Molecular Biology of the Cell
Vol. 8, 1175–1181, July 1997

Bet1p Activates the v-SNARE Bos1p

Shelly Stone,* Michael Sacher,* Yuxin Mao,* Chavela Carr, Patrick Lyons, Anne Marie Quinn, and Susan Ferro-Novick†

Howard Hughes Medical Institute and Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

Submitted November 5, 1996; Accepted April 2, 1997
Monitoring Editor: David Botstein

Bet1p is a type II membrane protein that is required for vesicular transport between the endoplasmic reticulum and Golgi complex in the yeast *Saccharomyces cerevisiae*. A domain of Bet1p, that shows potential to be involved in a coiled-coil interaction, is homologous to a region of the neuronal protein SNAP-25. Here, we used in vitro binding studies to demonstrate that Bet1p plays a role in potentiating soluble NSF attachment protein receptor (SNARE) interactions. Mutational analysis points to the coiled-coil region as necessary for Bet1p function, and circular dichroism experiments support this theory. In vitro binding studies were also used to demonstrate that a direct interaction between Bet1p and Bos1p is required for the efficient interaction of the vesicle SNARE with its SNARE target. Genetic studies suggest that the interactions of Bet1p with Bos1p are regulated by the small GTP-binding protein Ypt1p.

INTRODUCTION

In exocytic fusion events, SNAP-25 and its homologues potentiate the interaction of a synaptobrevin-like protein on a transport vesicle (v-SNARE) with a syntaxin-like receptor on the target organelle (t-SNARE; Söllner *et al.*, 1993a; Pevsner *et al.*, 1994; Ferro-Novick and Jahn, 1994). These three proteins comprise a docking/fusion complex (also referred to as a SNARE complex) that includes soluble factors such as NSF (*SEC18* in yeast) and NSF attachment proteins (α-SNAP, *SEC17* in yeast, and γ-SNAP). To date, no analogue of SNAP-25 that functions in endoplasmic reticulum (ER) to Golgi membrane traffic has been described. Here we report that Bet1p, which is required for vesicular traffic between the ER and Golgi complex in yeast, contains a domain that is homolgous to a region of SNAP-25. Our findings indicate that Bet1p potentiates SNARE interactions and that this domain of homology is important for this interaction.

* The first three authors made equal contributions to the manuscript.

† Corresponding author: Department of Cell Biology, Boyer Center for Molecular Medicine, Howard Hughes Medical Institute, 295 Congress Avenue, Room 254B, New Haven, CT 06510.

MATERIALS AND METHODS

DNA Constructions

The cytoplasmic domains of Bet1p, Bos1p, and Sed5p were fused to either 6-histidine (His_6) or a glutathione *S*-transferase (Gst) tag and expressed in *Escherichia coli*. The region of *BET1*, *BOS1*, and *SED5* encoding their cytoplasmic domain was amplified by PCR with the use of the following primers: 5′ oligo for *BET1*, GATCGGGGCCATATGAGTTCAAGATTTGCAGGGGGAAACGCTTATC; 3′ oligo for *BET1*, GCGCCGGGATCCTTAAGATCTTCTGGCCATCTCCATC; 5′ oligo for *BOS1* (His_6), CGGGGCGCCATGGGCCATCATCATCATCATCATAACGCTCTTTACAACCATGCT; 3′ oligo for *BOS1* (His_6), CGGGGCGGATCCTTATTTATCTTTGAACACCCGTTTATTGAT; 5′ oligo for *BOS1* (Gst), CTAGATGGATCCAAACGCTCTTTACAACCATG; and 3′ oligo for *BOS1* (Gst), AGACTCCCGGGTTTATCTTTGAACACCCGTTTATTGA. The PCR product for Bos1p(L190S)-Gst was digested with *Bam*HI and *Sma*I and ligated into the corresponding sites of the vector pGEX-1N (Pharmacia, Pistcataway, NJ): 5′ oligo for *SED5*, GCGCGGGGATCCATGAACATAAAGGATAGAACTTCAG and 3′ oligo for *SED5*, AGCCCCGAATTCTTACTTTGCGGCTAACCATCTATTACTC. The *BET1* PCR product was digested with *Nde*I and *Bam*HI and ligated into the corresponding sites of pET14b (Novagen) to allow for the expression of a His_6-tagged polypeptide. The *BOS1* PCR product was digested with *Nco*I and *Bam*HI and ligated into the corresponding sites of pET11d (Novagen, Madison, WI). The region encoding the His_6-tag was contained in the 5′ oligo used to amplify the coding region. The *SED5* PCR product was digested with *Bam*HI and *Eco*RI and ligated into the corresponding sites of pGEX2T (Pharmacia).

