*, *BET1::LEU2*), SFNY571 (*MATα, sed5–1, ura3–52, leu2–3, 112, his3-Δ200, trp1-Δ901, lys2–801, suc2-Δ9*), and NY426 (*MAT***a**, *ura3–52*, *sec22–3*).

Tetrad analysis and yeast transformations were performed as described before (10). All molecular biology reagents were from New England BioLabs except for *Taq* DNA polymerase, which was from Boehringer Mannheim. The *sec22–3* mutant was sequenced by subcloning the *SEC22* gene into the yeast shuttle vector pRS316 (*URA3, CEN6*; Ref. 11). The open reading frame was excised with *Aat*II and *Bsg*I, and the resulting linearized DNA was transformed into SFNY411 to allow for repair of the gapped gene (12). Plasmid DNA was rescued and sequenced to identify the site of the mutation.

DNA Constructions—The cytoplasmic domains of Bet1p, Bos1p, and Sec22p were amplified by polymerase chain reaction using the appropriate primers as described before (6). Additional oligonucleotides used for polymerase chain reaction in this study were as follows: 3′ oligonucleotide for His_6-Bos1p(1–150): 5′-ATACTAGGATCCCTGCGGTAGTCCCCCACCGTTGC-3′; 3′ oligonucleotide for His_6-Bos1p(1–135): 5′-ATACTAGGATCCCTAACCACCAACGTTCCTTTTATTC-3′; 5′ oligonucleotide for His_6-Sec22p(41–194): 5′-ATACTACATATGTTGACACCACAGTCTGCCACG-3′; 3′ oligonucleotide for His_6-Sec22p(1–154); 5′-ATACTACTCGAGTAGGTCTTCGATGTTCTTGG-3′. His_6-Bos1p(1–150) and His_6-Bos1p(1–135) were ligated into the *Nco*I/*Bam*HI site of pET11d (Novagen). His_6-Sec22p(41–194) and His_6-Sec22p(1–154) were ligated into the *Nde*I/*Xho*I site of pET29a (Novagen).

GST fusions of the cytoplasmic domains of Bos1p, Bos1p(L190S), and Sed5p were amplified by polymerase chain reaction and cloned in-frame

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[1] The abbreviations used are: ER, endoplasmic reticulum; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; v, vesicular; t, target; GST, glutathione *S*-transferase.

at the *Bam*HI/*Xho*I sites of pGEX-5X-3 (6); Pharmacia Biotech Inc.). Additional oligonucleotides used in this study were: 3′ oligonucleotide for Bos1p(1–170)-GST: 5′-ATACTACTCGAGCTAATCTAATTGAGCGTTACCCCTTTC-3′; 3′ oligonucleotide for Sed5p(1–250)-GST: 5′-ATACTACTCGAGTTAGTAGACGTTATTGGATAACTG-3′; 5′ oligonucleotide for Sed5p(158–324)-GST: 5′-ATACTAGGATCCCCAAAGACGTATTGGAGGAAAGGC-3′; 5′ oligonucleotide for Sed5p(251–324)-GST: 5′-ATACTAGGATCCCCTTACAAGAAAGAAATAGGGCGG-3′. Bos1p(1–170)-GST was ligated into the *Bam*HI site of pGEX-5X-3 (Pharmacia). All Sed5p-GST truncations were ligated into the *Bam*HI/*Xho*I site of pGEX-5X-3.

Protein Expression and Purification—His_6 (6-histidine)-tagged proteins were expressed in BL21(DE3)pLysS cells and purified on Ni^{2+}-nitrilotriacetic acid-agarose beads (Novagen) as described by the manufacturer except the tagged protein was eluted with 800 mM imidazole in 1-ml fractions. Fractions containing the peak of eluted protein were dialyzed against binding buffer (10 mM HEPES-NaOH, pH 7.4, 25 mM NaCl, 115 mM KCl, 2 mM $MgCl_2$) containing 30% glycerol. GST fusion proteins were purified by a batchwise procedure and eluted using 25 mM glutathione. The fusion protein was dialyzed overnight against phosphate-buffered saline (154 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.5), and the concentration was determined by the BCA assay (Pierce). The protein that failed to rebind to the resin was estimated, and the amount that bound to the beads was determined as the difference in values obtained before and after rebinding. Beads were stored at 4 °C in the presence of 1 mg/ml bovine serum albumin. The purity of each protein was determined by amino-terminal sequence analysis, and all concentrations reported take into account the percent purity.

