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BET3 Encodes a Novel Hydrophilic Protein that Acts in Conjunction with Yeast SNAREs

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Here we report the identification of *BET3*, a new member of a group of interacting genes whose products have been implicated in the targeting and fusion of endoplasmic reticulum (ER) to Golgi transport vesicles with their acceptor compartment. A temperature-sensitive mutant in *bet3–1* was isolated in a synthetic lethal screen designed to identify new genes whose products may interact with *BET1*, a type II integral membrane protein that is required for ER to Golgi transport. At 37°C, *bet3–1* fails to transport invertase, α-factor, and carboxypeptidase Y from the ER to the Golgi complex. As a consequence, this mutant accumulates dilated ER and small vesicles. The SNARE complex, a docking/fusion complex, fails to form in this mutant. Furthermore, *BET3* encodes an essential 22-kDa hydrophilic protein that is conserved in evolution, which is not a component of this complex. These findings support the hypothesis that Bet3p may act before the assembly of the SNARE complex.

INTRODUCTION

Proteins are transported from the ER to the cell surface through a series of membrane-bound compartments. In the case of endoplasmic reticulum (ER) to Golgi transport, specific carrier vesicles have been shown to mediate membrane traffic at this stage of the secretory pathway. Two soluble yeast gene products, *SEC18* and *SEC17*, have been implicated in vesicle fusion (Wilson *et al.*, 1989; Griff *et al.*, 1992). *SEC18*, which was originally identified as a gene whose product is required for vesicular transport between the ER and Golgi complex (Novick *et al.*, 1980), also plays a role in intra-Golgi and post-Golgi membrane traffic events (Graham and Emr, 1991). The mammalian homologue of *SEC18* is the *N*-ethylmaleimide–sensitive factor (NSF; Wilson *et al.*, 1989), a cytosolic protein that mediates the fusion of vesicles with their acceptor compartment (Malhotra *et al.*, 1988). The association of NSF with membranes is dependent upon the presence of three soluble NSF attachment proteins (α, β, and γ SNAPs) and a set of SNAP receptors that reside on the transport vesicle and target membrane (Clary *et al.*, 1990). In yeast, *SEC17* encodes a homologue of α-SNAP (Clary *et al.*, 1990). The SNAP receptors, called SNAREs, form a complex with NSF and the SNAPs (α and γ). In neurons, the vesicle SNARE (v-SNARE) is synaptobrevin, while the SNAREs on the target membrane (t-SNARE) are syntaxin and SNAP-25. Each intracellular fusion event may have one set of v-SNAREs and t-SNAREs that interact with each other to ensure the specificity of vesicular transport (Söllner *et al.*, 1993; Warren, 1993; Ferro-Novick and Jahn, 1994).

BET1, *BOS1*, *SEC21*, *SEC22*, and *YPT1* are members of a group of interacting yeast genes (Newman *et al.*, 1990; Dascher *et al.*, 1991). The *SEC21* gene product is a component of yeast coatomer (Hosobuchi *et al.*, 1992), while *BET1*, *BOS1*, and *SEC22* encode cytoplasmically oriented type II integral membrane proteins that are required for ER to Golgi transport (Newman and Ferro-Novick, 1987; Dascher *et al.*, 1991; Shim *et al.*, 1991). Ypt1p is a small GTP-binding protein that also functions at this stage of the secretory pathway (Ferro-Novick and Novick, 1993). Previous studies have shown that Bos1p, the v-SNARE of ER to Golgi transport vesicles, physically interacts with Sec22p on

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vesicles and not on other compartments. This interaction, which appears to facilitate the activity of Bos1p, is catalyzed by Ypt1p (Lian *et al.*, 1994). Based on these data it was proposed that Ypt1p, and other small GTP-binding proteins, regulate the specificity of vesicular transport by selectively activating the v-SNARE on vesicles.

In an effort to identify additional components that may functionally interact with this group of proteins, we have used the *bet1–1* mutant in a synthetic lethal screen. Synthetic lethality results when the effect of combining two mutations in the same haploid strain causes cell death under growth conditions that are permissive for either single mutant. Thus, the combined effect of both mutations impairs a specific process to a greater extent than either one alone. Here we report the use of such a screen to identify a new temperature-sensitive (ts) secretory mutant that is blocked in ER to Golgi transport, *bet3–1*. Like *BET1*, *BET3* interacts genetically with *BOS1*, *SEC21*, *SEC22*, *YPT1*, and encodes an essential hydrophilic 22-kDa protein that is highly homologous to a *Caenorhabditis elegans* protein (p20) of unknown function. Studies presented here suggest that Bet3p functions before the pairing of the v-SNARE with its t-SNARE.

MATERIALS AND METHODS

Mutant Isolation and Screen

SFNY185 was grown to early exponential phase at 25°C to obtain 5 $\times$ 10^7 cells/ml. Cells were washed twice with sterile water, resuspended in 0.1 M sodium phosphate buffer (pH 7.0) and mutagenized with 3% ethylmethanesulfonate (~70% killing). After shaking for 1 h at 30°C, the cells were washed with sterile water, transferred to a new tube, and washed two more times with 5% sodium thiosulfate. Afterward, the cells were allowed to recover during a 20-h incubation at 25°C in 100 ml of YPD medium (total OD_{599} of 25.5). Cells were centrifuged, resuspended in fresh YPD medium, and then shifted to 37°C for 3 h. Subsequent to this shift the cells were washed, resuspended in 0.5 ml of water, and layered onto a 12.5-ml Percoll gradient (90% Percoll prepared in yeast nitrogen base without amino acids) in a Beckman Quickseal centrifuge tube (16 $\times$ 76 mm; Fullerton, CA). The tubes were heatsealed and spun at 22,000 $\times$ *g* for 20 min at 4°C in a Beckman ultracentrifuge (TI50 rotor). Fractions (the densest 0.8–2.6% of the cells) were collected (1.0 ml) using an ISCO fraction collector, diluted 2 $\times$ 10^{-4} in sterile water, and 0.2 ml was plated onto selective plates that were grown for 3 days at 25°C as described previously (Novick *et al.*, 1980; Finger and Novick, 1992). The colonies were replica plated onto two different YPD plates and incubated at 25°C or 37°C for 1 day. Temperature-sensitive mutants were isolated, purified, and cells that failed to grow after 5 days at 25°C on 5-FOA (5-fluoroorotic acid) plates were screened for defects in the secretion of invertase.

Antibody Production, Immunoprecipitation, and Electrophoresis

Cells were grown overnight to an OD_{599} = 1.0 in yeast nitrogen base that was supplemented with the appropriate amino acids and 2% glucose. Mutant and wild-type cells were resuspended in fresh medium to an OD_{599} = 3.0 and then preshifted at 37°C for 30 min. At the end of this incubation, 200 μCi of ^{35}S In Vitro label (Amersham, Arlington Heights, IL) was added to each sample and the incubation was continued for 30 min longer at 37°C. To examine the secretion of invertase, cells were grown to an OD_{599} = 1.0 and then preshifted to 37°C for 30 min. Invertase synthesis was derepressed in minimal medium with 0.1% glucose at an OD_{599} = 2.0 during a 30-min incubation at 37°C in the presence of 330 μCi of ^{35}S Translabel. Cells were then washed with cold 10 mM sodium azide and resuspended in 1 ml of spheroplast buffer (1.4 M sorbitol, 50 mM potassium phosphate, pH 7.5, 10 mM sodium azide, 50 mM β-mercaptoethanol, 10 μg of zymolyase/1 OD unit of cells). Spheroplasts were formed during a 60-min incubation at 37°C and then lysed in 1 ml of lysis buffer (10 mM triethanolamine, pH 7.2, 1 mM EDTA, 0.8 M sorbitol, and 1$\times$ protease inhibitor cocktail; Ruohola *et al.*, 1988). The unbroken cells were removed during a 3-min spin at 450 $\times$ *g*, and the resulting supernatant was heated for 5 min at 100°C in the presence of SDS (1% final concentration). Samples (100 μl) were diluted with 900 μl of PBS in 1% Triton X-100 and centrifuged for 15 min in an Eppendorf microfuge. The supernatant was removed and incubated overnight on ice with one of the following antibodies: 2 μl of anti-invertase, 6 μl of anti-α-factor, or 2 μl of anti-carboxypeptidase Y. Immune complexes were precipitated onto protein A-Sepharose beads (30 μl of a 10% solution/μl of antibody) during a 90-min incubation at 4°C. The beads were washed twice with urea wash buffer (2 M urea, 200 mM NaCl, 100 mM Tris, pH 7.6, 1% Triton X-100), then twice with 1% β-mercaptoethanol, and the contents of the beads were solubilized in 70 μl of Laemmli sample buffer.