Purification of Fusion Proteins and Binding Assays

Gst and Sed5p-Gst were prepared as described previously (Smith and Johnston, 1988) with the following modifications. Protein

bound to the glutathione-agarose beads was eluted with 20 mM glutathione and dialyzed overnight against PBS (154 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.5). The concentration of the eluted protein was determined by the bicinchoninic acid method before it was rebound to beads. The protein that failed to bind to the resin was estimated, and the amount that bound to the beads was determined as the difference in values obtained before and after binding. His_6-Bos1p and His_6-Bet1p were purified on Ni^{2+}-NTA beads as described by the manufacturer with some modifications. Briefly, a lysate that was clarified during a spin at 39,000 × *g* was incubated with beads for 5 min on ice and washed four times with buffer A (20 mM Tris, pH 7.9, 500 mM NaCl, 5 mM imidazole). The beads were packed into a column and washed with 15 ml of 100 mM imidazole in buffer A. His_6-tagged protein was eluted in 800 mM imidazole in buffer A, and 1 ml fractions were collected and analyzed by SDS-PAGE. Peak fractions were pooled and dialyzed against 30% glycerol in binding buffer lacking Triton X-100. In vitro binding studies were performed with 1 μM Sed5p-Gst beads that were stored in 1 mg/ml bovine serum albumin and washed three times with 500 μl of binding buffer (10 mM HEPES-NaOH, pH 7.4, 25 mM NaCl, 115 mM KCl, 2 mM $MgCl_2$, 0.5% Triton X-100) before use. The appropriate amount of His_6-Bos1p and His_6-Bet1p was added, as indicated above, to a 100-μl reaction and incubated overnight at 4°C with mixing. The beads were washed six times with 500 μl of binding buffer, heated for 2 min at 100°C in 50 μl of sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.005% bromophenol blue), and electrophoresed on a 15% SDS-polyacrylamide gel. Bos1p and Bet1p were detected by Western blot analysis using the ^{125}I-labeled protein A method. Anti-Bos1p and anti-Bet1p sera were used at a 1:1500 dilution. The amount of Bos1p or Bet1p that bound to the beads was estimated from a standard curve (10–250 ng) of His_6-Bos1p or His_6-Bet1p that was electrophoresed on the same gel as the samples. Saturation was determined in Figures 2 and 3 by quantifying the bands and comparing them with the standard curve. The blots were exposed to a phosphoimaging plate (Molecular Dynamics, Sunnyvale, CA), scanned onto a phosphoimager, and quantitated with the use of Molecular Dynamics Image Quant software (version 3.15). Because approximately four times more Bos1p was bound to the beads when compared with Bet1p, we examined the washes to determine whether Bet1p was dissociating from the complex. The amount of Bet1p in the washes was the same if the beads contained either Gst or Sed5p-Gst. Thus, we conclude that only nonspecifically bound Bet1p was present in the washes.

Circular Dichroism (CD) Studies

His_6-tagged proteins were dialyzed against 50 mM sodium phosphate (pH 7.4) and 150 mM NaCl with 30% glycerol. CD spectra (Woody, 1985) were measured at 5 μM protein in a 0.2-cm pathlength cell with the use of an Aviv Circular Dichroism Spectrometer model 62DS running Aviv software (version 4.1). No concentration dependence of CD spectra was noted between 0.625 and 5 μM for either His_6-Bos1p or His_6-Bet1p. Samples were scanned from 260 to 202 nm in 1-nm steps with a sampling time of 1 s. The scans shown in Figure 5 represent the average of five scans and were corrected for buffer contributions by subtracting the spectrum of the buffer.

Sequencing the bet1–1 and bos1–1 Mutations

The *bet1–1* and *bos1–1* mutations were cloned by the gap-repair method (Orr-Weaver *et al.*, 1981). For *bet1–1*, a 2.7-kb fragment containing the *BET1* gene was inserted into the *Eco*RI/*Sal*I sites of pRS316 (*URA3*, *CEN6*). A *Sna*B1-*Afl*II fragment containing the *BET1* coding region was removed and the resulting plasmid, which includes DNA that flanks the 5′- and 3′-coding regions of *BET1*, was transformed into the yeast strain ANY 112 (*Mata*, *ura3–52*, *bet1–1*). To map the *bos1–1* mutation, a 1.4-kb fragment containing the *BOS1*-coding region was inserted into the *Kpn*I-*Nco*I sites of pRS316 (*URA3*, *CEN6*). A Tth111I-*Bcl*I fragment was removed and the resulting plasmid was transformed into the yeast strain SFNY 412 (*Mata*, *ura3–52*, *leu2–3,112*). Plasmid DNA was isolated from the yeast transformants, amplified in *E. coli*, and subjected to automated sequencing from the 5′ and 3′ ends of the *BET1* and *BOS1* genes.

