A High Copy Suppressor Screen Reveals Genetic Interactions Between *BET3* and a New Gene: Evidence for a Novel Complex in ER-to-Golgi Transport

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ABSTRACT

The *BET3* gene in the yeast *Saccharomyces cerevisiae* encodes a 22-kD hydrophilic protein that is required for vesicular transport between the ER and Golgi complex. To gain insight into the role of Bet3p, we screened for genes that suppress the growth defect of the temperature-sensitive *bet3* mutant at 34°. This high copy suppressor screen resulted in the isolation of a new gene, called *BET5*. *BET5* encodes an essential 18-kD hydrophilic protein that in high copy allows growth of the *bet3-1* mutant, but not other ER accumulating mutants. This strong and specific suppression is consistent with the fact that Bet3p and Bet5p are members of the same complex. Using PCR mutagenesis, we generated a temperature-sensitive mutation in *BET5* (*bet5-1*) that blocks the transport of carboxypeptidase Y to the vacuole and prevents secretion of the yeast pheromone α-factor at 37°. The precursor forms of these proteins that accumulate in this mutant are indicative of a block in membrane traffic between the ER and Golgi apparatus. High copy suppressors of the *bet5-1* mutant include several genes whose products are required for ER-to-Golgi transport (*BET1*, *SEC22*, *USO1* and *DSS4*) and the maintenance of the Golgi (*ANP1*). These findings support the hypothesis that Bet5p acts in conjunction with Bet3p to mediate a late stage in ER-to-Golgi transport. The identification of mammalian homologues of Bet3p and Bet5p implies that the Bet3p/Bet5p complex is highly conserved in evolution.

IN eukaryotic cells, the secretory pathway consists of a series of distinct membrane-bound compartments. The transport of proteins and lipids between these compartments is mediated by vesicles that dock and fuse with their acceptor membrane. In the neuron, membrane proteins called SNAREs (synaptobrevin, syntaxin and SNAP-25) are key players in these events (Söllner *et al.* 1993a). The SNAREs, which are evolutionarily conserved (Ferro-Novick and Jahn 1994), bind to each other to form a stable ternary complex that then becomes larger when the soluble factors NSF and α-SNAP bind (Söllner *et al.* 1993b). During membrane fusion (Söllner *et al.* 1993a), or after fusion, this complex is disassembled by NSF (Mayer *et al.* 1996) to prime the SNAREs for a new round of vesicular transport (Mayer *et al.* 1996). The recent finding that a v-SNARE can interact with more than one t-SNARE (Fischer Von Mollard *et al.* 1997) implies that the SNAREs are not sufficient to target vesicles to the right compartment.

In the yeast *Saccharomyces cerevisiae*, genetic and biochemical studies have led to the identification of a number of genes whose products function in the docking and fusion of ER-derived transport vesicles with the Golgi apparatus. Among them are the homologues of NSF and α-SNAP, which are encoded by the *SEC18* and *SEC17* genes, respectively (Wilson *et al.* 1989; Griff *et al.* 1992), as well as analogues of the neuronal SNAREs, synaptobrevin, syntaxin and SNAP-25. The synaptobrevin-like proteins Bos1p (Lian and Ferro-Novick 1993) and Sec22p (Lian *et al.* 1994; Søgaard *et al.* 1994) are classified as v-SNAREs (vesicle SNAREs), while a third SNARE, Bet1p, contains a domain that is homologous to SNAP-25 (Stone *et al.* 1997). The interactions of Bet1p with Bos1p and Sec22p are regulated by the small GTP-binding protein Ypt1p (Lian *et al.* 1994; Stone *et al.* 1997). Sed5p, a syntaxin related protein (Hardwick and Pelham 1992), is the t-SNARE (target SNARE) that acts at this stage of the secretory pathway (Søgaard *et al.* 1994; Sacher *et al.* 1997).

We recently identified Bet3p, a small hydrophilic protein that appears to mediate a late stage of ER-to-Golgi transport in yeast (Rossi *et al.* 1995). The importance of Bet3p in membrane traffic is marked by its essential role in this process and its genetic interactions with *YPT1* and known v-SNAREs (Rossi *et al.* 1995). Despite these interactions, however, Bet3p is not part of the SNARE complex that accumulates at 37° in *sec18* mutant cells when ER-to-Golgi transport is blocked (Rossi *et al.* 1995). In an attempt to understand the role of Bet3p in intracellular membrane traffic, we screened for genes that suppress the temperature-sensitive (ts) growth defect of the *bet3-1* mutant. This screen resulted in the identification of a new gene, *BET5*. *BET5* encodes an

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TABLE 1

Strains

Strain	Genotype	Source
ANY123	*MAT*α *bet1-1 ura3-52 his4-619*	Ferro-Novick Lab Collection
NY432	*MAT***a** *sec18-1 ura3-52*	Novick Lab Collection
NY920	*MAT***a**/α *ura3-52/ura3-52 leu2-3, 112/leu2-3, 112 his3-*Δ*200/his3*Δ*200*	Novick Lab Collection
SFNY26-6A	*MAT*α *his4-619*	Ferro-Novick Lab Collection
SFNY315	*MAT*α *bet3-1 his4-619*	Ferro-Novick Lab Collection
SFNY562	*MAT***a**/α *Gal*$^+$ *leu2-3, 112/leu2-3, 112 ura3-52/ura3-52*	Ferro-Novick Lab Collection
SGNY570	*MAT***a**/α *Gal*$^+$ *leu2-3, 112/leu2-3, 112 ura3-52/ura3-52 BET5/bet5*Δ*::URA3*	This study
SFNY576	*MAT*α *Gal*$^+$ *bet5*Δ*::URA3 urd3-52 leu2-3, 112::(LEU2 GAL1-BET5)*	This study
SFNY578	*MAT***a** *his4-619* p5.3 (*BET5* in YEp24)	This study
SFNY583	*MAT*α *ura3-52 bet5*Δ*::URA3 leu2-3, 112::(LEU2 BET5-2x-c-myc)*	This study
SFNY712	*MAT*α *Gal*$^+$ *ura3-52 leu2-3, 112 his3*Δ*200 bet5*Δ*::HIS3* pSFN470(*GAL1-BET5, URA3, CEN*)	This study
SFNY713	*MAT*α *Gal*$^+$ *ura3-52 leu2-3, 112:(LEU2 bet5-1) his3*Δ*200 bet5*Δ*::HIS3*	This study

essential 18-kD hydrophilic protein that is required for ER-to-Golgi membrane traffic. Here we show that Bet3p and Bet5p are members of a complex that is found in a variety of organisms from yeast to humans.