Polyclonal anti-Bet3p antibody was raised to a Bet3p-MBP fusion protein that was purified according to manufacturer's directions (New England Biolabs, Beverly, MA). The immunization protocol was described previously (Louvard *et al.*, 1982) and the serum was affinity purified on a column containing cross-linked Bet3p-GST fusion protein. To precipitate Bet3p, approximately 7 OD units of cells were resuspended in fresh medium to an OD_{599} = 3.00 and labeled at 24°C for 45 min with 0.7 mCi (200 μCi/ml) of ^{35}S In Vitro label. The protocol used was the same as described above with minor modifications. Spheroplasts were lysed in 0.7 ml of buffer (10 mM Tris, pH 8.0, 25 mM EDTA, pH 8.0, and 1% SDS) and then heated to 100°C for 5 min before they were diluted 1:20 in IP buffer (10 mM Tris, pH 8.0, 150 mM sodium chloride, 0.1 mM ethylenediaminetetraacetic acid, and 0.5% Tween). The diluted lysates were incubated with either 5 μl of pre-immune serum, 5 μl of anti-Bet3p antibody, or 5 μg/ml of affinity-purified anti-Bet3p polyclonal antibody. The immune complexes were precipitated with protein A-Sepharose and the beads were washed twice with IP buffer, twice with IP buffer containing 2 M urea, and twice with 1% β-mercaptoethanol.

All samples were subjected to SDS electrophoresis (12.5%). To compare strains, the volume of sample loaded onto the gel was proportional to the incorporation of ^{35}S Trans label in the lysate. Molecular weight standards were as follows: 97.4 kDa, phosphorylase B; 66 kDa, bovine serum albumin; 45 kDa, ovalbumin; 36 kDa, glyceraldehyde-3-phosphate dehydrogenase; 29 kDa, carbonic anhydrase; 24 kDa, trypsinogen; 20.1 kDa, trypsin inhibitor; and 14.2 kDa, α-lactoalbumin. Gels were stained for protein and fluorographed. Autoradiography was performed using Kodak XAR-5 film (Rochester, NY).

Genetic Techniques and Constructions

The *BET3* gene was cloned by complementation of the growth defect of the *bet3–1* mutant. The mutant was transformed with a high copy plasmid library (Carlson and Botstein, 1982) and the transformants were selected at 25°C on minimal agar plates lacking uracil. The colonies were then stamped onto YPD plates and incubated at 37°C. Complementing plasmids were recovered from yeast by the method of Strathern and Higgins (1991) and subcloned as described below.

To amplify plasmid DNA, recombinant plasmids were transformed into DH5α competent cells and isolated as described by

Maniatis *et al.* (1989). Plasmids used in this study (Figure 5) were constructed as follows. The original isolate pGR1 contained a 14.9-kb insert. Plasmid pGR3 was obtained by subcloning pGR1 as a 5.2-kb *Sal*I–*Sal*I fragment into the *Sal*I site in the polylinker of pRS426 (Christianson *et al.*, 1992) or the polylinker of pRS306 to yield pGR2. Plasmid pGR4 was constructed in an identical fashion except that the *Sal*I–*Sal*I fragment was inserted into the *Sal*I site in the polylinker of pRS316 (Christianson *et al.*, 1992). To isolate pGR5, the 2.6-kb *Sal*I–*Kpn*I insert from pGR4 was inserted into the *Kpn*I–*Sal*I sites of pRS316. Plasmid pGR6 was constructed by inserting the 1-kb *Kpn*I–*Pvu*II fragment from pGR4 into the *Kpn*I–*Sma*I sites in the polylinker region of pRS316. To isolate pGR7, pGR1 was digested with *Pvu*II and the vector was religated. Plasmid pGR8 was constructed by subcloning the 1.55-kb *Xba*I–*Xba*I fragment from pGR5 into the *Xba*I site in the polylinker of pRS315.

To place the *BET3* gene under the control of the regulatable *GAL1* promoter, polymerase chain reaction (PCR) was used to introduce restriction sites at the amino and carboxy terminus of *BET3*. A *Bam*HI site was placed two amino acids upstream from the start methionine of *BET3*, while an *Eco*RI site was introduced at the C-terminus of the gene. The PCR product was digested with *Bam*HI and *Eco*RI before it was subcloned into the *Bam*HI/*Eco*RI sites in the polylinker of pNB527. The resulting plasmid (pGR10) was linearized with *Cla*I and integrated into SFNY314 at the *LEU2* locus.

To disrupt *BET3*, the *Kpn*I–*Sal*I fragment shown in Figure 5, was first subcloned into a bluescript M13 vector in which the *Hin*dIII site was destroyed. The *BET3* gene was then excised from this subclone, by digesting the plasmid with *Hin*dIII, and replaced with a *Hin*dIII–*Hin*dIII fragment that contains the selectable marker *URA3*. The *Sal*I–*Kpn*I fragment was excised, transformed into NY1060, and tetrad analysis was performed.

The Bet3p-MBP fusion protein was constructed by introducing a *Bam*HI site two amino acids upstream from the start methionine of *BET3* using PCR. A *Bam*HI–*Hin*dIII fragment was then introduced into the polylinker site of pPR734 placing *BET3* in-frame with the 3′ end of the *malE* gene to yield pGR18. The Bet3-GST fusion protein was constructed (pGR20) by introducing a *Bam*HI site two amino acids from the start methionine of *BET3* using PCR. An *Eco*RI site was also introduced after the stop codon of *BET3* by PCR. The *Bam*HI–*Eco*RI fragment was then subcloned into the *Bam*HI–*Eco*RI sites of pGEX three times, placing the *BET3* gene in-frame with *GST*.

DNA Sequence Analysis and Homology Search

To sequence the *Sal*I–*Kpn*I fragment that contains *BET3*, the 1.6-kb *Sal*I–*Pvu*II fragment and the 1-kb *Pvu*II–*Kpn*I fragment were subcloned into sequencing vectors for single strand DNA sequencing. The 1.6-kb *Sal*I–*Pvu*II fragment was digested from pGR3 and inserted into the *Sma*I–*Sal*I sites in the polylinker of pUC118 and pUC119 (Vieira and Messing, 1987). The 1-kb *Pvu*II–*Kpn*I insert was subcloned into the same vectors using the *Hinc*II–*Kpn*I sites of the polylinker. The DNA sequence was determined by the method of Sanger *et al.* (1977) using the Sequenase version 2 DNA sequencing kit. The *BET3* sequence was compared with other sequences by searching databases with the TFASTA program.

Enzyme Assays and Other Procedures

Whole cell invertase assays were performed as described by Goldstein and Lampen (1975); the units of activity are reported as micromoles of glucose released per minute per OD_{599} of cells. Internal invertase was assayed as described earlier (Newman and Ferro-Novick, 1987).

