TRAPP, a highly conserved novel complex on the cis-Golgi that mediates vesicle docking and fusion

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We previously identified BET3 by its genetic interactions with BET1, a gene whose SNARE-like product acts in endoplasmic reticulum (ER)-to-Golgi transport. To gain insight into the function of Bet3p, we added three c-myc tags to its C-terminus and immunopurified this protein from a clarified detergent extract. Here we report that Bet3p is a member of a large complex (~800 kDa) that we call TRAPP (transport protein particle). We propose that TRAPP plays a key role in the targeting and/or fusion of ER-to-Golgi transport vesicles with their acceptor compartment. The localization of Bet3p to the cis-Golgi complex, as well as biochemical studies showing that Bet3p functions on this compartment, support this hypothesis. TRAPP contains at least nine other constituents, five of which have been identified and shown to be highly conserved novel proteins.

Keywords: membrane traffic/novel complex/vesicle targeting and fusion

Results

Bet3p is a component of a large complex
To begin to address the function of Bet3p, we added three c-myc tags to the C-terminus of this protein and then precipitated the associated proteins from a radiolabeled lysate with anti-c-myc antibody. Figure 1A demonstrates that in addition to Bet3p (~27 kDa with three c-myc tags), nine radiolabeled bands were specifically precipitated. These bands were only precipitated from a lysate that contained tagged Bet3p (compare lane 2 with lane 1 in Figure 1A), and co-purified during size exclusion and ion exchange chromatography (Figure 1B). The Bet3p-associated proteins included several low molecular weight polypeptides (18, 20, 23 and 33 kDa) and a 31 kDa species (see starred band below p33 in Figure 1B) that was not well resolved from the 33 kDa polypeptide. In addition, five high molecular weight bands (65, 85, 105, 120 and 130 kDa) were present. We determined, from gel filtration analysis, that none of the cellular Bet3p was monomeric, but instead was present with the radiolabeled

In yeast, analogs of NSF and α-SNAP are encoded by the SEC18 (Wilson et al., 1989) and SEC17 genes (Griff et al., 1992), respectively, while SNAREs on the vesicle and target membrane (t-SNAREs) can be found at every stage of the pathway. Using the yeast Saccharomyces cerevisiae as a model system, we have been studying the mechanism by which endoplasmic reticulum (ER)-derived transport vesicles target and fuse with the Golgi apparatus. In ER-to-Golgi transport, the v-SNAREs, Bos1p and Sec22p (Lian et al., 1994), bind to a domain on Sed5p (Sacher et al., 1997) that is homologous to syntaxin (Hardwick and Pelham, 1992). These v/t-SNARE interactions are potentiated by a third SNARE, Bet1p, that contains a domain which is homologous to SNAP-25 (Stone et al., 1997). The small GTP-binding protein Ypt1p (Rab1 in mammalian cells) acts upstream of these events (Dascher et al., 1991; Lian et al., 1994; Søgaard et al., 1994).

Bet3 encodes a 22 kDa hydrophilic protein that previously was identified in a synthetic lethal screen with the bet1-1 mutant (Rossi et al., 1995). While Bet3p interacts genetically with SNAREs, this gene product is not part of the SNARE complex that forms at 37°C in sec18 mutant cells (Rossi et al., 1995). Here we show that Bet3p is a component of a large complex, called TRAPP, which is highly conserved from yeast to man. This complex includes Bet5p, identified as a high-copy suppressor of the temperature-sensitive bet3-1 mutant (Jiang et al., 1998), and at least nine other proteins. TRAPP resides on the cis-Golgi complex where it acts prior to SNARE complex assembly.
bands in a complex of ~800 kDa that we call TRAPP (transport protein particle).

The Bet3p-associated proteins were purified from the c-myc-tagged strain by precipitating the complex from 1.2 g of lysate. This was achieved by incubating a clarified detergent extract with affinity-purified immunoglobulin (IgG) that was bound to Affigel. The bound protein was then eluted at low pH and analyzed by SDS–PAGE. A control strain that did not contain the tag was treated in the same way. Bands of the appropriate molecular weight, which were not observed in the control, were excised from a Coomassie Blue-stained gel, digested with trypsin and analyzed by mass spectrometry. The results of this analysis are presented in Table I.