RESULTS

Bet1p Is Related to SNAP-25

Bet1p was reported previously to be homologous to the synaptic vesicle protein synaptobrevin (Dascher *et al.*, 1991). In particular, amino acids 61–106 were found to be 21% identical to amino acids 50–102 of *Drosophila melanogaster* synaptobrevin after five gaps were introduced into the sequence (Figure 1). On the basis of this alignment, it was proposed that Bet1p functions as a v-SNARE. A more recent search of the database using the BLAST program (Altschul *et al.*, 1990) has revealed that this same approximate region (amino acids 55–93) is more significantly similar (36% identity and 61% similarity with no gaps) to amino acids 151–189 of *D. melanogaster* SNAP-25 (Figure 1). BLAST revealed that this region of SNAP-25 is highly conserved among all species (Figure 1). Furthermore, it is important for the function of Bet1p. A mutation in *bet1* (*bet1–1*) that blocks vesicle traffic changes the highly conserved leucine at amino acid 72 to a phenylalanine (L72F). This conserved change lowers the coiled-coil potential of this region. Because the identity of Bet1p is significantly stronger to SNAP-25 than to synaptobrevin, the role of Bet1p in membrane traffic may be more comparable to SNAP-25 than to synaptobrevin.

Bet1p Potentiates SNARE Interactions

Although Bet1p and SNAP-25 each contain a homologous domain, they are structurally different. Bet1p is a type II cytoplasmically oriented integral membrane protein (Dascher *et al.*, 1991; Newman *et al.*, 1992), whereas SNAP-25 lacks a transmembrane domain but is palmitoylated (Oyler *et al.*, 1989). Furthermore, Bet1p resides primarily on donor membranes (Newman *et al.*, 1992), whereas SNAP-25 (also called a t-SNARE) forms a complex with syntaxin on the target organelle (Söllner *et al.*, 1993b).

To address the possibility that Bet1p and SNAP-25 are performing related roles, we carried out in vitro binding studies. In the neuron, SNAP-25 potentiates the binding of synaptobrevin to syntaxin (Pevsner *et al.*, 1994). Because genetic studies (Newman *et al.*, 1990) have suggested a possible physical interaction between Bet1p and Bos1p (an essential synaptobrevin-like v-SNARE), we tested the possibility that Bet1p facilitates the pairing of Bos1p to its syntaxin-like t-SNARE, Sed5p (Hardwick and Pelham, 1992; Søgaard *et al.*, 1994). To execute these binding studies, the cytoplasmic domains of Bet1p, Bos1p, and Sed5p were expressed in *E. coli* as fusion

```
      SNAP-25   143 ENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRI 181
                    |.: :|.::.::. | .|: :.|.||:|| . |. ||.:
        Bet1p    55 ESQSEEQMGAMGQRIKA KSLSLKMGDEIRGSNQTIDQL 93
                    |.: ||.||.:. .| .|:.:.|.||.|: . |. ||.:
D. mel SNAP-25  151 EDEMEENMGQVNTMIGNLRNMALDMGSELENQNRQIDRI 189
```

	Bet1p	(no gaps)
D.melanogaster	36(61)%	
C.auratus	33(64)%	
torpedo	33(58)%	
human	30(61)%	
chicken	30(61)%	
D.mel. synapt.	21%	(5 gaps)

Figure 1. Bet1p contains a domain that is homolgous to a region of SNAP-25. The amino acid sequence of Bet1p (accession number P22804) from residues 55–93 is compared with residues 151–189 of *D. melanogaster* SNAP-25 (accession number P36975) and residues 143–181 of human SNAP-25 (accession number P13795) using the Genetics Computer Group BESTFIT program. Identity is indicated by a line and conserved changes are indicated by two dots (two corresponding bases in their codons) or one dot (one corresponding base in their codons) between the sequences. The leucine that is mutated to phenylalanine in the *bet1–1* mutant is shaded in the Bet1p sequence. The homology between residues 55–93 of Bet1p and other species of SNAP-25 is indicated in the table shown at the bottom of the Figure. Parentheses, percent similarity.

proteins (either a His_6 or Gst tag) and then purified. As anticipated, His_6-Bos1p bound to Sepharose beads containing immobilized Sed5p-Gst (Figure 2A) but not to Gst Sepharose (Figure 2B). Binding increased in a linear manner until saturation was achieved at 5 μM Bos1p (Figure 2A, lane 5). At that concentration, approximately 19 pmol of His_6-Bos1p bound to Sed5p-Gst. To investigate whether Bet1p could affect the binding of Bos1p to Sed5p, we measured the binding of His_6-Bos1p (0.5 μM) to Sed5p-Gst in the presence of increasing concentrations of His_6-Bet1p. Because binding of Bos1p to Sed5p was barely detectable at 0.5 μM His_6-Bos1p, this concentration was chosen for our studies. Saturable binding of His_6-Bos1p (approximately 18.6 pmol) to Sed5p-Gst was achieved as the concentration of His_6-Bet1p was raised to 5 μM (Figure 3A, lane 5). This represents approximately 30% of the His_6-Bos1p that was added to the reaction mix. To determine whether the region of Bet1p that is homologous to SNAP-25 is important for its activity, we tested the ability of His_6-Bet1p (L72F), a recombinant form of Bet1p that contains the *bet1–1* mutation, to stimulate the binding of Bos1p to Sed5p. This experiment suggested that His_6-Bet1p (L72F) lacks the ability to enhance v-SNARE/t-SNARE interactions (Figure 3B). When the effect of the *bet1–1* mutation on the interaction of Bet1p with Bos1p and Sed5p was assessed independently, we found that His_6-Bet1p (L72F) failed to bind to Bos1p-Gst as well as Sed5p-Gst (our unpublished observations).