Samples were prepared for electron microscopy using a modification of protocols described previously (Newman and Ferro-Novick, 1987). Briefly, early log phase cells that were grown in YPD medium were shifted for 2 h at 37°C. The cells were washed in 0.1 M cacodylate buffer (pH 6.8), fixed in buffer A (0.1 M cacodylate buffer, pH 6.8, 3% glutaraldehyde) for 1 h at room temperature, and then resuspended in fresh buffer A before they were incubated overnight at 4°C. Fixed cells were washed in 50 mM potassium phosphate (pH 7.5) and converted to spheroplasts in buffer B (50 mM potassium phosphate, pH 7.5, 0.25 mg/ml of Zymolyase 100T) during a 40-min incubation at 37°C. The spheroplasts were washed twice with ice cold 0.1 M cacodylate buffer and post-fixed for 1 h at 4°C in 0.1 M cacodylate buffer with 2% osmium tetroxide. The pellet was washed three times with distilled water and then treated with a 2% uranyl acetate solution for 1 h at room temperature. The samples were washed twice in water, dehydrated with ethanol, pre-embedded in a 1:1 mixture of acetone with spurr resin, and embedded in spurr resin. Thin sections (60–80 nm) were cut, stained with uranyl acetate and lead citrate, and analyzed with a JEOL-(JEM-100 CX II) electron microscope at 80 kV.

RESULTS

Isolation of bet3–1

To identify new genes whose defective products are lethal in combination with *BET1*, a *bet1–1* mutant strain (SFNY185; Table 1), that harbors *BET1* on a *CEN* plasmid (pAN102) was mutagenized with ethylmethanesulfonate. Because the enrichment of dense cells leads to the selection of a higher percentage of secretory mutants, we used a density enrichment protocol in our screen (Novick *et al.*, 1980; see MATERIAL

Table 1. Yeast strains used in this study

Strain	Genotype
ANY113	*MATα, ura3-52, his4-619, bet1-1*
SFNY26-3A	*MAT***a**, *ura3-52*
SFNY26-6A	*MATα, his4-619*
SFNY26-12C	*MATα, ura3-52*
SFNY185	*MATα, ura3-52, his4-619, bet1-1*, pAN102 (*BET1, URA3, CEN*)
SFNY312	*MAT***a**, *leu2-3,112, bet3-1*
SFNY313	*MATα, leu2-3,112, bet3-1*
SFNY314	*MATa, ura3-52, leu2-3,112, bet3-1*
SFNY315	*MATα, his4-619, bet3-1*
SFNY316	*MATα, ura3-52, his4-619, bet3-1*
SFNY317	*MAT***a**, *ura3-52, his4-619, bet3-1*
SFNY446	*MAT***a**, *ura3-52, ypt1-3*
SFNY473	*MAT***a**, *ura3-52*, pGR3 (*BET3, URA3*, 2μm)
NY4	*MATα, his4-619, sec1-1*
NY13	*MAT***a**, *ura3-52*
NY55	*MATα, his4-619, sec9-4*
NY133	*MATα, his4-619, sec2-41*
NY179	*MAT***a**, *ura3-52, leu2-3,112*
NY180	*MATα, ura3-52, leu2-3,112s*
NY404	*MATα, his4-619, sec4-8*
NY411	*MATα, his4-619, sec8-9*
NY413	*MATα, ura3-52, sec13-1*
NY416	*MATα, ura3-52, sec16-2*
NY417	*MATα, ura3-52, sec17-1*
NY419	*MATα, ura3-52, sec19-1*
NY424	*MATα, ura3-52, sec21-1*
NY425	*MATα, ura3-52, sec22-3*
NY432	*MATα, ura3-52, sec18-1*
NY738	*MAT***a**, *ura3-52, sec12-4*
NY1060	*MAT***a**/α, *GAL/GAL, ura3-52/ura3-52, leu2-3, 112/leu2-3, 112*

AND METHODS). Of the 21,800 colonies analyzed, 2,120 were temperature sensitive (ts) for growth at 37°C. These mutants were tested for their ability to lose the *BET1*-containing plasmid (pAN102) on 5-FOA plates. 5-FOA is converted to a toxic compound (5-fluorouracil) by the product of the *URA3* gene (orotidine-5′-phosphate decarboxylase). In the presence of 5-FOA, *ura3* (but not *URA3*) cells will grow. Of the 2,120 ts colonies that were tested on these plates, 66 failed to grow at 25°C. The remaining strains were assayed for defects in the secretion of invertase, a glycoprotein that is transported into the yeast periplasm (Goldstein and Lampen, 1975). This analysis revealed that 12 of the 66 ts mutants secreted a significantly smaller portion of invertase than wild type (≤ 50%) at 37°C. Additionally, all the mutants accumulated active invertase.

Linkage studies and plasmid complementation analysis were performed with the remaining mutants to determine whether any were defective in previously identified genes whose products have been implicated in secretion. These studies indicated that ts 44 was allelic with *sec22*, while five other mutants (ts 14, 20a, 40, 60, and 75) harbored mutations in three known *sec* genes (*sec3*, *sec4*, and *sec17*). These strains, as well as the remaining mutants, were mated to wild-type cells. Further genetic analysis was performed by displacing plasmid pAN102 and sporulating the diploid cells. The *bet1–1* mutation was then separated from the newly identified ts mutation during tetrad analysis. Only one mutant, ts29, was found to be defective in a gene that is lethal in combination with *bet1–1*. In contrast, the *sec3*, *sec4*, and *sec17* alleles that were identified by this selection were not lethal with *bet1*, suggesting that their isolation was fortuitous. The remaining five mutants that accumulated invertase did not harbor mutations that were lethal in combination with *bet1–1*.

To determine whether the defect in ts29 was due to one mutation, this strain was crossed to wild type, sporulated, and subjected to tetrad analysis. Of the 50 tetrads examined, the growth defect segregated 2:2 in all cases, indicating that it was due to a single ts mutation. Furthermore, the secretion defect co-segregated with the growth defect in three of the tetrads examined. To demonstrate that ts29 is lethal in combination with *bet1–1*, we crossed one of the ts colonies to *bet1–1* and performed tetrad analysis. Twelve tetrads were examined. In each case, the double mutants were inviable (see Table 3), demonstrating that ts29 is defective in a new gene whose product interacts genetically with *BET1*. We called this new mutant *bet3–1*. Subsequent analysis of *bet3* was performed with a strain that was backcrossed twice to wild type.

The Secretion of Invertase Is Rapidly Blocked in bet3–1 upon a Shift to 37°C

Our analysis of invertase indicated that this marker protein failed to be transported to the periplasm at 37°C (Table 2), although transit appeared to be normal (our unpublished results) at the permissive temperature (24°C). If *BET3* plays a direct role in secretion, then cells harboring a mutation in this gene should rapidly block the export of invertase after a shift to the restrictive temperature. To assess the speed at which the *bet3* mutant phenotype is expressed at 37°C, cells were first incubated for 35 min at 25°C in low glucose-containing medium to derepress the synthesis of invertase. Mutant cells were then shifted to 37°C and internal and external levels of the enzyme were measured at various timepoints. We found that active invertase accumulated within the *bet3–1* mutant 5 min after it was incubated at its restrictive temperature (Figure 1A). Fur-

Table 2. Reversibility of accumulated invertase (U/OD_{599})

		40 min (37°C)		40 min (37°C) →3 h (25°C)	25°C
Strain	Description	External	Internal	External	Release
SFNY 26-6A	Wild-type	0.39	0.08	...	...
NY 432	*sec 18-1*	0.02	1.1	0.92	82%
ANY 113	*bet 1-1*	0.1	0.53	0.49	74%
SFNY 312	*bet 3-1*	0.12	0.9	0.39	30%

Cells were grown overnight in YPD medium at 25°C to an early exponential phase and then incubated at 37°C for 10 min. At the end of this incubation, the cells were resuspended in 2.0 ml of prewarmed YP medium containing 0.1% glucose (OD_{599}=1.0) and incubated for 40 min longer at the same temperature. Aliquots (1.0 ml) were pelleted, resuspended in prewarmed YPD medium with 0.1 mg/ml of cycloheximide, and incubated at 25°C for 3 h; the remaining cells were placed on ice. Subsequent to all incubations, the cells were washed with cold 10 mM sodium azide, and stored on ice until they were assayed for internal and external levels of invertase. The percent release at 25°C was calculated by the following equation: [external (37°C–25°C)−40 min external invertase (37°C)]/40 min internal invertase (37°C).

thermore, only a small amount of invertase escaped the initial block (Figure 1B). Thus, as previously reported for *bet1–1* (Newman *et al.*, 1990), the *bet3–1* mutant phenotype is rapidly displayed upon a shift to the restrictive temperature.