In Vitro Binding Assays and Quantitation of Bound Bos1p and Sec22p—In vitro binding assays were performed by incubating varying amounts of His_6-tagged proteins with 1 μM GST fusion protein (immobilized on beads) in a 100-μl reaction containing binding buffer (see above) and 0.5% Triton X-100. Samples were incubated overnight at 4 °C, and the beads were washed four times with binding buffer. The bound His_6-tagged protein was eluted in SDS-sample buffer and electrophoresed on a 15% SDS-polyacrylamide gel. Western blot analysis was performed with anti-Bos1p (1:1,500 dilution) or anti-Sec22p (1:1,250 dilution) antiserum using ^{125}I-protein A. Truncated forms of Bos1p and Sec22p were tested to ensure immunoreactivity with their respective antiserum. To determine the amount of His_6-tagged fusion protein bound to the beads, samples were compared with standards of the appropriate recombinant protein (either His_6-Bos1p or His_6-Sec22p) and, following immunodetection, exposed to a PhosphorImaging plate and scanned onto a PhosphorImager. The Bos1p and Sec22p standard curves were linear in the range used (0–250 ng of protein). Antisera to Bos1p and Sec22p were raised against cytoplasmic domains of these proteins tagged with six histidine residues to facilitate antigen purification.

Estimation of Bos1p and Sec22p in a Yeast Lysate—Wild type yeast (SFNY26-6A) were grown to an OD_{599} = 1.0 and converted to spheroplasts during a 1-h incubation at 37 °C. The spheroplasts were lysed in 1% SDS (0.14 OD_{599} units/μl) and heated to 100 °C. The protein concentration of the lysate was determined by the method of Bradford using bovine serum albumin as a standard. Quantitative immunoblotting was performed on known amounts of lysate with an anti-Bos1p (1:1,500) or anti-Sec22p antiserum (1:1,250), and His_6-Bos1p or His_6-Sec22p (prepared as described above) were used to establish the standard curve. Western blotting was performed using ^{125}I-protein A. The blots were exposed to a PhosphorImaging plate, scanned onto a PhosphorImager, and analyzed with Molecular Dynamics Image Quant software (version 3.15).

RESULTS

The v-SNARE Contains More Than One Subunit—Cells disrupted for the *SEC22* gene display no growth defect at 30 °C but are cold-sensitive and temperature-sensitive for growth. This conditional lethal growth defect is suppressed by the overexpression of *BET1* or *BOS1* (3, 9), indicating that an increase in either gene product partially compensates for the loss of Sec22p. Previous studies have shown that Bet1p enables Bos1p to interact more efficiently with Sed5p (6). If Bet1p and Sec22p have related functions, then Sec22p may also play a role in facilitating the activity of Bos1p. To begin to address the function of Sec22p, we performed *in vitro* binding studies. These studies were executed by fusing the cytoplasmic portion of Sec22p to a six-histidine tag (His_6-Sec22p) and then testing its ability to bind to a Sed5p-GST hybrid protein that was immobilized on beads. As shown in Fig. 1 (*lanes 1–3*), His_6-Sec22p bound to Sed5p-GST but not to beads that contained GST (*lanes 4–6*). Saturable binding of Sec22p (19 pmol) was achieved as the concentration was raised to 5 μM. This is the same concentration at which His_6-Bos1p binding saturates Sed5p-GST (6).

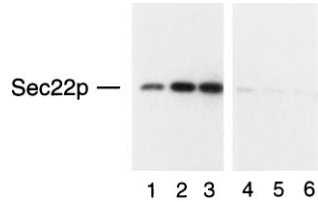

FIG. 1. **Sec22p binds to Sed5p.** Glutathione-agarose beads containing either 1 μM Sed5p-GST (*lanes 1–3*) or GST (*lanes 4–6*) were incubated with His_6-Sec22p. His_6-Sec22p was used at 0.1 μM (*lanes 1* and *4*), 0.5 μM (*lanes 2* and *5*), and 1 μM (*lanes 3* and *6*). Binding increased until the concentration of His_6-Sec22p was raised to 5 μM. No increase in binding was observed at 10 μM His_6-Sec22p (not shown). Bound protein was eluted and detected by Western blot analysis using polyclonal anti-Sec22p antiserum (1:1,250 dilution).