SNAP-25 is an integral component of a SNARE complex that includes synaptobrevin and syntaxin. If Bet1p is related to SNAP-25, it should form a ternary complex with Bos1p and Sed5p. The presence of Bet1p in the Bos1p/Sed5p complex was quantitated by determining the amount of His_6-Bet1p that bound to Sed5p-Gst beads in the presence of increasing concentrations of His_6-Bos1p (Figure 3C). These experiments demonstrated that at saturation, approximately 4.8 pmol of Bet1p bound to Sed5p. His_6-Bet1p also bound to Sed5p-Gst in the absence of His_6-Bos1p (our unpublished observations). Thus, like SNAP-25, Bet1p interacts with both a synaptobrevin-like and syntaxin-related protein. Although synaptobrevin does not stimulate the binding of SNAP-25 to syntaxin, Bos1p enhances the binding of Bet1p to Sed5p. Therefore, although Bet1p shares some similarity to SNAP-25, it also differs in several ways.

Bet1p Facilitates the Activity of Bos1p

To determine whether Bos1p must be present for Bet1p to exert its effects, an in vitro binding study

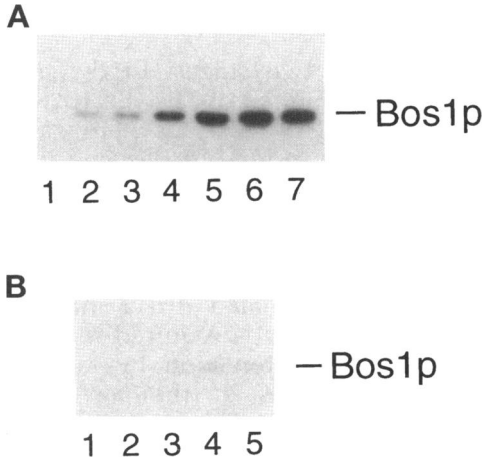

Figure 2. Bos1p binds to Sed5p. Increasing concentrations of His_6-Bos1p were incubated in the presence of 1 μM Sed5p-Gst Sepharose (A) or 1 μM Gst Sepharose (B). The Bos1p that bound to the beads was eluted and detected by Western blot analysis. Reagents were prepared as described in MATERIALS and METHODS. The concentration of His_6-Bos1p in A was varied from 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 2 (lane 4), 5 (lane 5), 10 (lane 6), and 15 (lane 7) μM. In B, the concentration varied from 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 5 (lane 4), and 15 (lane 5) μM.

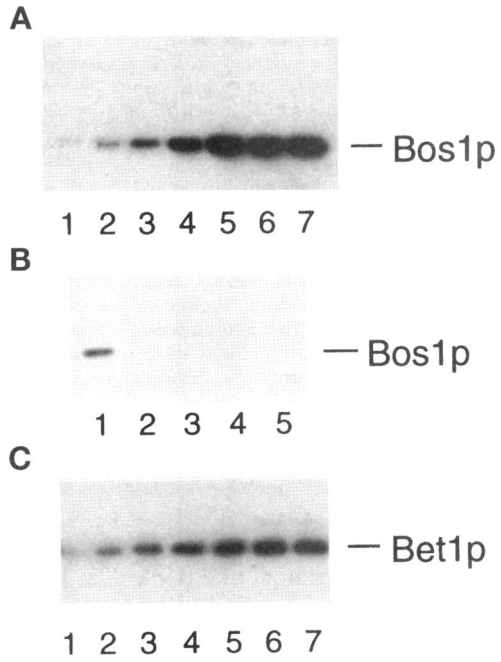

Figure 3. Bet1p facilitates the binding of Bos1p to Sed5p. (A) 0.5 μM His_6-Bos1p was incubated with an increasing amount of His_6-Bet1p in the presence of 1 μM Sed5p-Gst Sepharose. His_6-Bet1p was used at 0 (lane 1), 0.5 (lane 2), 1 (lane 3), 2 (lane 4), 5 (lane 5), 10 (lane 6), and 15 (lane 7) μM. (B) Bos1p and Sed5p were incubated as in A, but with increasing amounts of His_6-Bet1p (L72F). The concentration of His_6-Bet1p (L72F) was varied from 0 (lane 2), 0.5 (lane 3), 1 (lane 4), and 2 (lane 5) μM. The migration of His_6-Bos1p is shown in lane 1 of B. (C) His_6-Bet1p (0.5 μM) was incubated with increasing concentrations of His_6-Bos1p in the presence of 1 μM Sed5p-Gst Sepharose. His_6-Bos1p was used at 0 (lane 1), 0.2 (lane 2), 0.5 (lane 3), 1 (lane 4), 2 (lane 5), 5 (lane 6), and 10 (lane 7) μM.