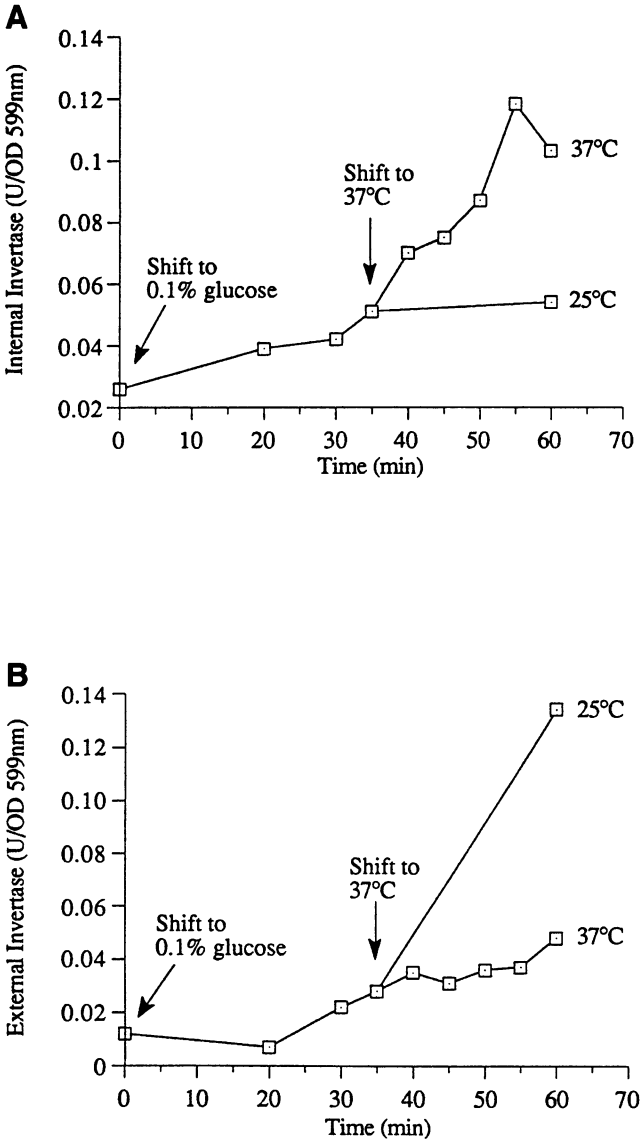

Figure 1. The *bet3–1* mutant quickly displays a block in invertase secretion upon a shift to the restrictive temperature. Cells were grown overnight at 25°C in YPD medium to early exponential phase. Samples were resuspended in YP medium containing 0.1% glucose at 25°C. At the indicated time points, 1.0-ml aliquots were removed, washed, resuspended in cold 10 mM sodium azide, and stored on ice. After 35 min at 25°C, the remaining cells were pelleted, resuspended in prewarmed YP medium containing 0.1% glucose, and incubated at 37°C. Aliquots were again removed at the indicated time points, washed, and resuspended in 10 mM sodium azide. Internal (A) and external (B) levels of invertase were assayed as described in MATERIALS AND METHODS.

The bet3–1 Mutant Pleiotropically Blocks the Transit of Proteins from the ER to the Golgi Complex

A prediction of the synthetic lethal screen that we employed is that *bet3* should be defective in ER to Golgi transport. Because the precursor form of a protein that accumulates intracellularly is indicative of the stage at which transport is blocked (Novick *et al.*, 1981; Newman and Ferro-Novick, 1987), we radiolabeled yeast cells during the derepression of invertase and examined the form that is retained within the cell (Carlson and Botstein, 1982). The secreted form of invertase is initially synthesized as a 62-kDa protein. In the lumen of the ER, the signal sequence is cleaved and 9–10 N-linked oligosaccharide units are added to invertase to yield an ~80-kDa species. As has been shown for *bet1–1* (Newman and Ferro-Novick, 1987) and other ER-accumulating mutants, such as *sec18–1* (Novick *et al.*, 1981; see control in Figure 2A, lane 3), the invertase that failed to be secreted in the *bet3–1* mutant at 37°C accumulated as an 80-kDa species (Figure 2A, compare lane 2 with wild type in lane 1).

To determine the extent to which the 80-kDa form of invertase is reversibly secreted, *bet3–1* mutant cells were first shifted to 37°C in low glucose-containing medium to accumulate precursor intracellularly (Table 2). Subsequent to this incubation, the cells were shifted to 24°C for 3 h in the presence of cycloheximide, a drug that inhibits further protein synthesis. To control for the leakage of accumulated enzyme, a parallel experiment was performed at 37°C (our unpublished results). The data in Table 2 indicates that the *bet3–1* mutant secreted invertase upon a shift to the permissive temperature. The secretion of invertase was greater at 24°C than 37°C, indicating that the defect in transport was partially reversible.

Transport of the yeast pheromone α-factor and the vacuolar protease carboxypeptidase Y (CPY) were also examined in *bet3–1*. α-Factor is synthesized as a 19-kDa precursor that is converted to a 26-kDa species in the lumen of the ER. The 26-kDa ER form of α-factor accumulates in the *bet1–1*, *sec18–1* (Newman and Ferro-Novick, 1987; Figure 2B, lanes 3 and 4), and *bet3–1* mutants at 37°C (Figure 2B, compare lanes 2, 3, and 4 with wild type in lane 1). A partially glycosylated form of α-factor, which contains two instead of three N-linked oligosaccharide units, was also observed in these mutants. Furthermore, like *bet1–1* (Figure 2C, lane 3) and *sec18–1* (Figure 2C, lane 4), *bet3–1* (Figure 2C, lane 2) accumulates the p1 form (67 kDa) of CPY. p1 CPY is further modified in the Golgi apparatus to p2 CPY (69 kDa) before it is processed into the mature form (61 kDa; Figure 2C, lane 1) in the vacuole (reviewed by Klionsky *et al.*, 1990). Thus, at 37°C *bet3–1* displays a pleiotropic block in membrane traffic.