MATERIALS AND METHODS

Strains, plasmids and media: Yeast cells were grown in either YP or minimal medium that was supplemented with the appropriate nutrients (20 μg/ml of histidine, 40 μg/ml of uracil). The growth medium contained either 2% glucose or 0.5% galactose and 2% raffinose as a carbon source.

Isolation of the *BET5* gene: The *BET5* gene was isolated by screening a 2-μm yeast genomic library (Carlson and Botstein 1982) for genes that suppress the growth defect of the ts *bet3-1* mutant. Plasmid DNA was transformed into the *bet3-1* mutant strain, and Ura$^+$ transformants were selected on minimal medium lacking uracil. After 3 days at 25°, transformants were replica plated onto YPD plates and then incubated overnight at 30°. Of the 11,146 Ura$^+$ transformants examined, 166 grew at 30°. These transformants were then tested for growth at 34°. Plasmids from the 8 transformants that grew at 34° were retrieved and reintroduced into the *bet3-1* mutant to confirm suppression. Restriction analysis indicated that two of these plasmids contained the *BET3* gene, while the other six shared a common region of DNA that conferred suppression. The plasmid with the smallest insert (4.3 kb) was subcloned further and tested for activity. This analysis indicated that the region that includes the *Xho*I site is critical for suppression. A 1.0-kb *Bsu*36I-*Sma*I genomic fragment, which fully suppressed *bet3-1*, was sequenced. The open reading frame (ORF) that spanned the *Xho*I site was named *BET5*.

Disruption of the *BET5* gene and construction of SFNY576: A chromosomal deletion of *BET5* was created by replacing all but the last 12 amino acids of the coding sequence with the *URA3* gene. Plasmid pSFNB387 (Figure 2), which contains a disruption of the *BET5* gene, was constructed as described below. First, a 1.6-kb *Kpn*I-*Sac*I DNA fragment containing *BET5* was subcloned into pBluescript. (The *Kpn*I and *Sac*I sites were generated by PCR.) The *URA3* gene was then inserted between the *Bsa*BI and *Xho*I sites that encode the first 140 amino acids of *BET5* to yield pSFNB387. A diploid strain, with one disrupted copy of *BET5*, was created by digesting pSFNB387 with *Kpn*I and *Sac*I and transforming the linear fragment into SFNY562 (Table 1). The transformants were sporulated, and tetrad analysis was performed. After 3 days at 25°, the 46 tetrads examined displayed 2:2 segregation for viability. All viable spores were Ura$^-$.

SFNY576 (Table 1) was constructed in several steps. First, *BET5* was fused to the *GAL1* promoter in plasmid pNB527 (*GAL1, LEU2*) by cloning the ORF into the *Bam*HI site adjacent to *GAL1*. The resulting construct was linearized with *Afl*II and transformed into SFNY570 (Table 1). As the *Afl*II site is internal to *LEU2*, the linearized plasmid was expected to integrate at the *LEU2* locus. The transformants were sporulated and tetrads were dissected on YP plates that contained 0.5% galactose and 2% raffinose as a carbon source. Colonies that were Ura$^+$ and Leu$^+$ (SFNY576) contained *BET5* under the control of the *GAL1* promoter.

Construction of yeast strains containing epitope-tagged *BET5*: A strain (SFNY583; see Table 1) in which the sole copy of *BET5* is tagged with c-myc was constructed as described below. Briefly, *BET5* was cloned into an integrating vector (pRS305, *LEU2*; Sikorski and Hieter 1989), and site-directed mutagenesis was used to replace the last codon in the gene with two c-myc epitopes (Kunkel *et al.* 1987). The tagged *BET5* gene encodes a protein containing the following sequence at the C-terminus: AEQKLISEEDLAEQKLISEEDLA-STOP. After confirming that the mutagenesis was done correctly by DNA sequence analysis, the plasmid was digested with *Afl*II and transformed into SFNY570 (Table 1), a strain in which the *BET5* gene is disrupted by *URA3*. The transformants were sporulated and tetrad analysis was performed. Colonies that contained c-myc-tagged Bet5p as the sole copy were selected (SFNY583) by screening for Ura$^+$, Leu$^+$ transformants.

Isolation of the *bet5-1* mutant: A ts mutation in *BET5* (*bet5-1*) was generated by PCR mutagenesis (Muhlrad *et al.* 1992). Briefly, SFNY712 (Table 1) was transformed with a gapped plasmid (pSFN404) and mutagenized *BET5* DNA (Muhlrad *et al.* 1992). Plasmid pSFN404 (*BET5, CEN, LEU2*) was gapped by excising a *Bsu*36I-*Bsa*BI fragment that contains the *BET5* gene. Transformants, selected on minimal plates containing glucose but lacking leucine, were screened for their inability to grow on YPD plates at 37°. Plasmid pSFN470 (*GAL1-BET5, URA3, CEN*) was then displaced from the transformants on 5-FOA (orotidine-5′-phosphate decarboxylase) plates at 25°. One transformant, whose ts growth defect was complemented by plasmid pSFN469 (*BET5, URA3, CEN*) was analyzed further. The mutated plasmid was retrieved from the host strain and subcloned into an integrating vector (pRS305) that contained the *LEU2* gene. The resulting plasmid was then linearized

with *Eco*RV and integrated at the *LEU2* locus of SFNY712. A Leu$^+$ transformant was incubated on a plate containing 5-FOA to displace pSFN470. The resulting strain was SFNY713 (Table 1). DNA sequence analysis revealed that the *bet5-1* mutant contained nine mutations (C13R; I32V; M47T; K57R; N65D; D66A; R68P; F88L; S98P).