To address the possibility that Sec22p may affect the binding of Bos1p to Sed5p, we incubated His_6-Bos1p with increasing concentrations of His_6-Sec22p. These studies were performed at a concentration of His_6-Bos1p (0.5 μM) in which binding to Sed5p was barely detectable (Fig. 2, *lane 1*). Saturable binding of His_6-Bos1p (19.5 pmol) was achieved as the concentration of His_6-Sec22p was raised to 5 μM (*lanes 2–6*). Thus, Sec22p greatly enhances the affinity of Bos1p for Sed5p. To determine if Bos1p facilitates the binding of Sec22p to Sed5p, we performed the reciprocal of the experiment described above. When His_6-Sec22p (0.5 μM) was incubated with increasing amounts of His_6-Bos1p, saturable binding of Sec22p (18.5 pmol) to Sed5p was observed as the concentration of His_6-Bos1p was raised to 5 μM (data not shown). Thus, efficient v-SNARE/t-SNARE interactions require the presence of Bos1p as well as Sec22p. Although Bos1p binds to Sec22p *in vitro*,[2] we did not detect an interaction between these proteins at the lower concentrations used in this experiment. Thus, the most likely interpretation of our data is that Bos1p and Sec22p cooperatively interact with Sed5p-GST.

The Synaptobrevin-like Domain of Bos1p Contains Regions That Interact with Different SNAREs—The ER to Golgi v-SNAREs and their t-SNARE receptor contain domains that are homologous to their neuronal equivalents. Are these the regions where the SNAREs bind to each other? Bos1p, for example, contains a domain (amino acids 136–197) which is 25% identical to the region of synaptobrevin which binds to syntaxin (13). Previous findings have shown that the leucine at position 190, which lies within this domain, is required for binding to Bet1p but not Sed5p (6). To determine if other regions of this domain are critical for binding to Sed5p, we created truncations of Bos1p which lie within it and then tested their ability to bind to Sed5p. Whereas His_6-Bos1p(1–150), which deletes a significant portion of the synaptobrevin-like domain, was able to bind to Sed5p-GST efficiently (Fig. 3, compare *lane 2* with *lane 4*), the His_6-Bos1p(1–135) construct, which lacks the entire region, failed to bind (Fig. 3, compare *lane 6* with the GST control in *lane 5*). Furthermore, the presence of His_6-Sec22p did not stimulate the binding of His_6-Bos1p(1–135) to Sed5p-GST (not shown). This result was not a consequence of the inability of the antibody to recognize His_6-Bos1p(1–135), as His_6-Bos1p, His_6-Bos1p(1–150), and His_6-Bos1p(1–135) were all recognized readily by the anti-Bos1p serum (Fig. 3, *lanes 7–9*). Similar results were obtained when these constructs were tested for their ability to bind to Sec22p-GST (not shown).

[2] M. Sacher and S. Ferro-Novick, unpublished observations.

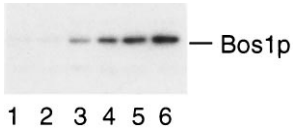

FIG. 2. **Sec22p facilitates the interaction between Bos1p and Sed5p.** His_6-Bos1p at a concentration of 0.5 μM was incubated with increasing concentrations of His_6-Sec22p in the presence of 1 μM Sed5p-GST. Protein bound to Sed5p-GST was eluted and detected by Western blot analysis using anti-Bos1p antiserum (1:1,500 dilution). His_6-Sec22p was used at 0 μM (*lane 1*), 0.01 μM (*lane 2*), 0.1 μM (*lane 3*), 1 μM (*lane 4*), 2 μM (*lane 5*), and 5 μM (*lane 6*). More Bos1p bound to Sed5p as the concentration of His_6-Sec22p was raised to 5 μM. At higher concentrations of His_6-Sec22p (10 μM) there was no increase in the amount of Bos1p that bound (not shown).