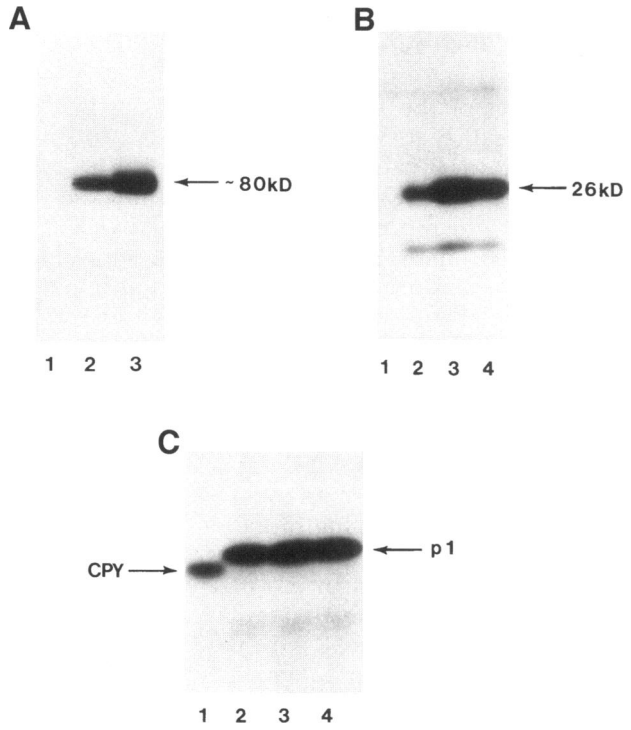

Figure 2. The *bet3–1* mutant fails to transport invertase, α-factor, and CPY from the ER to the Golgi complex. Mutant (lanes 2, 3, and 4) and wild-type (lane 1) cells were labeled with ^{35}S Trans label at 37°C. Radiolabeled cells were converted to spheroplasts and lysed as described in MATERIALS AND METHODS. Invertase (A), α-factor (B), and CPY (C) were immunoprecipitated from each sample and analyzed on a 12.5% SDS polyacrylamide gel. ER forms of invertase (~80 kDa), α-factor (26 kDa), and CPY (p1) were found to accumulate in *bet3–1* (lane 2), *bet1–1* (lane 3 in panels B and C), and *sec18–1* (lane 3 in panel A and lane 4 in panels B and C) mutant cells, but not wild type (lane 1).

The bet3–1 Mutant Accumulates ER and Vesicles at Its Restrictive Growth Temperature

The *bet1–1* mutant accumulates ER membrane at its restrictive growth temperature (Newman and Ferro-Novick, 1987). To morphologically examine *bet3–1* at 37°C, thin sections of this mutant were compared with wild type. In wild-type cells, tubules of ER are occasionally found at the periphery of the cell or juxtaposed to the nuclear envelope. In contrast, when *bet3–1* was incubated at 37°C for 2 h, the ER membrane was elaborated (Figure 3, compare A and B) and some small vesicles (average size ~60 nm) were found to accumulate in the cytoplasm (Figure 3B). The lumen of the ER was dilated and sometimes the nucleus appeared to be multi-lobed. The *bet1–1* mutant also accumulates small vesicles at 37°C. These vesicles are comparable in size to the vesicles that accumulate in *sec17*, *sec18*, *sec22* (Novick *et al.*, 1980), and Bos1p-depleted cells (Shim *et al.*, 1991). Thus, the *bet3–1* mutant is phenotypically similar to *bet1–1* at its restrictive growth temperature (Newman and Ferro-Novick, 1987). In addition to small vesicles, *bet3–1* also accumulated a number of large vesicles (average size ~110 nm). Vesicles of this size have been seen before in secretory mutants that disrupt post-Golgi secretion (Novick *et al.*, 1980). Both small and large vesicles accumulate in *bet2–1* and *sec19–1*, two mutants that function at more than one stage of the yeast secretory pathway (Newman and Ferro-Novick, 1987; Rossi *et al.*, 1991; Garrett *et al.*, 1994).

Like BET1, BET3 Interacts Genetically with BOS1, SEC21, SEC22, and YPT1

Previous studies have shown that *BET1* interacts genetically with *BOS1*, *SEC21*, *SEC22*, and *YPT1* (Newman *et al.*, 1990, 1992; Dascher *et al.*, 1991). To determine whether *BET3* also interacts with members of this group of genes, we transformed the *bet3–1* mutant with either the *BOS1*, *SEC21*, *SEC22*, *YPT1*, or *BET1* gene on a multicopy plasmid and compared the growth of the transformed strain to the parent at various temperatures. These studies revealed that although *bet3–1* failed to grow at 30°C, the overproduction of either *BOS1*, *SEC22*, *YPT1*, or *BET1* facilitated growth at this temperature (Figure 4). In contrast, it was difficult to transform the *bet3–1* mutant with the *SEC21* gene on a multicopy plasmid. However, when transformants were obtained, they grew slower than the parent strain (our unpublished results). Thus, the overproduction of either of these genes enhances or inhibits the growth of the *bet3–1* mutant, suggesting that they interact with *BET3*. In support of these findings, we have also found that the following haploid double mutant combinations were inviable at 25°C: *bet3–1* and *bet1–1*; *bet3–1* and *sec21–1*; *bet3–1* and *sec22–3*; and *bet3–1* and *ypt1–3* (see Table 3). A ts mutant in *bos1* was not available for this analysis. These synthetic lethal interactions are specific as mutations in two genes (*sec13–1* and *sec16–2*), whose products are required for vesicle budding, are not lethal in combination with *bet1–1*.

Because thin section electron microscopy has revealed that the *bet3–1* mutant accumulates both small and large vesicles (Figure 3), the *BET3* gene product may function at more than one stage of the yeast secretory pathway. To explore this possibility further, we crossed the mutant to three ER-accumulating mutants, *sec17–1*, *sec18–1*, and *sec19–1* that have been proposed to function at multiple stages of the pathway, as well as several post-Golgi mutants (*sec2–41*, *sec4–8*, *sec8–9*, and *sec9–4*). This analysis has shown that haploid double mutants containing *bet3–1* and *sec17–1*, *bet3–1* and *sec18–1*, *bet3–1* and *sec2–41*, and *bet3–1* and *sec4–8* were either sick or inviable at 25°C. Thus, these genetic studies support the morphological

studies that suggest Bet3p may function in more than one transport event.

Cloning the BET3 Gene

To clone a wild-type copy of the *BET3* gene, the *bet3–1* mutant (SFNY316) was transformed with a library of yeast genomic inserts (Carlson and Botstein, 1982) cloned into a high copy vector, YEp24. Of the 30,000 colonies screened, seven grew at 37°C. Plasmids recovered from six of these strains fully complemented the growth defect of *bet3–1* at 37°C. The analysis of these six plasmids defined a common 5.2-kb *Sal*I–*Sal*I fragment (pGR4 in Figure 5). To determine whether this insert contained the *BET3* structural gene, an integration experiment was performed. The 5.2-kb *Sal*I–*Sal*I fragment was subcloned into an integrating vector (pGR2) and digested with *Kpn*I to direct integration into the genome of the *bet3–1* mutant (SFNY316). This event placed *URA3* adjacent to the cloned gene. The resulting strain was backcrossed to wild type (NY179) and the diploid was sporulated. Of the 16 tetrads examined, the Ts^+ colonies were always Ura^+, demonstrating that the cloned locus was tightly linked to *BET3*.

Subcloning studies (see Figure 5) revealed that *BET3* was contained within a smaller 2.6-kb *Sal*I–*Kpn*I fragment (pGR5). When this region was further subcloned to a 1-kb *Pvu*II–*Kpn*I fragment (pGR6), there was a loss of complementing activity. Furthermore, when the 3.6-kb region to the right of the *Pvu*II site was deleted from pGR4 (see Figure 5), activity was also abolished. Thus, the 1.6-kb *Sal*I–*Pvu*II fragment (pGR7) was not sufficient to complement the growth defect of the *bet3–1* mutant. Based on these subcloning studies, we conclude that the *Pvu*II site is contained within the *BET3* gene. In support of this proposal, we have shown that the 1.55-kb *Xba*I–*Xba*I fragment (pGR8 in Figure 5), which contains this *Pvu*II site, is sufficient to completely restore growth to *bet3–1* at 37°C.