Preparation of anti-Bet5p serum: Bet5p antiserum was raised against recombinant Bet5p that contained a six-histidine (His_6) tag at its amino terminus. The His_6-tagged construct was made by cloning a PCR product, that contained the *BET5* ORF, into a T7 expression vector (pET15b from Novagen, Madison, WI). The cloning sites (*Nco*I/*Bam*HI) of the PCR product were generated so that, after ligation, the initial ATG of *BET5* was fused in-frame with the coding sequence for the His_6 tag. Bet5p was expressed in BL21 (DE3) cells and gel purified from a cell extract. The immunization protocol used was described before (Louvard *et al.* 1982).

***In vivo* labeling and immunoprecipitation:** Yeast cells were grown at 25° to early exponential phase in minimal medium containing 2% glucose. The cells were harvested and resuspended in 1 ml of medium to an OD_{600} = 3.00 before they were radiolabeled for 30 min with 200 μCi of ^{35}S Trans label. SFNY576 was incubated for 15 hr at 25° in glucose containing medium prior to the addition of label. Subsequent to this incubation, the cells were labeled for 30 min at 25°. The *bet1-1*, *sec18-1*, and *bet5-1* (Table 1) mutants were preincubated for 30 min at 37° and then labeled at the same temperature. Radiolabeled cells were washed with 10 mm sodium azide, converted to spheroplasts, and lysed as described before (Shim *et al.* 1991). CPY and α-factor were precipitated from clarified extracts as described by Shim *et al.* (1991).

For the Bet3p/Bet5p coprecipitation studies, yeast cells were grown to early exponential phase in YPD medium at 25°. Eight OD_{600} units of cells were collected, washed with cold sodium azide, and resuspended in 2 ml of spheroplast buffer (1.4 m sorbitol, 50 mm potassium phosphate, pH 7.5, 10 mm sodium azide, 50 mm β-mercaptoethanol and 10 μg zymolase/OD unit of cells). Spheroplasts formed during a 60-min incubation at 37° were lysed in 250 μl of lysis buffer (20 mm HEPES, pH 7.4, 500 mm KCl, 1 mm DTT, 1 mm $MgCl_2$, 2% Triton X-100, 1× Protease Inhibitor Cocktail also called PIC [see Ruohola *et al.* 1988]) and then centrifuged for 15 min in an Eppendorf centrifuge. The supernatants were removed and diluted with 750 μl of Buffer A (20 mm HEPES (pH 7.4), 100 mm KCl, 1 mm DTT, 1 mm $MgCl_2$, 0.5% Triton X-100, 1× PIC). Affinity-purified anti-Bet3p antibody (320 μg), preimmune serum (20 μl), or anti-c-myc antibody (20 μl) was added to samples before they were incubated overnight at 4°. The antigen-antibody complexes were precipitated onto protein A Sepharose beads during a 90-min incubation at 4° and the beads were washed twice with Buffer B (20 M HEPES (pH 7.4), 500 mm KCl, 1 mm DTT, 1 mm $MgCl_2$, 0.5% Triton X-100, 1× PIC), six times with Buffer A and once with 20 mm Tris-Cl (pH 7.0). The antigen-antibody complexes were solubilized by heating the beads for 5 min in the presence of 100 μl of sample buffer. The beads were pelleted by centrifugation and 30 μl of the supernatant was electrophoresed on a 15% SDS polyacrylamide gel. Western blot analysis was performed using anti-Bet5p antiserum (1:1000 dilution).

Cloning the human *BET5* homolgue: Using sequence AA203173, primers were constructed to amplify the full-length coding region for human Bet5p from a CLONTECH (Palo Alto, CA) fetal liver cDNA library (catalog no. 7403-1). As for human Bet3p, plasmid clones were sequenced at the Keck Foundation at Yale University. Sequence analysis and the database search were performed using the Wisconsin Genetics Computer Group (GCG) software version 8.1.

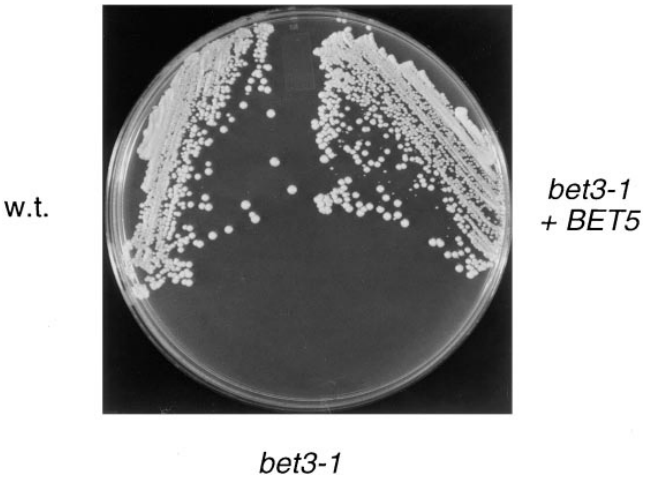

Figure 1.—*BET5* is a high copy suppressor of the *bet3-1* mutant. Yeast cells were grown on a YPD plate and incubated at 34° for 3 days.