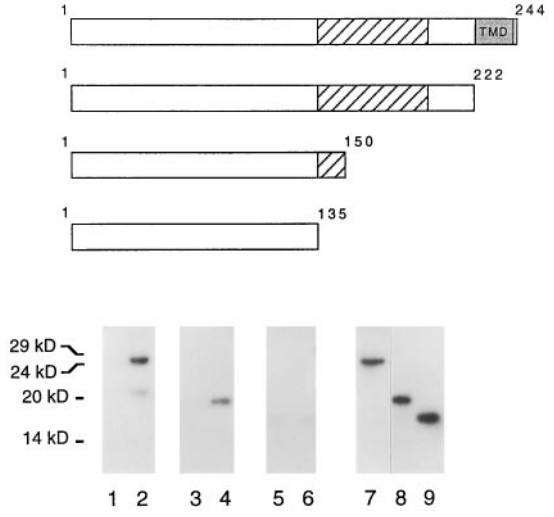

FIG. 3. **The synaptobrevin-like domain of Bos1p binds to Sed5p.** The full-length cytoplasmic domain of Bos1p (His_6-Bos1p; *lanes 1* and *2*) as well as His_6-Bos1p(1–150) (*lanes 3* and *4*) and His_6-Bos1p(1–135) (*lanes 5* and *6*) were incubated overnight at a concentration of 2 μM with beads containing 1 μM Sed5p-GST (*lanes 2*, *4*, and *6*) or 1 μM GST (*lanes 1*, *3*, and *5*). The beads were washed, and bound protein was eluted and detected on Western blots. The region of synaptobrevin homology is indicated by the *stippled area*, and the transmembrane domain (*TMD*) is *shaded*. In *lanes 7–9*, 50 ng of His_6-Bos1p, His_6-Bos1p(1–150), and His_6-Bos1p(1–135), respectively, were probed with anti-Bos1p antiserum to demonstrate that the truncated forms of Bos1p were still efficiently recognized by the antibody. Note the presence of a breakdown product of His_6-Bos1p(1–222) at ~20 kDa in *lane 2*. This band is sometimes seen when His_6-Bos1p is incubated overnight with beads.

Thus, these and previous findings (6) define two regions of the synaptobrevin-like domain of Bos1p. The extreme amino-terminal portion is required for binding to Sed5p (or Sec22p), whereas the carboxyl terminus interacts with Bet1p (6).

The Synaptobrevin-like Domain of Sec22p Binds to Sed5p—To define the domain of Sec22p which interacts with Sed5p, we focused on amino acids 130–194 because of its homology (32% identity) with *Drosophila melanogaster* synaptobrevin (Fig. 4 and Ref. 3). We began our analysis by deleting ~40 amino acids (155–194) of this region (Fig. 4*C*) and examining the ability of the truncated protein to bind to Sed5p-GST. As a control, a truncated form of Sec22p which lacks the first 40 residues was tested (Fig. 4*B*). Although this construct bound as efficiently as the full-length protein (Fig. 4, *A* and *B*), the carboxyl-terminal truncation failed to bind (Fig. 4*C*). Thus, as was observed for Bos1p, the region of synaptobrevin homology is essential for the interaction of Sec22p with Sed5p.

We reported previously that the *bet1–1* mutation maps to a domain of Bet1p which is homologous to SNAP-25, whereas *bos1–1* lies within the synaptobrevin-like region of Bos1p (6). *In vitro* studies have demonstrated that these mutations disrupt