Nucleotide Sequence of the BET3 Gene

As a first step toward determining the function of *BET3*, we cloned and sequenced the gene that encodes this protein. Because the *Pvu*II site described above is contained within *BET3*, we subcloned the *Sal*I–*Pvu*II (1.6 kb) and *Pvu*II–*Kpn*I (1 kb) fragments into the pUC118 and pUC119 vectors and sequenced to the left and right of the *Pvu*II site. This analysis revealed that one open reading frame spans the *Pvu*II site. The sequence of the ~0.6-kb *Hin*dIII–*Hin*dIII fragment, which contains this open reading frame, is located on chromosome XI (accession number Z28292–4; Dujon *et al.*, 1994). A search of the data bank revealed that *BET3* is 36.4% identical and 60% similar to a 20-kDa (p20) nematode protein (accession number P34605) of unknown function (Figure 6), indicating that homologues of Bet3p can be found in a variety of organisms.

To confirm that the identified open reading frame encodes *BET3*, its ability to complement the growth defect of the *bet3–1* mutant was tested. This was done by cloning the open reading frame behind the regulatable *GAL1* promoter. Transcription from this promoter is repressed in the presence of glucose, but not galactose. The resulting plasmid (pGR10 in Figure 5) was integrated at the *LEU2* locus of SFNY314 (see Table 1) and two Leu^+ transformants were tested for their ability to grow on plates containing galactose or glucose. These transformants only grew on YP galactose plates at 37°C, confirming that the *Hin*dIII–*Hin*dIII fragment that we sequenced encodes the *BET3* gene.

Table 3. Tetrad analysis of crosses with *bet3-1*

Cross	No. of crosses						
bet3-1 crossed with:	Four viable		Three viable			Two viable	
	4−:0+[a]	3−:1+	3−:0+	2−:1+	1−:2+	1−:1+	0−:2+
bet1-1	1			11			
sec2-41	2			9			1
sec4-8[b]	1			9			1
sec17-1[b]				11			
sec18-1[b]		1		10			1
sec21-1	8	1	1	8	1	1	3
sec22-3	3			4		2	3
ypt1-3	5		1	10		2	6

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

[b] Double mutants were either sick or lethal.

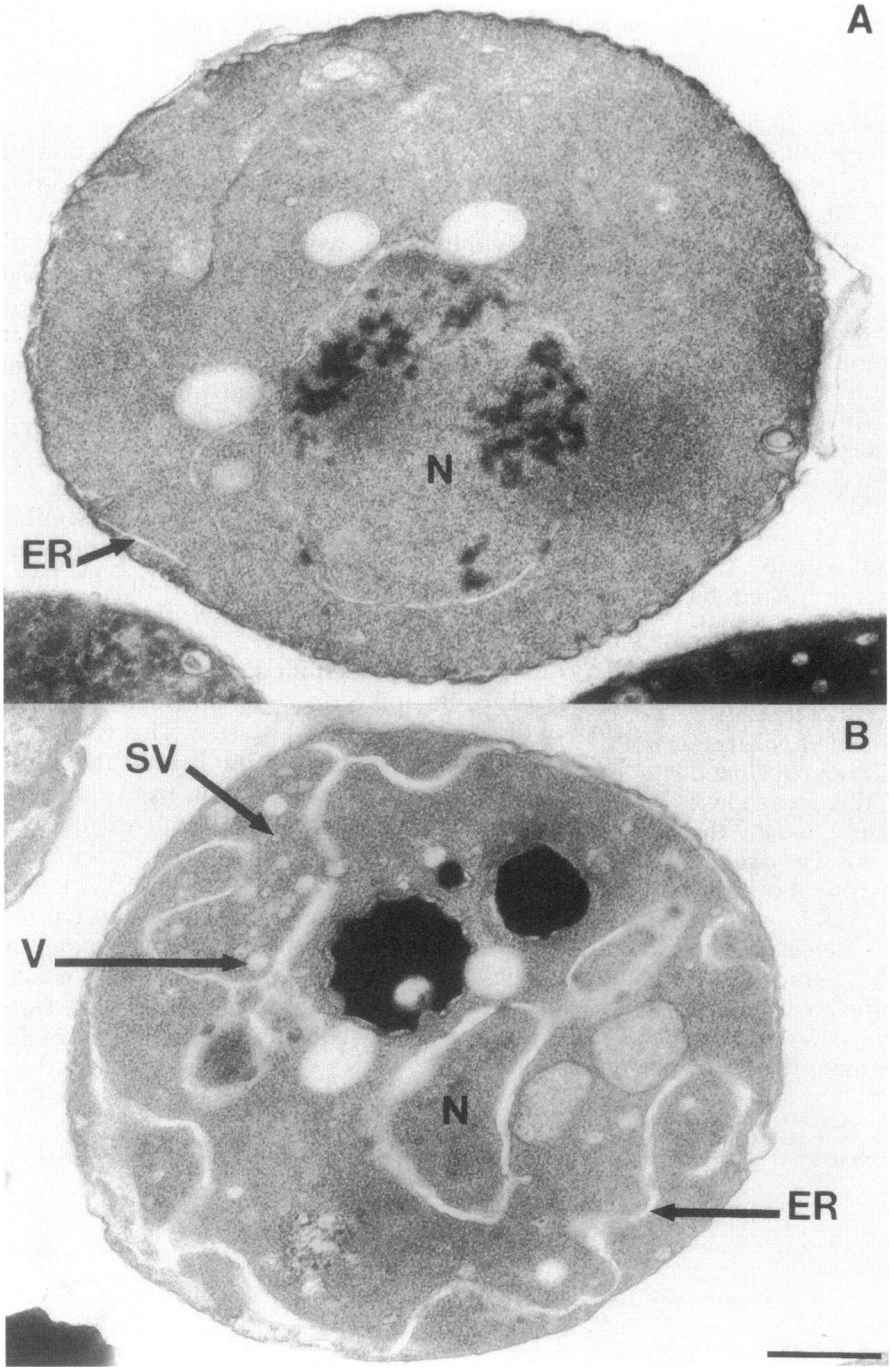

Figure 3. EM analysis of *bet3–1* mutant cells. Samples were prepared for electron microscopy as described in MATERIALS AND METHODS. Thin-section analysis of wild-type (A) and *bet3* mutant cells (B). ER, endoplasmic reticulum; N, nucleus; V, vesicles; SV, small vesicles; Bar, ~0.5 μm.

BET3 Is Essential for Function

To determine whether *BET3* encodes a protein that is essential for the vegetative growth of yeast cells, we disrupted the *BET3* open reading frame in a diploid strain (NY1060) using *URA3* as a selectable marker. A series of constructions were performed before disrupting the genomic copy of *BET3*. First, we subcloned the 2.6-kb *Sal*I–*Kpn*I fragment shown in Figure 5 into a bluescript vector that lacked a *Hin*dIII site (see MATERIALS AND METHODS). Subsequently, this vector was digested with *Hin*dIII, removing the 0.6-kb *Hin*dIII–*Hin*dIII fragment that contains *BET3*. The *URA3* gene was then inserted in its place to yield plasmid pGR 9. This plasmid was digested with *Kpn*I and *Sal*I, integrated into a diploid strain (NY1060), sporulated, and tetrad analysis was performed. Of the 24 tetrads