RESULTS

Isolation of *BET5* as a high copy suppressor of the *bet3-1* mutant: We previously reported the isolation of a ts *bet3-1* mutant that fails to grow at 30° and higher temperatures (Rossi *et al.* 1995). To identify new genes whose products may interact with Bet3p, we transformed the *bet3-1* mutant with a high copy yeast genomic library and screened for genes that suppress the growth defect of *bet3-1*. Of the 11,146 transformants examined, only 8 grew at 34°. Plasmids retrieved from these transformants rendered *bet3-1* temperature resistant, indicating that suppression was plasmid dependent. Two of these plasmids restored growth at 37° and were subsequently shown to carry the *BET3* gene. The other six plasmids enabled *bet3-1* to grow as well as wild type at 34°, but failed to do so at 37°. Restriction enzyme analysis demonstrated that these plasmids contained a common region of DNA. The plasmid with the smallest insert (4.3 kb) was subcloned to a 1.0-kb *Bsu*36I-*Sma*I fragment that retained full suppression ability (Figure 1). An internal *Xho*I site was found to be critical for this activity. Sequence analysis revealed that one ORF spanned the *Xho*I site (Figure 2). We called this ORF *BET5*. A comparison of *BET5* with other sequences in the GenBank revealed that it resides on yeast chromosome *XIII* (accession number PIR S48820). The *BET5* gene encodes a hydrophilic polypeptide of 159 amino acids with a predicted molecular mass of 18.4 kD. A search of the yeast database did not reveal any significant homologies.

***BET5* is an essential gene:** To determine whether *BET5* encodes a protein that is essential for the vegetative growth of yeast cells, we constructed a plasmid in which the first 147 codons of *BET5* were replaced with *URA3*. The disrupted *BET5* gene (Figure 2) was then transformed into a diploid strain to replace one chromosomal copy (see materials and methods). The Ura$^+$ transformants were sporulated and tetrad analysis was

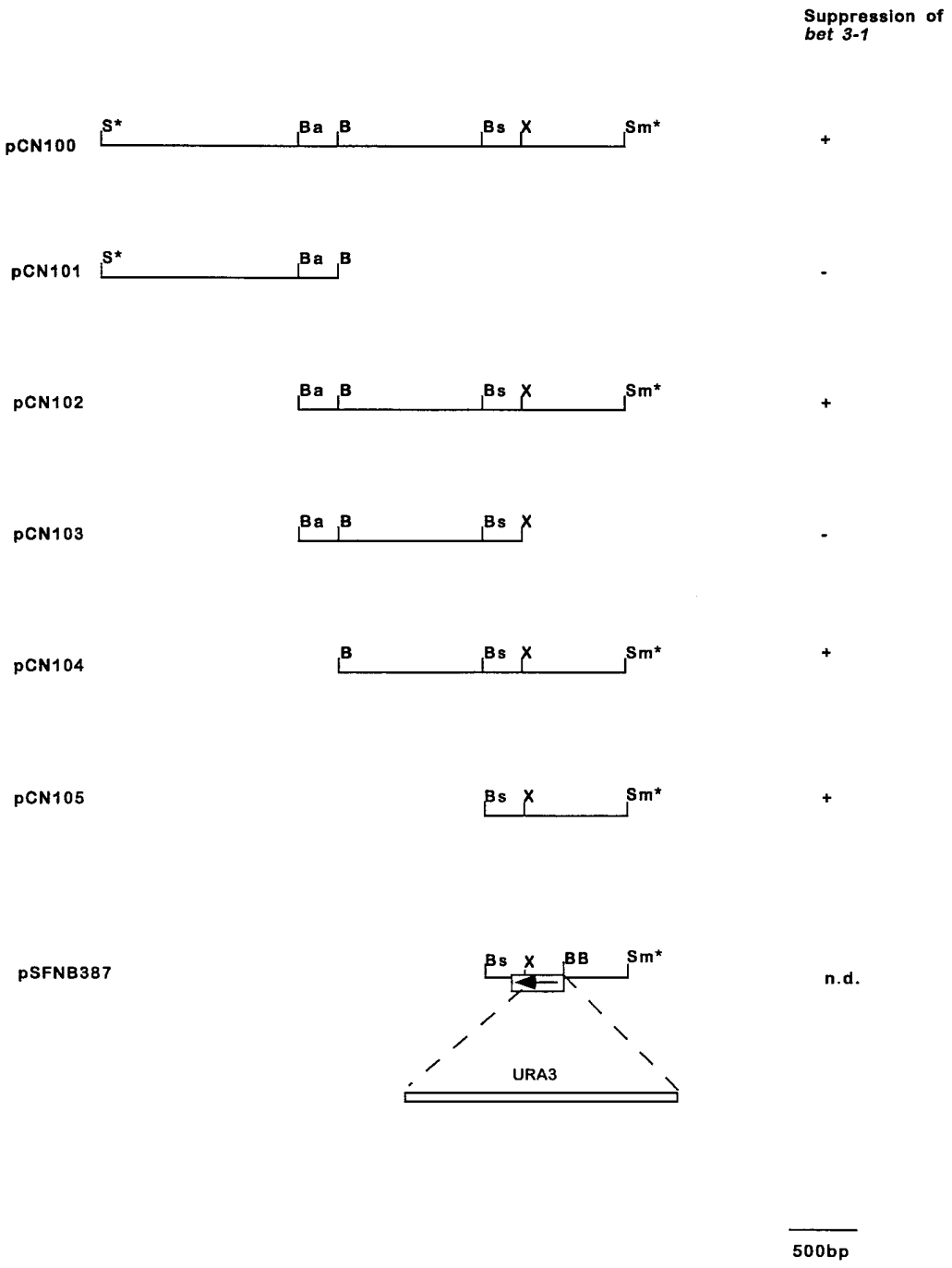

Figure 2.—*BET5* is contained within a 1.0-kb *Bsu*36I-*Sma*I fragment. S, *Sal* I; Ba, *Bam*HI; B, *Bgl* II; Bs, *Bsu*36I; X, *Xho*I; Sm, *Sma*I; BB, *Bsa*BI.

performed. Of the 48 tetrads examined, all displayed 2:2 segregation for viability. In addition, the viable spores were found to be Ura$^-$, suggesting that spores containing a disrupted copy of *BET5* could not give rise to viable colonies. Microscopic examination revealed that growth ceased in the disrupted cells after 3–4 cell divisions (8–12 cells). Thus, *BET5* is an essential gene whose product is required for the vegetative growth of yeast cells.