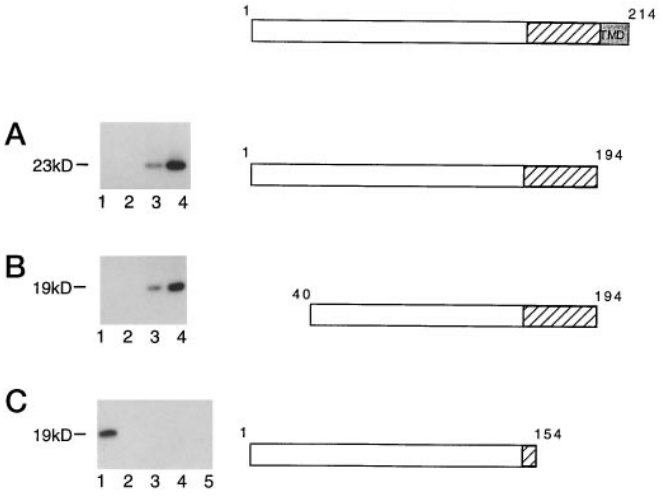

FIG. 4. **The carboxyl terminus of Sec22p is required for binding to Sed5p.** The cytoplasmic domain of Sec22p (*panel A*) as well as amino-terminal (*panel B*; amino acids 40–194) and carboxyl-terminal (*panel C*; amino acids 1–154) truncations were incubated in the presence of 1 μM Sed5p-GST. The full-length cytoplasmic domain of Sec22p (23 kDa) and the two truncations (19 kDa each) were detected by immunoblotting with anti-Sec22p antiserum. The His_6-tagged proteins were used at 0 μM (*lane 1* in *panels A* and *B*; *lane 2* in *panel C*), 0.5 μM (*lane 2* in *panels A* and *B*; *lane 3* in *panel C*), 1 μM (*lane 3* in *panels A* and *B*; *lane 4* in *panel C*), and 2 μM (*lane 4* in *panels A* and *B*; *lane 5* in *panel C*). *Lane 1* in *panel C* shows that truncated Sec22p (amino acids 1–154; 20 ng) is recognized by the anti-Sec22p antiserum. The *stippled* region indicates the region of synaptobrevin homology in Sec22p, and the transmembrane domain (*TMD*) is *shaded*.

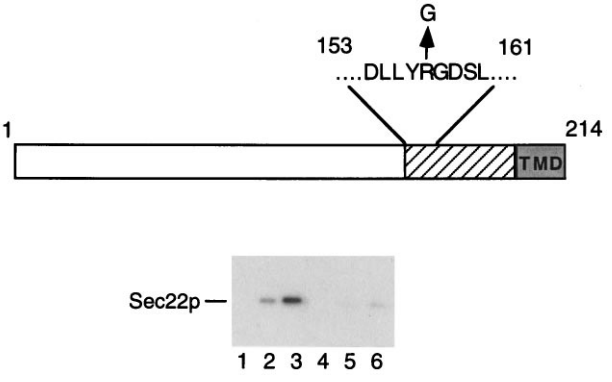

FIG. 5. **A mutation in the synaptobrevin-like domain of Sec22p disrupts binding to Sed5p.** His_6-Sec22p (*lanes 1–3*) and His_6-Sec22p(R157G) (*lanes 4–6*) were incubated with 1 μM Sed5p-GST. The His_6-tagged constructs were used at 0 μM (*lanes 1* and *4*), 0.5 μM (*lanes 2* and *5*), and 1 μM (*lanes 3* and *6*). Bound protein was eluted from the beads and detected by Western blot analysis using anti-Sec22p antiserum. *TMD*, transmembrane domain.

the binding of these proteins to other SNAREs (6). As shown in Fig. 5, the *sec22–3* mutation, which blocks transport *in vivo* (14), changes the arginine at position 157 to a glycine. Based on earlier findings, we hypothesized that the *sec22–3* lesion may block the interaction of Sec22p with Sed5p. To address this possibility, we constructed a His_6-tagged form of Sec22p (His_6-Sec22p(R157G), which contains the *sec22–3* mutation, and tested its ability to interact with Sed5p-GST. As shown in Fig. 5, His_6-Sec22p(R157G) failed to bind to Sed5p-GST (compare *lanes 4–6* with *1–3*). Circular dichroism spectroscopy indicated that the spectra of the mutant was the same as wild type (data not shown). Therefore, the failure of His_6-Sec22p(R157G) to interact with Sed5p-GST was not a consequence of a change in the secondary structure of the mutant protein.