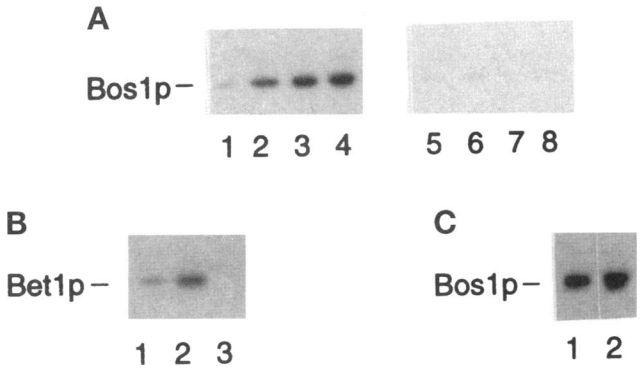

Figure 4. Bet1p Activates the v-SNARE Bos1p. (A) His_6-Bos1p (lanes 1–4) and His_6-Bos1p (L190S; lanes 5–8) were incubated at a concentration of 0.5 μM with 1 μM Sed5p-Gst and His_6-Bet1p at 0 (lanes 1 and 5), 0.5 (lanes 2 and 6), 1 (lanes 3 and 7) and 2 (lanes 4 and 8) μM. (B) Bos1p-Gst (lanes 1 and 2) and Bos1p (L190S)-Gst (lane 3) at 1 μM were incubated with 1 μM His_6-Bet1p (lane 1) and 2 μM His_6-Bet1p (lanes 2 and 3). In C, His_6-Bos1p (lane 1) and His_6-Bos1p (L190S) (lane 2) at 2 μM were incubated with 1 μM Sed5p-Gst.

was performed in two stages. In the first stage, increasing concentrations of His_6-Bet1p were incubated with Sed5p-Gst. The beads were washed, and then His_6-Bos1p was added in a second incubation. In this situtation, Bet1p could not potentiate the binding of Bos1p to Sed5p (our unpublished observations). Thus, this event may require the simultaneous presence of all three components. We were able to demonstrate that a direct interaction between Bet1p and Bos1p is required for an efficient v-SNARE/t-SNARE interaction by taking advantage of the properties of the *bos1–1* mutation (Wuestehube *et al.*, 1996). This mutation changes the leucine at amino acid 190 to a serine (L190S) and maps to the region of synaptobrevin homology (amino acids 136–197). As shown in Figure 4C, although His_6-Bos1p (L190S) bound (lane 2) as efficiently as wild-type Bos1p (lane 1) to Sed5p-Gst, His_6-Bet1p failed to facilitate the binding of mutant Bos1p to Sed5p-Gst (Figure 4A, compare lanes 5–8 with the control in lanes 1–4). This was a direct consequence of the inability of mutant Bos1p to interact with Bet1p because His_6-Bet1p was unable to bind to Bos1p (L190S)-Gst (Figure 4B, compare lane 3 with lanes 1 and 2). Thus, Bet1p potentiates the v-SNARE/t-SNARE interaction through Bos1p. However, these findings do not exclude the possibility that Bet1p also acts on Sed5p.

Bet1p and Bos1p Are Helical Proteins

To address the structural basis for the inability of Bet1p (L72F) to interact with Bos1p and Sed5p, we examined His_6-Bos1p, His_6-Bet1p, and His_6-Bet1p (L72F) by CD spectroscopy. CD spectroscopy is a sensitive means of detecting α-helical secondary structure in proteins. The double minima observed for His_6-Bos1p at 208 and 222 nm (Figure 5B) and the helical contribution at 222 nm for His_6-Bet1p (Figure 5A) are characteristic of structures that are predominantly helical. The helical nature of Bos1p and Bet1p is consistent with the proposal that protein–protein interactions between SNAREs are mediated by coiled-coils. By contrast, His_6-Bet1p (L72F) showed a clear loss of the helical signal at 222 nm and a more random coil contribution below 208 nm (Figure 5A). Because His_6-Bos1p (L190S) failed to bind to Bet1p-Gst, we also examined its structure by CD spectroscopy and found that it was unchanged (our unpublished observations). Thus, the inability of a mutant form of a SNARE to interact with another SNARE does not always correlate with a change in the secondary structure of the mutant protein. Taken together, these data suggest that the failure of mutant Bet1p to function in vivo stems from a loss of α-helical structure, which may disrupt its binding to other SNAREs.