Yeast cells depleted of Bet5p fail to transport proteins between the ER and Golgi complex: Previous studies have shown that Bet3p is required for vesicular transport between the ER and Golgi complex. As a suppressor of *bet3-1*, we hypothesized that Bet5p may also play a role in membrane traffic at this stage of the secretory pathway. To test this notion, we determined the consequences of depleting yeast cells of Bet5p. This was done by constructing a strain (SFNY 576) in which the sole copy of *BET5* was placed under the control of the regulatable *GAL1* promoter. In glucose containing growth medium, the expression of *BET5* is repressed in this strain. Growth of SFNY 576 was reduced subsequent to a 13-hr incubation in YPD medium and ceased after 15 hr. At the 15-hr time point, no Bet5p was detected (data not shown).

To determine if Bet5p is required for intracellular

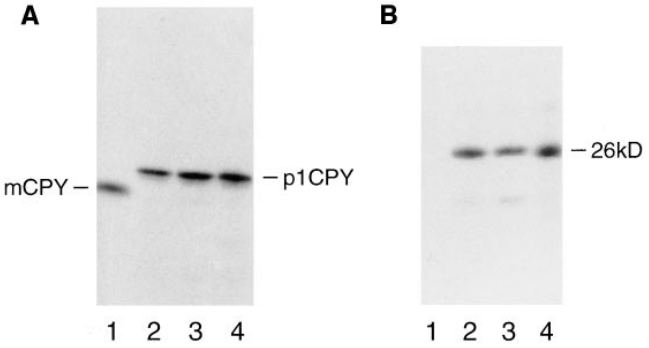

Figure 3.—Depletion of Bet5p blocks ER-to-Golgi transport. SFNY576 was grown to stationary phase in YP medium that was supplemented with 2% raffinose and 0.5% galactose. At the zero time point, cells were inoculated into YP medium containing 2% glucose and incubated at 25° for 15 hr. The *bet1-1* and *sec18-1* mutants were grown overnight in YPD medium at 25° and then incubated at 37° for 30 min. Cells were radiolabeled as described in materials and methods and incubated with anti-CPY (A) or anti-α-factor (B) antibody. The immunoprecipitates were analyzed on a 12.5% SDS gel. To compare strains, the volume of sample loaded onto the gel was proportional to the incorporation of ^{35}S label in the lysate. Lane 1, wild-type (SFNY26-6A); lane 2, *bet1-1* (ANY123); lane 3, *sec18-1* (NY432); lane 4, Bet5p-depleted cells (SFNY576).

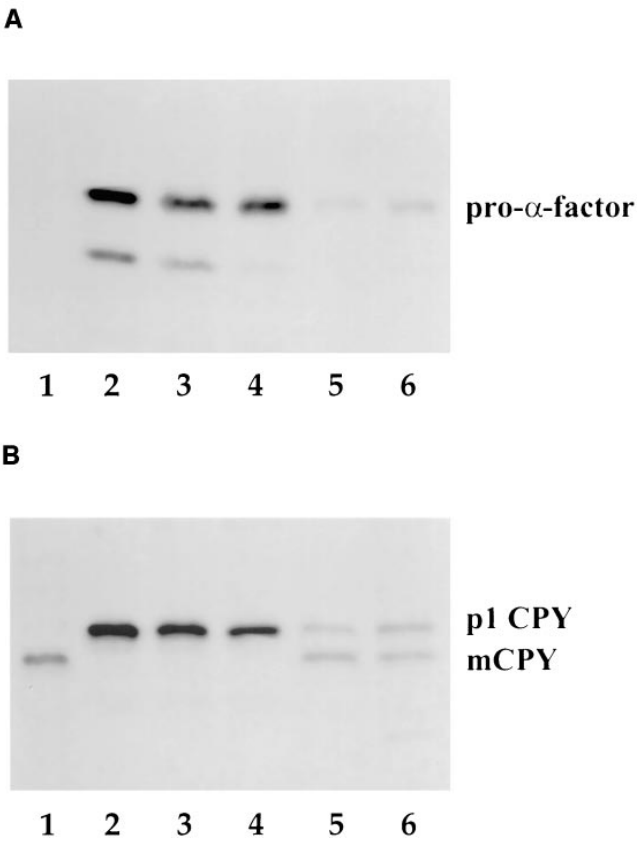

Figure 4.—The overexpression of *YPT1* and *BOS1* suppresses the secretion defect of the *bet5-1* mutant. Samples were radiolabeled, prepared as described in materials and methods, and incubated with either anti-α-factor (A) or anti-CPY (B) antibody. Lane 1, wild-type; lane 2, *sec18-1* (NY432); lane 3, *bet1-1* (ANY123); lane 4, *bet5-1* (SFNY713); lane 5, *bet5-1* with *YPT1*, 2 μm; lane 6, *bet5-1* with *BOS1*, 2 μm.

membrane traffic, we examined the transport of the vacuolar protease carboxypeptidase Y (CPY) and the yeast pheromone α-factor in SFNY576. CPY, which is initially synthesized as a 59-kD precursor, is processed to a 67-kD species (p1CPY) in the ER and then modified in the Golgi complex (p2CPY), before it is proteolytically cleaved in the vacuole to yield the mature form (mCPY) (for a review see Jones *et al.* 1997). As shown in Figure 3A, when Bet5p-depleted cells were radiolabeled with ^{35}S Trans label for 30 min, p1CPY was observed (lane 4). This form of CPY was also present in *bet1-1* and *sec18-1*, two mutants that fail to support ER-to-Golgi transport at 37° (lanes 2 and 3). The mature form of CPY was only found in wild-type cells (lane 1).