The Syntaxin-like Domain of Sed5p Is Sufficient for Interac-

tion with v-SNAREs—The v-SNAREs Bos1p and Sec22p bind to Sed5p via a domain that is homolgous to synaptobrevin. To define the site on Sed5p which binds these v-SNAREs, we focused our attention on amino acids 252–324. This domain of Sed5p is highly homologous (~54% identity) to the region of syntaxin which binds to synaptobrevin (15). Our analysis was performed by testing the ability of His_6-Bos1p and His_6-Sec22p to bind to portions of Sed5p which were fused to GST. Both His_6-tagged proteins bound to Sed5p-GST (Fig. 6, *A* and *D*). However, neither interacted with a truncated form of the protein (amino acids 1–251) which lacks the syntaxin-like domain (Fig. 6, *B* and *E*). This domain is sufficient for v-SNARE/t-SNARE interactions, since His_6-Bos1p and His_6-Sec22p bound efficiently to a GST fusion protein that only contains amino acids 251–324 of Sed5p (Fig. 6, *C* and *F*). Thus, the regions of Bos1p and Sec22p which are related to synaptobrevin bind to the domain of Sed5p which is homologous to syntaxin.

Sed5–1 Displays Synthetic Lethal Interactions with bos1–1 and sec22–3—To correlate our *in vitro* binding studies with interactions that take place *in vivo*, we determined if *bos1–1* and *sec22–3* display synthetic lethality with *sed5–1*. Synthetic lethality, or inviability of double mutants, is another means of documenting interactions between gene products. It results when the effect of combining two mutations in the same haploid strain causes cell death under normally permissive conditions. The explanation for this event is that the mutated genes encode proteins that have a related function. In some cases, the products may even physically interact with each other (16). Thus, the combined effect of both mutations is to disrupt a process to a greater extent than either mutation alone. When *sed5–1* was crossed to either *bos1–1* or *sec22–3*, a pattern of synthetic lethality was observed (Table I); that is, the majority of the tetrads had three or two viable spores. This is the anticipated result if the double mutant is inviable. Given our *in vitro* studies, we conclude that this synthetic lethal pattern is indicative of a physical interaction.

Sec22p May Concentrate in Budding Vesicles—Although Bos1p and Sec22p colocalize to the ER and ER to Golgi carrier vesicles (5, 17), they only form a complex on ER-derived transport vesicles (9). As shown above, recombinant Bos1p and Sec22p interact readily with each other *in vitro*, and equimolar amounts are present in a complex that forms with Sed5p. *In vivo*, these v-SNAREs form a 1:1 complex on vesicles before they bind to Sed5p on the Golgi apparatus. If Bos1p and Sec22p both reside on the ER, what prevents them from binding to each other on this compartment? One possibility is that the ER contains an excess of one v-SNARE and because the other is limiting, the Bos1p-Sec22p complex fails to accumulate on this membrane. Since transport vesicles are transient intermediates, the steady-state levels of Bos1p and Sec22p on the ER can be estimated by determining the concentration of these proteins in a total cell lysate. Quantitative immunoblotting revealed that although yeast cells contain 4.25 fmol of Bos1p/μg of lysate there is only 0.30 fmol of Sec22p. Thus, there is approximately 14 times more Bos1p on the ER than Sec22p. Since equal amounts of these proteins are found on vesicles (9), a likely interpretation of these data is that the Bos1p-Sec22p complex forms as Sec22p concentrates in budding ER-derived transport vesicles.