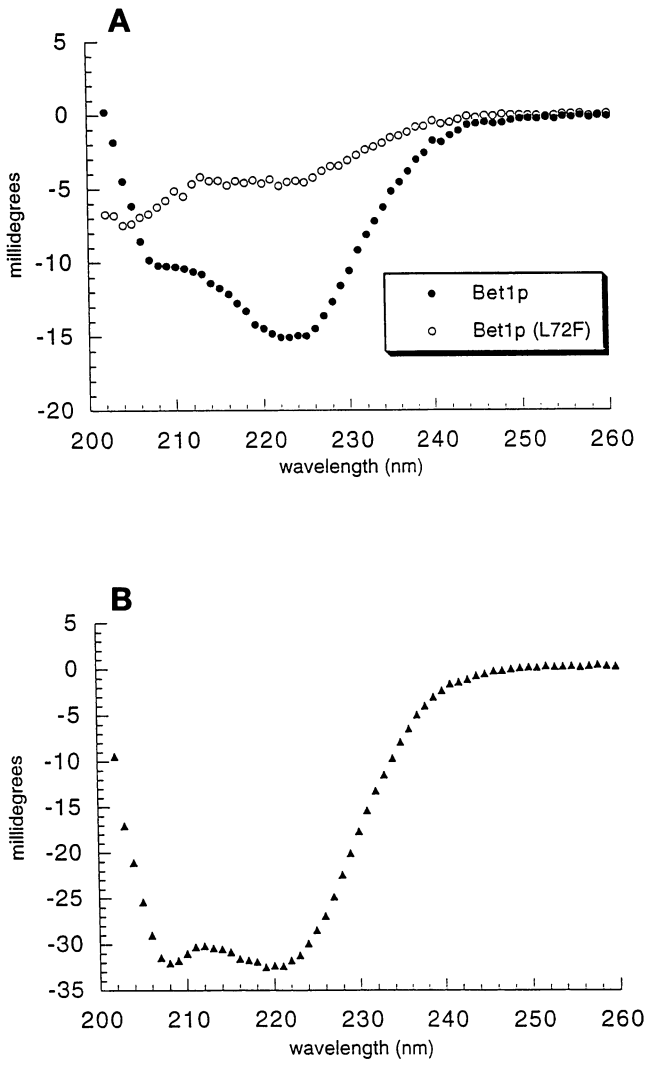

Figure 5. His_6-Bet1p (L72F) is less helical than His_6-Bet1p. The CD spectra of (A) His_6-Bet1p (●), His_6-Bet1p (L72F; ○) and (B) His_6-Bos1p, all at 5 μM, were measured from 260 to 202 nm as described (Woody, 1995). Note the increased minima at 222 nm and the decreased signal below 208 nm for His_6-Bet1p (L72F) versus His_6-Bet1p. This is indicative of a less α-helical structure for the mutant protein. His_6-Bos1p shows strong and equal minima at 222 and 208 nm, as well as an increased signal below 208 nm, which is characteristic of a predominanlty α-helical protein. The measured values at 222 nm adjusted for polypeptide length indicate that the mutant protein was one-third as helical as the wild-type protein and that Bos1p was 1.5 times more helical than Bet1p.

The Co-Overexpression of BOS1 and BET1 Efficiently Bypasses the Loss of YPT1

Genetic studies have shown that Bet1p acts downstream of the Ras-like GTP-binding protein Ypt1p (Dascher *et al.*, 1991). Ypt1p may regulate the specificity of vesicular traffic by specifically activating the v-SNARE on vesicles (Lian *et al.*, 1994). This is a prerequisite for an efficient v-SNARE–t-SNARE interaction. Here, we have demonstrated that, in the presence of Bet1p, Bos1p interacts with Sed5p more efficiently. Given these and previous findings, one could speculate that *BET1* may bypass the loss of *YPT1* more efficiently if it is co-overexpressed with *BOS1*. To address this possibility, we transformed a diploid strain disrupted for one copy of the *YPT1* gene (NY921) with the *BOS1* (pPL101) and *BET1* (pSFN100) genes on high copy plasmids and then performed tetrad analysis. As a control, we also transformed NY921 with either pPL101 or pSFN100. *BOS1* alone could not compensate for the loss of Ypt1p (our unpublished observations), and the suppression by *BET1* was so weak that the microcolonies formed after 6 d are not apparent in Figure 6 II. The only *YPT1*-disrupted spores, which grew to a significant extent, co-overexpressed both *BOS1* and *BET1* (Figure 6, I see colonies 1A, 2A, 2C, 3A, 3B, 4A, and 4C).