Bet5p-depleted cells also failed to secrete α-factor. A precursor form of α-factor is converted to a 26-kD species (called pro-α-factor) in the ER before it is processed in the Golgi and post-Golgi secretory vesicles to a mature form that is secreted into the medium. As shown in Figure 3B, wild type failed to accumulate α-factor (lane 1), while the 26-kD species accumulated in *bet1-1* (lane 2), *sec18-1* (lane 3) and Bet5p-depleted cells (lane 4). Thus, in the absence of Bet5p, CPY and α-factor are blocked in transit between the ER and Golgi complex.

Isolation and characterization of the *bet5-1* mutant: To address the role of Bet5p more directly, we used PCR mutagenesis (Muhlrad *et al.* 1992) to construct a ts allele of *bet5*. The *bet5-1* mutant was constructed by introducing mutagenized *BET5* DNA and a gapped plasmid (*CEN*, *LEU2*) into a strain (SFNY712) in which the genomic copy of *BET5* was disrupted by *HIS3*. SFNY712 grows in the absence, but not the presence, of glucose because it harbors a copy of *BET5* that is under the control of the inducible *GAL1* promoter (pSFN470). Leu$^+$ transformants that grew on YPD plates at 25°, but not 37°, were selected. The growth defect of one transformant was fully complemented at 37° by pSFN469 (*BET5*, *CEN*, *URA3*), indicating that this strain was ts for growth because it contained a mutant copy of *bet5*. The *bet5-1* mutant was retrieved from the mutagenized plasmid and subcloned into a vector that was integrated at the *leu2* locus of SFNY712. Plasmid pSFN470 (*GAL1-BET5*, *URA3*, *CEN*) was then displaced on 5-FOA plates to yield SFNY713.

SFNY713 was assayed for its ability to secrete α-factor and transport CPY to the vacuole. Mutant and wild-type cells, grown at 25°, were shifted to 37° for 30 min before they were incubated with ^{35}S Trans label. As shown in Figure 4, at 37°, the *bet5-1* mutant failed to process CPY (Figure 4B, lane 4) and accumulated the 26-kD ER form of α-factor (also called pro-α-factor), as well as a partially glycosylated ER form (Figure 4A, compare lane 1 with lane 4). These forms were also found in *sec18-1* (Figure 4A, lane 2) and *bet1-1* (Figure 4A, lane 3) mutant cells. Like *sec18-1* (Figure 4B, lane 2) and *bet1-1* (Figure 4B, lane 3), the CPY that accumulated in *bet5-1* was the ER