We found that the low molecular weight bands immunopurified more efficiently than the higher molecular weight species and, as a consequence, sufficient protein was obtained to identify the smaller constituents of the complex. The p20 subunit was identified as YBR254c, p23 as YDR246w and p33 as YOR115c. These three open reading frames (ORFs) predicted proteins of the appropriate molecular weight. The gene encoding p18, BET5 (YML077w), was identified previously as a high-copy suppressor of the bet3-1 mutant (Jiang et al., 1998). The only peptides obtained for p31 (Figure 1B, starred band below p33) were derived from YOR115c, suggesting that the 31 kDa band may be a breakdown product of p33. However, we cannot exclude the possibility that p31 is a distinct, but incompletely resolved, protein. By knowing the cysteine and methionine content of the smaller subunits, we were able to estimate their stoichiometry by quantitating the radiolabeled bands shown in Figure 1A on a phosphorimager. This analysis revealed that these subunits are present in the complex in approximately equimolar amounts. All of the identified subunits are hydrophilic, but none are homologous to known proteins in the yeast database.

Table I. Peptides identified by mass spectrometry of TRAPP subunits

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Sequence name</th>
<th>Peptides identified</th>
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<tbody>
<tr>
<td>p20</td>
<td>YBR254c</td>
<td>(K)DNPVYEIEFTANPQGPQPQLK (M)PQYPAIGK (R)SFQYEHELHVK</td>
</tr>
<tr>
<td>p23</td>
<td>YDR246w</td>
<td>(M)AIETILINK (K)ALQLTQIENNTIPYVPRASNNR (K)LNSNEYLIALSLHGVFAIASQTPK (K)ISGLIYOR (R)SNLFDEK</td>
</tr>
<tr>
<td>p33</td>
<td>YOR115c</td>
<td>(R)AQQFOFITENSLPK (R)GTFYLLDYDPQPSFLEDAK (R)GTFYLLDYDPQPSFLEDANL (K)IEEHTVDIR (K)ILSELLKSNPPL (K)MIPEPFLPVIGIR (R)QISGDISSDNSVTSENGNINMK (R)RSHNHHLYK (R)RSHNHHLYKADVKEEK (K)VSQSVYQKMLNEMVPLMG</td>
</tr>
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Like BET5, p20, p23 and p33 are high-copy suppressors of the bet3-1 mutant

The overexpression of BET5 (p18) was shown previously to suppress bet3-1, but not other mutants blocked in ER-to-Golgi transport (Jiang et al., 1998). A prediction of these earlier findings is that other subunits of TRAPP may also act as high-copy suppressors of the temperature-sensitive bet3-1 mutant. To test this hypothesis, we cloned the genes that encode each of the newly identified subunits (p20, p23 and p33) into a high-copy vector and tested their ability to suppress bet3-1. Like BET5, the overexpression of p23 was found to suppress bet3-1 at 30°C and 34°C, while p20 and p33 suppressed this mutant at 30°C (Figure 2A). The growth defect of a temperature-sensitive mutant in p18 (bet5-1), that blocks transport from the ER to the Golgi complex (Jiang et al., 1998), was also suppressed by the overexpression of p33 (our unpublished data). As was found for BET5, p20, p23 and p33 did not suppress other ER-accumulating mutants (sec12, sec13, sec16, sec17, sec18, sec21, sec22, sec23, bos1 and bet1).
conclude that the specific suppression displayed by these genes is indicative of the fact that they encode components of the same complex.

**Membrane-bound Bet3p resides on the cis-Golgi complex**

When metabolically active yeast spheroplasts were lysed in a buffer that supports vesicular transport in vitro, most of the cellular Bet3p was present on membranes (Figure 5D, compare lanes 2–4). To localize membrane-bound Bet3p, we fused Bet3p to the green fluorescent protein (GFP) (Prascher et al., 1992; Chalfie et al., 1994). The Bet3p–GFP fusion protein that we constructed is functional, as it complemented the bet3-1 mutant at 37°C and supported the growth of yeast cells that lacked the BET3 gene. When we examined wild-type living cells that contained the Bet3p–GFP fusion protein by confocal microscopy, brightly stained punctate structures were found throughout the cytoplasm (Figure 3A). These structures were not visible when the fusion protein was absent (Figure 3D). Furthermore, GFP alone exhibited a cytoplasmic staining pattern (Figure 3C), indicating that Bet3p targets the fusion protein to the punctate structures.