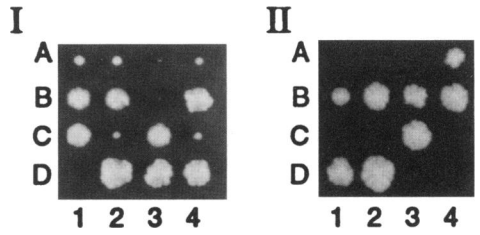

Figure 6. The co-overexpression of *BOS1* and *BET1* efficiently bypasses the loss of *YPT1*. In I, a diploid strain (*MATa/α, ura3–52/ura3–52, leu2–3, 112/leu2–3, 112, his3-Δ200/his3-Δ200, ypt1Δ::HIS3*) disrupted for one copy of the essential *YPT1* gene was transformed with pPL101 (*BOS1, LEU2*, 2 μM) and pSFN100 (*BET1, URA3*, 2 μM), sporulated, and then tetrad analysis was performed. The tetrads were photographed after 6 d of growth at 25°C as in II. Of the 42 tetrads analyzed, 22 colonies were His^+, Leu^+, and Ura^+ (in I, see colonies 1A, 2A, 2C, 3A, 3B, 4A, and 4C). The disrupted colonies that contain both plasmids were of variable size because high-copy plasmids are lost at a high frequency on nonselective media. In II, the same diploid strain was transformed with pSFN100, sporulated, and then tetrad analysis was performed.

DISCUSSION

Here we used in vitro binding studies to demonstrate that Bet1p enhances the affinity of Bos1p for its receptor, Sed5p. Bet1p contains a domain that is significantly similar to a highly conserved region of SNAP-25. Furthermore, mutational analysis has shown that this region is important for function.

A yeast docking/fusion (SNARE) complex that includes Bos1p, Bet1p, and Sed5p has been shown previously to form in vivo (Søgaard *et al.*, 1994). This complex accumulates in *sec18* (Søgaard *et al.*, 1994) and *sec17* mutant (Jiang *et al.*, 1995) cells when ER to Golgi vesicle targeting and fusion is blocked. The M ratio of Bos1p to Bet1p, in the complex that is formed in vivo, is approximately the same (4:1) as we ob-

served in vitro. The ratio of Sed5p to Bos1p in vitro, however, was almost 5:1 instead of 2:1 (Søgaard *et al.*, 1994). Sed5p may recognize Bos1p less efficiently because it is fused to Gst and immobilized on Sepharose beads. Nonetheless, the stoichiometries of Bos1p/Bet1p/Sed5p in the in vitro-formed SNARE complex closely parallel what is found in vivo, implying that the interactions we observe here are physiologically relevant.

One way that Bet1p could increase the affinity of Bos1p for Sed5p is to form higher ordered structures of Bos1p. In an attempt to address this possibility, we first analyzed recombinant forms of these proteins on a sieving column. Though the peaks were broad, molecular sieving is most consistent with Bos1p forming a tetramer and Bet1p a dimer. However, coiled-coil proteins tend to run aberrantly on sieving columns because of their elongated shape, so these sizes may not accurately reflect the stoichiometry of these proteins. To obtain the stoichiometry of Bos1p and Bet1p that we observed in our binding studies, one would hypothesize that two tetramers of Bos1p would bind to a dimer of Bet1p. When Bos1p and Bet1p were mixed together in the presence of Sed5p higher molecular weight complexes were detected, but the peak was too broad to estimate the stoichiometry of the complex.

We previously reported that immunoisolated ER to Golgi transport vesicles, which are formed in vitro, contain equivalent amounts of Bos1p and a second synaptobrevin-like protein, Sec22p (Lian *et al.*, 1994). Detectable amounts of Bet1p, however, were not found in these vesicles (Lian and Ferro-Novick, 1993). In light of the fact that there is nearly four times more Bos1p than Bet1p in the yeast SNARE complex, we conclude that small but functionally significant amounts of Bet1p, which may be difficult to detect, could be present in ER to Golgi transport vesicles. Bet1p was readily found in vesicles that were formed in vitro from *ypt1* mutant cells (Rexach *et al.*, 1994). In fact, Ypt1p deficient vesicles are equally enriched for Bet1p and Sec22p (see Rexach *et al.*, 1994). Given that yeast cells contain equivalent amounts of Bet1p and Sec22p (Sacher and Ferro-Novick, our unpublished observations), this would imply that the ratio of Bet1p/Sec22p on vesicles increases in the absence of functional Ypt1p. Hence, an increase in Bet1p on this compartment may enhance the activity of the v-SNARE (Bos1p), which in turn partially compensates for the loss of Ypt1p.