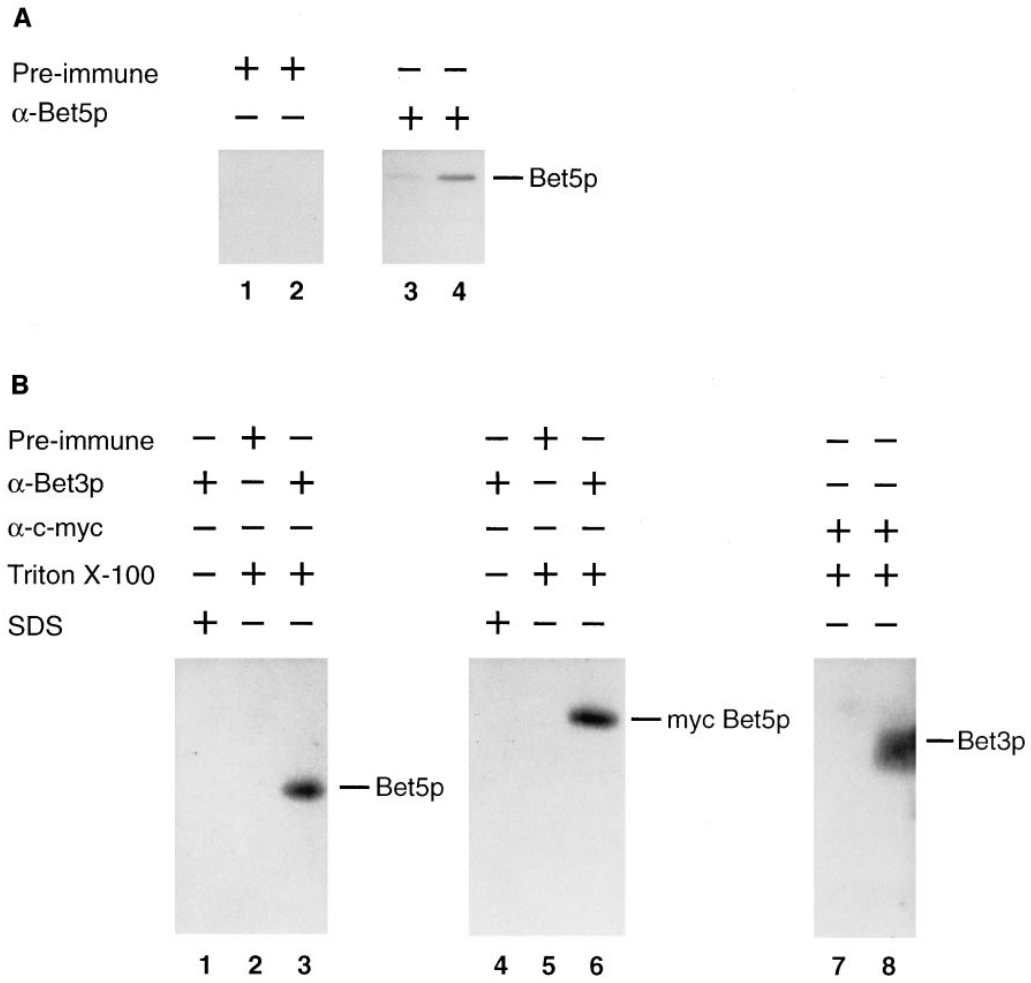

Figure 5.—Bet5p coprecipitates with Bet3p. (A) Cell extracts from wild-type yeast cells (lanes 1 and 3) or wild-type cells containing *BET5* on a 2μ plasmid (lanes 2 and 4) were electrophoresed on a 15% SDS gel and blotted with either anti-Bet5p serum (lanes 3 and 4), or preimmune serum (lanes 1 and 2). (B) Wild-type yeast cells (lanes 1, 2, 3, and 7) or cells containing c-myc-tagged *BET5* (lanes 4, 5, 6, and 8) were grown in YPD medium to early exponential phase. Cells were then converted to spheroplasts and lysed with lysis buffer containing either 1% SDS (lanes 1 and 4) or 2% Triton X-100 (lanes 2, 3, 5, 6, 7, and 8). The lysates were incubated with either anti-Bet3p antibody (lanes 1, 3, 4, and 6), preimmune serum (lanes 2 and 5), or anti-c-myc antibody (lanes 7 and 8). The precipitates were subjected to SDS gel electrophoresis (15%) and blotted with anti-Bet5p or anti-Bet3p serum. Note that the Bet3p band tends to migrate heterogeneously.

or p1 form (Figure 4B, compare lane 1 with lane 4). These findings indicate that, upon a short incubation at the restrictive temperature, *bet5-1* fails to transport proteins from the ER to the Golgi complex. These findings clearly indicate that like Bet3p, Bet5p plays a role in membrane traffic at this stage of the secretory pathway.

Bet5p coprecipitates with Bet3p: To analyze the product of the *BET5* gene, we raised polyclonal antibody to a recombinant form of Bet5p that contains a six-histidine epitope tag at its amino terminus. Anti-Bet5p antibody recognized an 18-kD polypeptide (Figure 5A, lane 3) that was overproduced in a strain that overexpresses *BET5* (lane 4). In contrast, preimmune serum failed to identify this band (lanes 1 and 2). To further characterize this antibody, we also constructed an epitope-tagged version of Bet5p (SFNY583) that contains two carboxy-terminal c-myc tags. Anti-Bet5p antibody, as well as anti-c-myc antibody, recognized this form of Bet5p which migrates at 21 kD (Figure 5B, lane 6).

Previous studies have shown that the overexpression of *YPT1*, *BET1*, *SEC22*, and *BOS1* suppressed the growth defect of *bet3-1* at 30° (Rossi *et al.* 1995), but not at 34° as *BET5* does. However, overexpression of Bet5p failed to suppress other ER-to-Golgi mutants. These findings indicate that the genetic interactions between *BET5* and *BET3* are strong and specific, implying a stable physical association between the products of these genes. To test this notion, we quantitatively precipitated a yeast cell extract with affinity-purified anti-Bet3p antibody and analyzed proteins of the precipitate with anti-Bet5p serum. As is shown in Figure 5B, Bet5p was detected in the anti-Bet3p precipitates (lane 3). Quantitation of this precipitate revealed that all of the cellular Bet5p was present. When this experiment was performed with a strain (SFNY583) in which the sole copy of Bet5p contained two c-myc tags, the 18-kD Bet5p band was shifted to 21 kD (lane 6). Bet5p did not coprecipitate with Bet3p if preimmune serum replaced anti-Bet3p antibody (lanes 2 and 5), or if the extract was denatured prior to the addition of anti-Bet3p antibody (lanes 1 and 4). Bet3p also coprecipitated with Bet5p if a cell lysate, prepared from SFNY583, was precipitated with anti-c-myc antibody (compare lane 8 with control in lane 7). These findings conclusively demonstrate that Bet5p and Bet3p associate with each other to form a complex.

High copy suppressors of the *bet5-1* mutant: We previously demonstrated that *BET3* interacts genetically with *BOS1*, *BET1*, *SEC22*, and *YPT1* (Rossi *et al.* 1995). This observation suggested that Bet3p functions in con-

TABLE 2

Summary of high copy suppression of *bet5-1*

Strains	2-μm plasmid	25°	35°	37°
w.t.	Vector	+++++	+++++	+++++
bet5-1	Vector	+++	—	—
bet5-1	*YPT1*	++++	++++	++++
bet5-1	*BOS1*	++++	++++	+++
bet5-1	*DSS4*	++++	++++	+++
bet5-1	*SEC22*	+++	+++	++
bet5-1	*BET1*	+++	+++	++
bet5-1	*USO1*	+++	+++	++
bet5-1	*ANP1*	+++	++	++

junction with v-SNAREs and the GTPase that regulates their activity (Lian *et al.* 1994; Rossi *et al.* 1995). Since Bet5p and Bet3p are components of a stable complex, we anticipated that *BET5* would display a similar pattern of genetic interactions. Indeed, as shown in Table 2, the overexpression of *BOS1*, *BET1*, *SEC22*, and *YPT1* suppressed the growth defect of the *bet5-1* mutant at 35° and 37°. *YPT1* and *BOS1* were the strongest suppressors of *bet5-1* at 35°, while *YPT1* displayed the strongest suppression at 37°. To confirm that the suppression we observed was due to the restoration of intracellular membrane traffic, we examined the transport of α-factor and CPY in *bet5-1* mutant cells overexpressing *YPT1* or *BOS1*. The overexpression of either gene partially restored the maturation of CPY (Figure 4B, compare lane 4 with lanes 5 and 6) and the secretion of α-factor, and as a consequence, less of the 26-kD form of α-factor accumulated within mutant cells (Figure 4A, compare lane 4 with lanes 5 and 6).