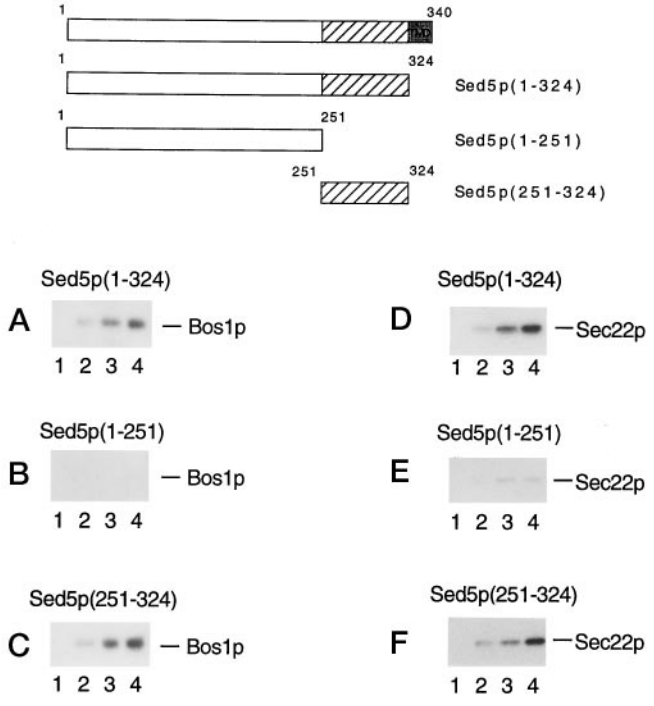

FIG. 6. **The v-SNAREs bind to the syntaxin-like domain of Sed5p.** Sed5p-GST (1 μM) or truncations of Sed5p which were fused to GST (as indicated above each *panel*) were incubated with 0 μM (*lane 1*), 0.5 μM (*lane 2*), 1 μM (*lane 3*), and 2 μM (*lane 4*) His_6-Bos1p (*panels A–C*) or His_6-Sec22p (*panels D–F*). The beads were washed, and bound protein was eluted and detected on Western blots with either anti-Bos1p (*panels A–C*) or anti-Sec22p (*panels D–F*) antiserum. The *stippled* region indicates the region of syntaxin homology in Sed5p, and the transmembrane domain (*TMD*) is *shaded*.

DISCUSSION

Here we used *in vitro* binding studies to show that in ER to Golgi membrane traffic, efficient SNARE interactions require a v-SNARE that is composed of more than one subunit. Furthermore, we have demonstrated for the first time that one of these SNAREs, Sec22p, binds directly to Sed5p. Although Bos1p and Sec22p were shown to be components of the same complex that includes Sed5p (7), it was not known if either of these proposed v-SNAREs binds directly to this putative t-SNARE. Our *in vitro* binding studies indicate that Sec22p (Fig. 1) as well as Bos1p (6) come in direct contact with Sed5p. The inability of His_6-Sec22p(R157G) to bind to the t-SNARE *in vitro* is consistent with *in vivo* data showing that the SNARE complex fails to form in the *sec22–3* mutant (7, 17).

In addition to containing synaptobrevin-like and syntaxin-related proteins, SNARE complexes contain a SNAP-25 like component. In the ER to Golgi SNARE complex, Bet1p, which contains a domain that is related to SNAP-25, potentiates v-SNARE/t-SNARE interactions via direct contact with Bos1p (6), or Sec22p.[2] Thus, Bet1p appears to act on the different subunits of the v-SNARE to enhance their interaction with the t-SNARE. Although Bet1p, Bos1p, and Sec22p may function in concert with each other, the combined effects of Sec22p and Bet1p on the Bos1p/Sed5p interaction are additive and not

TABLE I
sed5–1 lethal interactions with bos1–1 and sec22–3

Crossed to	Four viable			Three viable			Two viable			One viable	
	4−:0+[a]	3−:1+	2−:2+	3−:0+	2−:1+	1−:2+	2−:0+	1−:1+	0−:2+	1−:0+	0−:1+
bos1–1	2	0	0	0	15	0	0	1	3	0	1
sec22–3	1	0	0	0	15	1	0	0	5	0	0

[a] Designates the number of spores that grew (+) or did not grow (−) at 37 °C.

synergistic.[3] Previous studies have shown that *in vivo*, these interactions are regulated by the ras-like GTP-binding protein Ypt1p (6, 9).

Our findings illustrate that regions of yeast SNAREs, which are homologous to their neuronal counterparts, are functionally significant. Although Sed5p, Sec22p, and Bos1p were initially referred to as SNAREs because of this homology and their subcellular distribution, here we have demonstrated the relevance of these homologies at a molecular level. Although we have begun to address the mechanism by which the activity of the v-SNARE is regulated, clearly many other interactions that contribute to this process may take place. For example, Bos1p contains a region from amino acids 1 to 108 which, according to the Lupas algorithm (18), has nearly a 100% probability of being involved in a coiled-coil interaction. If this region does not interact with Sed5p, Sec22p, or Bet1p, then some other component, perhaps as yet unknown, may interact with it. Could this coiled-coil domain play a role in the retrieval of Bos1p from the Golgi complex? High copy suppressor analysis or synthetic lethal screens, in addition to *in vitro* binding studies, may aid in identifying other Bos1p-interacting proteins that regulate v-SNARE activity. Our data, however, underscore the remarkable evolutionary conservation of the basic secretory mechanism from yeast to mammals.

[3] S. Stone and S. Ferro-Novick, unpublished observations.

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