We propose that Bet1p modulates vesicle fusion by facilitating v-SNARE–t-SNARE interactions. The helical structure of Bet1p, which is maintained via the domain that is homologous to SNAP-25, seems to play a pivotal role in this event. We speculate that Bos1p, residing on the ER membrane (Newman *et al.*, 1992), fails to recognize Sed5p. However, as a vesicle is formed, Bet1p interacts with Bos1p. This interaction, which seems to be regulated by Ypt1p, facilitates the pairing of the v-SNARE with its cognate t-SNARE receptor. Such a regulated activation step may be necessary to prevent the ER from directly fusing with the Golgi complex (Lian *et al.*, 1994). In addition to the role discussed above, Bet1p may have a second function. Because it is a resident of the ER and related to the t-SNARE SNAP-25, Bet1p may also serve as a receptor (t-SNARE) for vesicles that recycle from the Golgi back to the ER. Further experiments should enable us to address this possibility.

ACKNOWLEDGMENTS

We thank Judy Burston for outstanding technical assistance, Uwe Krüger for assistance in the purification of the His_6- and Gst-tagged fusion proteins, Ed Chapman for advice on the binding studies, Yu Jiang for assistance and advice in the construction of His_6- and Gst-tagged proteins, and Randy Schekman for the *bos1–1* mutant. M.S. was supported as an Associate of the Howard Hughes Medical Institute. This work was supported by a grant from the National Institutes of Health and the National Cancer Institute.

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Dascher, C., Ossig, R., Gallwitz, D., and Schmitt, H.D. (1991). Identification and structure of four yeast genes (*SLY*) that are able to suppress the functional loss of *YPT1*, a member of the RAS superfamily. Mol. Cell. Biol. *11*, 872–885.

Ferro-Novick, S., and Jahn, R. (1994). Vesicle fusion from yeast to man. Nature *370*, 191–193.

Hardwick, K.G., and Pelham, H.R.B. (1992). *SED5* encodes a 39-kD integral membrane protein required for vesicular transport between the ER and Golgi complex. J. Cell Biol. *119*, 513–521.

Jiang, Y., Sacher, M., Singer-Krüger, B., Lian, J.P., Stone, S., and Ferro-Novick, S. (1995). Factors mediating the late stages of ER-to-Golgi transport in yeast. CSH Symp. Quant. Biol. *60*, 119–126.

Lian, J.P., and Ferro-Novick, S. (1993). Bos1p, an integral membrane protein of the ER to Golgi transport vesicles, is required for their fusion competence. Cell *73*, 735–745.

Lian, J.P., Stone, S., Jiang, Y., Lyons, P., and Ferro-Novick, S. (1994). Ypt1p implicated in v-SNARE activation. Nature *372*, 698–701.

Newman, A.P., Shim, J., and Ferro-Novick, S. (1990). *BET1*, *BOS1* and *SEC22* are members of a group of interacting genes required for transport from the ER to the Golgi complex. Mol. Cell. Biol. *10*, 3405–3414.

Newman, A.P., Groesch, M., and Ferro-Novick, S. (1992). Bos1p, a membrane protein required for ER to Golgi transport in yeast, co-purifies with the carrier vesicles and with Bet1p and the ER membrane. EMBO J. *12*, 3609–3617.

Orr-Weaver, T.L., Szostak, J.W., and Rothstein, R.J. (1981). Yeast transformation: a model system for the study of recombination. Proc. Natl. Acad. Sci. USA *78*, 6354–6358.

Oyler, G.A., Higgins, G.A., Hart, R.A., Battenberg, E., Billingsley, M., Bloom, F.E., and Wilson, M.C. (1989). The identification of a

novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. J. Cell Biol. *109*, 3039–3052.

Pevsner, J., Hsu, S.-C., Braun, J.E. A., Calakos, N., Ting, A.E., Bennett, M.K., and Scheller, R.H. (1994). Specificity and regulation of a synaptic vesicle docking complex. Neuron *13*, 353–361.

Rexach, M.F., Latterich, M., and Schekman, R.W. (1994). Characteristics of endoplasmic reticulum-derived transport vesicles. J. Cell Biol. *126*, 1133–1148.

Smith, D.B., and Johnston, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. Gene *67*, 31–40.

Søgaard, M., Tani, K., Ye, R.R., Geromanos, S., Tempst, P., Kirchhausen, T., Rothman, J.E., and Söllner, T. (1994). A rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. Cell *78*, 937–948.

Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J.E. (1993a). SNAP receptor implicated in vesicle targeting and fusion. Nature *362*, 318–324.

Söllner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H., and Rothman, J.E. (1993b). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell *75*, 409–418.

Woody, R.W. (1985). Circular dichroism of peptides. In: The Peptides, ed. S. Udenfriend, J. Meienhofer, and V.J. Hruby, Orlando, FL: Academic Press. *7*, 15–114.

Wuestehube, L.J., Duden, R., Eun, A., Hamamoto, S., Korn, P., Ram, R., and Schekman, R. (1996). New mutants of *Saccharomyces cerevisiae* affected in the transport of proteins from the endoplasmic reticulum to the Golgi complex. Genetics *142*, 393–406.