To identify additional genes that interact genetically with *BET5*, we screened for high copy suppressors of the *bet5-1* mutant. Of the 20,000 transformants examined, 31 grew at 37°. Plasmids retrieved from these transformants suppressed *bet5-1*, indicating that suppression was linked to the plasmid. The 12 plasmids that suppressed *bet5-1* the best were analyzed further, and the genes contained within them were identified by sequencing the ends of each insert and comparing the sequence with known sequences in the yeast database. As anticipated, *BET1*, *SEC22*, and *BET5* were among the suppressors. Several genes whose products have been implicated in membrane traffic either directly (*USO1*) or indirectly (*DSS4* and *ANP1*) were also isolated. To demonstrate that suppression was conferred by *BET1*, *SEC22*, *USO1*, *DSS4*, and *ANP1*, these genes were cloned by PCR into high copy expression vectors. The resulting plasmids were transformed into the *bet5-1* mutant and found to retain full suppression activity (Table 2), indicating that the identified genes were indeed the suppressors. It is noteworthy that we did not isolate either *YPT1* or *BOS1*, which are strong suppressors of *bet5-1*. Thus, our screen has not yet reached saturation.

Bet5p is highly conserved: We previously reported that Bet3p is homologous to a *Caenorhabditis elegans* protein of unknown function (Rossi *et al.* 1995), suggesting that Bet3p may be highly conserved. To identify homologues of Bet5p, we searched the dbEST database with TBLASTN using the yeast protein. Fifty sequences with a probability score (*P* value) of less than 0.01 were identified. Two of these sequences contained full-length coding regions for human (AA203173) and murine (AA041907) Bet5p. Pairwise alignment in Figure 6 shows that the mammalian proteins (145 amino acids) are shorter than the yeast protein (159 amino acids). In addition, the human and mouse coding regions differ only by two amino acids, while the yeast protein is 53.8% similar and 29% identical to the human homologue. These findings indicate that Bet3p and Bet5p are highly conserved proteins that are present in a variety of species.

DISCUSSION

We previously identified *BET3* by its interactions with *BET1* (Rossi *et al.* 1995), a gene whose SNARE-like prod-

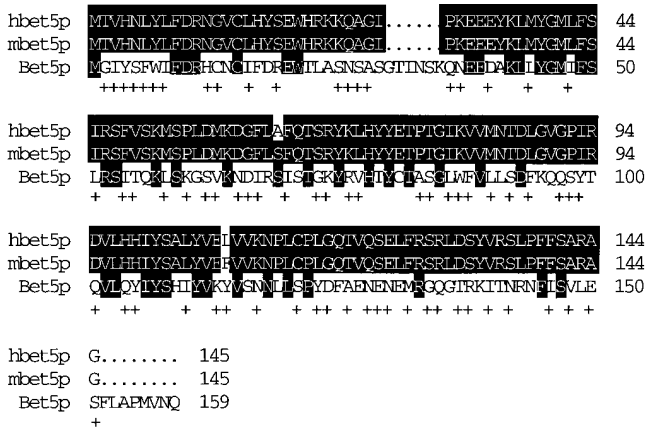

Figure 6.—Pairwise alignment of human, mouse, and yeast Bet5p. Sequences were matched with the PileUp program using the Wisconsin Genetics Computer Group (GCG) software version 8.1. Conserved amino acids that were determined by the BestFit program in GCG are marked with a +.

uct acts in ER-to-Golgi transport (Stone *et al.* 1997). The observation that a block in ER-to-Golgi transport immediately ensues upon inactivation of Bet3p implies that this protein plays a critical role in this process (Rossi *et al.* 1995). In an effort to gain insight into the function of Bet3p, we isolated high copy suppressors of the *bet3-1* mutant. This screen resulted in the identification of *BET5*, a novel gene whose product is required for ER to Golgi transport. The strong and specific interactions observed between *BET3* and *BET5* implies that their products physically interact with each other. This hypothesis was confirmed by the demonstration that Bet5p coimmunoprecipitates with Bet3p. More recently we have shown that Bet3p and Bet5p are members of a large complex (~800 kD) that includes at least eight other proteins (Sacher *et al.* 1998).

Previous studies indicate that cells lacking functional Bet3p accumulate ER and small vesicles, suggesting that this gene product acts in vesicle targeting or fusion (Rossi *et al.* 1995). This finding was corroborated by the isolation of *USO1*, *BET1*, *SEC22*, and *DSS4* as high copy suppressors of the *bet5-1* mutant. These genes all encode proteins that act at a late stage of ER-to-Golgi transport. Uso1p is a large globular cytosolic protein (206 kD) that has been proposed to tether transport vesicles to a putative receptor on the Golgi (Sapperstein *et al.* 1996; Barlowe 1997), while *BET1* and *SEC22* encode SNAREs (Sacher *et al.* 1997; Stone *et al.* 1997). Dss4p may activate the small GTP-binding protein Ypt1p by promoting nucleotide displacement (Collins *et al.* 1997). Finally, *ANP1*, which was also isolated as a high copy suppressor of *bet5-1*, encodes a 58-kD type II membrane protein that resides on the ER and is required to maintain a functional Golgi apparatus (Chapman and Munro 1994). Taken together, these findings imply that the Bet3p/Bet5p complex mediates a late stage of ER-to-Golgi transport and that the maintenance of the Golgi may be important for its function.

Although Bet3p and Bet5p are unrelated to previously identified proteins required for membrane traffic, they are highly conserved. In an earlier study (Rossi *et al.* 1995), we found that Bet3p is 36% identical to a *C. elegans* protein of unknown function. A more recent search of the dbEST database has led to the identification of a human homologue of Bet3p (Sacher *et al.* 1998) and Bet5p (Figure 6). Human Bet3p (Hbet3p) is 180 amino acids and is highly homologous (54% identity, 72% similarity) to its yeast counterpart, while human and mouse homologues of Bet5p are ~29% identical to the yeast protein. This finding reinforces the notion that the role of the Bet3p/Bet5p complex in membrane traffic is highly conserved. Further characterization of Bet3p and Bet5p in both yeast and mammalian cells will enable us to determine precisely how these proteins function together to mediate a late stage in ER-to-Golgi transport.

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