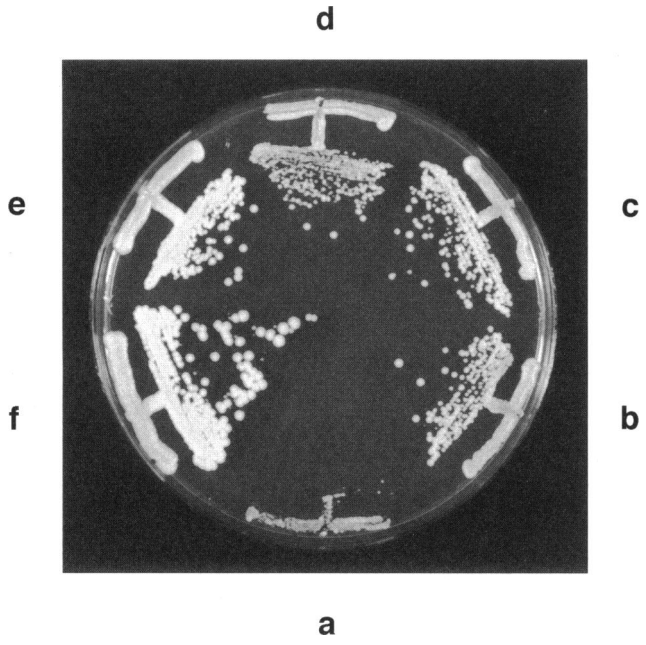

Figure 4. The overexpression of *BET1*, *BOS1*, *SEC22*, or *YPT1* suppresses the growth defect of the *bet3–1* mutant at 30°C. The *bet3–1* mutant (SFNY316) was transformed with either (a) pRB307 (vector alone; *URA3*, 2 μm); (b) pAN109 (*BOS1*, *URA3*, 2 μm); (c) pFN100 (*BET1*, *URA3*, 2 μm); (d) pJG103 (*SEC22*, *URA3*, 2 μm); or (e) pNB167 (*YPT1*, *URA3*, 2 μm). The growth of the transformants were compared with wild type (f) on a YPD plate at 30°C.

examined, all displayed 2:2 segregation for viability. In addition the viable spores were Ura$^-$, indicating that *BET3* is required for the viability of yeast cells. To demonstrate that the inviable spores failed to grow because they lacked Bet3p, we introduced pGR10 (*GAL1*::*BET3*) into cells disrupted for *BET3*. Growth was observed on YP galactose plates, but not on YPD plates. Thus, the growth defect was only rescued when *BET3* was expressed.

BET3 Encodes a 22-kDa Protein

The nucleotide sequence of *BET3* predicts a hydrophilic protein of 22-kDa with a net negative charge of 6.9 at neutral pH. To confirm the size of Bet3p, polyclonal antibody was raised to a Bet3p-MBP fusion protein. Lysates prepared from cells that were radiolabeled by the protocol described in MATERIALS AND METHODS were precipitated with pre-immune serum (Figure 7, lane 1 and 2) or α-Bet3p (Figure 7, lane 3). This analysis revealed that α-Bet3p recognizes a 22-kDa band, which was also overproduced in a strain that overproduces Bet3p (Figure 7, lanes 4 and 6). Affinity-purified α-Bet3p cross-reacted with the same protein species (Figure 7, lane 5). A c-myc–tagged version of Bet3p, of approximately the same molecular weight, was also recognized by this antibody (our unpublished results).

The SNARE Complex Does Not Form in bet3–1 Mutant Cells

Genetic and morphological studies imply that Bet3p may play a role in the late stages of ER to Golgi transport. With antibody in hand, we directly tested the possibility that Bet3p is a component of the SNARE complex. When vesicle fusion is blocked at 37°C in *sec18* mutant cells, the SNARE complex accumulates. This complex has been immunoisolated from detergent extracts of *sec18* with affinity-purified anti-Bos1p antibody (Sogaard *et al.*, 1994). It contains the t-SNARE, which is proposed to be Sed5p, the

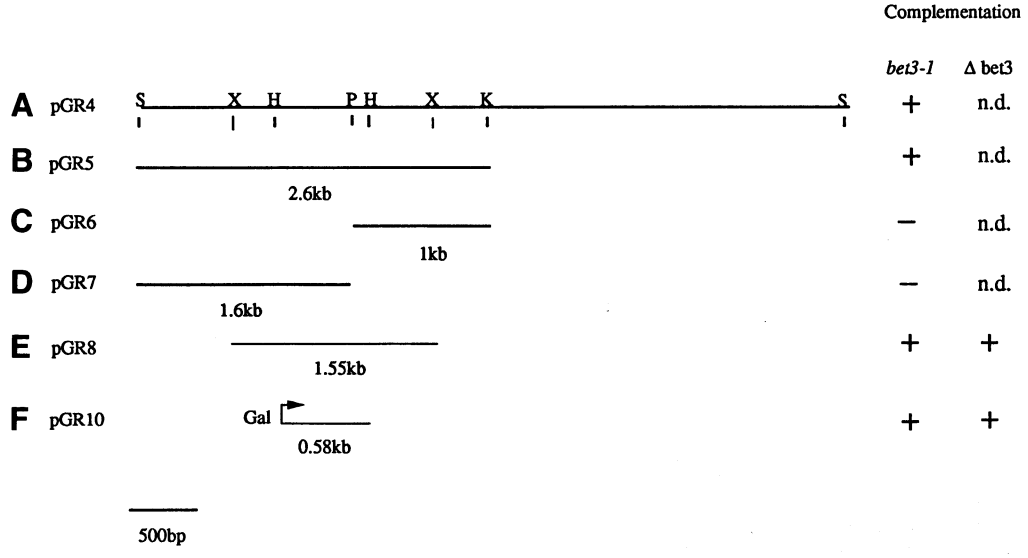

Figure 5. Complementing activity of cloned inserts. Plasmids were prepared as described in MATERIALS AND METHODS. Only the cloned inserts are shown. S, *Sal*I; X, *Xba*I; H, *Hin*dIII; P, *Pvu*II; K, and *Kpn*I. bp, base pairs.

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                .         .         .         .         .
Bet3p      13 MGEEIWKNKTE..KINTELFTLTYGSIVAQLCQDYERDFNKVNDHLYSMG 60
              |:..|..| .:  |:..|||.||||.:|.:: .||| | ..|. :| .||
  p20       1 MSKAIKQNLADSKKMSAELFCLTYGAMVTEMLKDYE.DPKDVTIQLDKMG 49
                .         .         .         .         .
Bet3p      61 YNIGCRLIEDFLARTA.LPRCENLVKTSEVLSKCAFKIFLNITPNITNWS 109
              :|:|.|| :||||:.| :||| :  ...:||:: |:..:|.|.:. ..|.
  p20      50 FNMGTRLADDFLAKNANVPRCVDTRQIADVLCRNAIPCYLGISATASSWT 99
                .         .         .         .         .
Bet3p     110 HNKDTFSLILDENPLADFVELPMDAMK.SLWYSNILCGVLKGSLEMVQLD 158
               .. .|.:.|:.|||.::|::| . :. :|.||.::.|.::|.|| |::.
  p20     100 SGDREFTITLEANPLTELVQVPAHLVSAGLSYSQLIAGAIRGALEAVHFK 149
                .         .         .
Bet3p     159 CDVWFVSDILRGDSQTEIKVKLNRILKDEIPIGED 193
              .   :.|..  .:..|||:::::.:|||.:| |||
  p20     150 V...YASAT.DTGANTEIRIRFDQVLKDSLPAGED 180
```

Figure 6. A comparison of Bet3p with the *C. elegans* protein p20. The amino acid sequence of Bet3p is compared with p20 using the Bestfit program (GCG software package). Identity is indicated by a line between the sequences and conserved changes are indicated by two dots (two corresponding bases in a codon) or one dot (one corresponding base in a codon). Gaps are shown as dots within the sequence. Bet3p and p20 are 36.4% identical at the amino acid level.

v-SNARE, and several unidentified proteins that are similar in molecular weight to Bet3p. In performing our studies, we prepared a detergent extract of *sec18* mutant cells and precipitated it with α-Bos1p. As a control, we also examined wild-type extracts that do not accumulate this docking/fusion complex. The precipitate was then blotted with antibodies to Sec22p and Sed5p, which are components of the SNARE complex, as well as Bet3p. As expected, α-Bos1p precipitated Sec22p and Sed5p from the *sec18* mutant extract, but not wild type (Figure 8A, lanes 1 and 2). Bet3p was not found in this precipitate (Figure 8A, lanes 1, 2, and 3), however, it was recovered in the supernatant fraction (our unpublished results).

Although the findings described above indicate that Bet3p is not a component of the SNARE complex, active Bet3p may be required to form this complex. To test this possibility, we precipitated an extract of *bet3–1* mutant cells with α-Bos1p and blotted the precipitate with α-Sec22p and α-Sed5p. As shown in Figure 8B, Bos1p co-precipitated with Sec22p and Sed5p from the *sec18–1* mutant extract (Figure 8B, lane 2), but not from wild type (Figure 8B, lane 1) or the *bet3–1* extract (Figure 8B, lane 3). Thus, the SNARE complex fails to form in *bet3–1* mutant cells. Furthermore, the Bos1p/Sec22p complex, which accumulates in secretory mutants that are blocked in vesicle targeting and fusion (Lian *et al.*, 1994), could not be detected in the

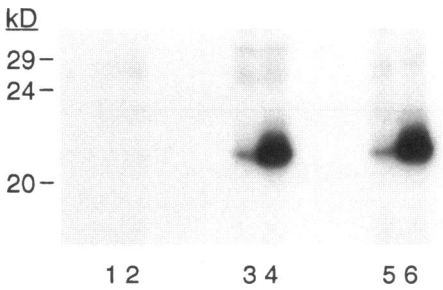

Figure 7. *BET3* encodes a 22-kDa protein. Wild-type cells (NY13) or cells overexpressing the *BET3* gene (SFNY473) were radiolabeled, spheroplasted, and lysed as described in MATERIALS AND METHODS. The lysates were incubated with either pre-immune serum (lanes 1 and 2), anti-Bet3p antibody (lanes 3 and 4), or affinity-purified anti-Bet3p antibody (lanes 5 and 6) and the precipitates were analyzed on a 12.5% SDS gel. A 22-kDa protein was detected by the anti-Bet3p antibody, but not by the pre-immune serum. The 22-kDa band was also overproduced in a strain that overproduces *BET3* (lanes 4 and 6).

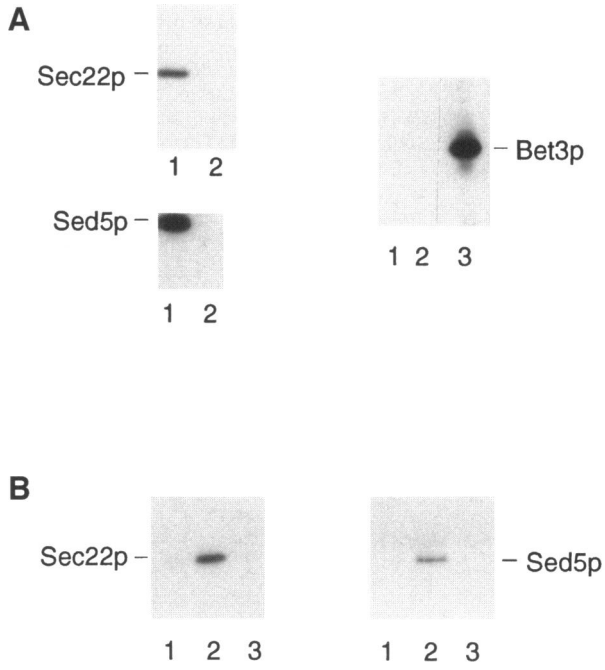

Figure 8. Bet3p is not a component of the yeast SNARE complex. Wild type (lane 2 in panel A and lane 1 in panel B), *sec18–1* (lane 1 in panel A and lane 2 in panel B), and *bet3–1* (lane 3 in panel B) cells were grown in YPD medium to early log phase. Cells were converted to spheroplasts at 25°C using protocols described previously (Ruohola *et al.*, 1988) and the spheroplasts were regenerated for 1 h at 37°C. Regenerated spheroplasts were lysed, and processed as described elsewhere (Lian *et al.*, 1994). The clarified lysates were precipitated with 40 μg of affinity-purified α-Bos1p and the solubilized precipitates were blotted with α-Sec22p and α-Sed5p. A lysate that overproduces Bet3p marks the location of this protein in panel A (lane 3).

bet3–1 mutant extract (Figure 8B, compare lanes 1 and 3 with lane 2). The formation of the Bos1p/Sec22p complex correlates with the ability of Bos1p to pair with Sed5p (Lian *et al.* 1994).

DISCUSSION

Here we report the use of a synthetic lethal screen to identify an additional gene whose defective product is lethal in combination with *bet1–1*. This analysis has resulted in the isolation of *bet3–1*, a new ts yeast secretory mutant that fails to transport proteins from the ER to the Golgi complex at 37°C. The phenotypic consequences of mutations in *bet3–1* are similar to those that have been observed previously for *bet1–1*. Furthermore, like *BET1*, *BET3* displays genetic interactions with *BOS1*, *SEC21*, *SEC22*, and *YPT1*. Thus, Bet3p may act in conjunction with Bet1p to mediate the same step in ER to Golgi transport.

Although *BET3* interacts genetically with a group of genes whose products are components of the SNARE complex, the Bet3 protein is not physically associated with this complex. Furthermore, the Bos1p/Sec22p complex, as well as the SNARE complex, does not accumulate in *bet3–1* mutant cells. These findings support the hypothesis that Bet3p acts upstream of this docking/fusion complex. A clue to the role of Bet3p has come from studies with Bet1p and Bos1p. Bos1p specifically functions on vesicles where it forms a complex with Sec22p, a protein that modulates its activity (Lian and Ferro-Novick, 1993; Lian *et al.*, 1994). These interactions are catalyzed by the ras-like GTP-binding protein Ypt1p. Bet1p may interact with Bos1p on the ER before Bos1p associates with Sec22p on vesicles. Thus, it is possible that Ypt1p stimulates the formation of the Bos1p/Sec22p complex through Bet1p, and Bet3p may act in concert with it. An analysis of Bet3p in an in vitro reaction that reconstitutes transport from the ER to the Golgi complex (Ruohola *et al.*, 1988; Groesch *et al.*, 1990) will enable us to address this possibility.

Two findings suggest that Bet3p may function at more than one stage of the secretory pathway. First, a morphological analysis of *bet3–1* has revealed that small (mean size ~60 nm) and larger (average size ~110 nm) vesicles accumulate in this mutant at 37°C. The larger vesicles are similar to those seen previously in mutants that are blocked in post-Golgi transport (Novick *et al.*, 1980). Second, genetic studies have revealed that *bet3–1* is lethal in combination with *sec2–41* or *sec4–8*, two mutants that are defective in the fusion of post-Golgi transport vesicles with the plasma membrane (Novick *et al.*, 1980). A prediction of these observations is that *BET3* may encode a soluble protein that functions in both ER to Golgi and post-Golgi transport. In support of this hypothesis is the finding that *BET3* encodes a highly conserved 22-kDa hydrophilic protein. Further characterization of Bet3p will allow us to determine whether this protein and its homologues function at more than one stage of the secretory pathway in yeast and higher cells.

The findings described here have shown that synthetic lethal screens can be used to isolate secretory mutants that are phenotypically related to each other. The advantage of such a screen is that it facilitates the identification of physically interacting components, or gene products that function on the same or parallel pathways. Now that we have demonstrated that this approach will lead to the isolation of new *bet* mutants, we are currently using similar screens to find additional genes whose products functionally interact with Bos1p and Sec22p.

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