The fluorescence pattern of Bet3p–GFP resembles that of the cis-Golgi marker Sed5p (Banfield et al., 1994) and not the resident ER protein Kar2p (yeast BiP) (Rose et al., 1989). Sed5p is found in punctate structures throughout the cytoplasm (Hardwick and Pelham, 1992), while Kar2p stains the nuclear envelope and ribbon-like structures at the cell periphery (see Figure 3E as an example). Attempts to demonstrate that Bet3p and Sed5p co-localize to the same compartment by double labeling were hampered as the usual fixation protocols resulted in a background of autofluorescence that masked the GFP signal. To show that Bet3p resides on the Golgi, we took advantage of the observation that the Golgi forms stacks in sec7 mutant cells that have been shifted to 37°C in low glucose-containing medium (Novick et al., 1981). If the Bet3p–GFP fusion protein resides on the Golgi, the punctate structures that we observed should become larger and less numerous in the sec7 shifted cells. Similar experiments have been used before to show that Ypt1p resides on the Golgi complex in yeast (Segev et al., 1988; Brennwald and Novick, 1993). When the Bet3p–GFP fusion was localized in sec7 mutant cells subsequent to a 2 h incubation at 37°C in YP medium containing 0.1% glucose, fewer and larger punctate structures were observed (Figure 3B). At 25°C, these structures were smaller and more numerous, as in wild-type (not shown). Thus, Bet3p appears to be associated with the Golgi.
Novel complex in ER-to-Golgi transport

Fig. 3. A Bet3p–GFP fusion protein localizes to punctate structures. The images shown were obtained on a Bio-Rad confocal microscope. (A) Wild-type cells containing the Bet3p–GFP fusion protein (SFNY696). (B) sec7-1 mutant cells with the Bet3p–GFP fusion protein (SFNY710) after a 2-hour incubation at 37°C in 0.1% glucose. (C) Wild-type cells with GFP under the control of the BET3 promoter (SFNY709). (D) Wild-type cells without the GFP fusion protein. (E) Wild-type cells with anti-Kar2 antibody.

We also found that sec7-1 mutant cells (Figure 3B) were slightly larger than wild-type (Figure 3A). This difference is likely to be due to the strain background, since mutant cells grown at 25°C were also larger.

Subcellular fractionation studies were performed to determine the subcompartment of the Golgi that contains Bet3p. In the protocol we employed, a homogenate was centrifuged to yield P2 (10,000 g pellet) and P3 (100,000 g pellet) fractions that were then subfractionated on sucrose step gradients. The location of medial- and trans-Golgi subcompartments was followed by enzyme assays for GDPase (Abeijon et al., 1989) and Kex2p (Cunningham and Wickner, 1989), respectively, while the cis-Golgi marker Sed5p (Banfield et al., 1994) and Bet3p were monitored on Western blots. Sed5p and Bet3p were distributed equally between the P2 and P3 fractions, while most of the GDPase (74 versus 26% in P2) and Kex2p (67 versus 17% in P2) were found in the P3 fraction. Bet3p, in the P2 fraction, co-fractionated with Sed5p on sucrose gradients (Figure 4C) and was clearly separated from the trans- (Figure 4B) and medial-Golgi markers (Figure 4A). The same result was obtained when the P3 fraction was subfractionated.

Bet3p is required for the targeting/fusion competence of the Golgi complex

To begin to address the function of TRAPP, we used an in vitro transport assay that was developed in our laboratory (Ruohola et al., 1988; Grosech et al., 1990; Lian and Ferro-Novick, 1993). In this assay, the 26 kDa form of α-factor marks vesicles that bud from the ER retained within permeabilized yeast cells (PYCs). The vesicles formed in vitro do not bind and fuse with post-ER
membranes retained within the PYCs, instead they are released from cells to fuse with exogenously added Golgi. In the Golgi, the 26 kDa form of α-factor is converted to a heterogeneous high molecular weight product. Conversion to high molecular weight α-factor is measured with an antibody (anti-outer chain) that specifically recognizes outer chain carbohydrate which is only added to yeast glycoproteins in the Golgi complex. In our assay, soluble factors and Golgi membranes are provided by an S1 fraction (1000 g supernatant). These factors are then separated during high-speed centrifugation. Resolution of cytosolic factors (HSS) from Golgi (HSP) and donor membranes (PYCs) makes it feasible to assess the transport activities of each of these compartments in vitro.

One way of assessing the function of a protein in vitro is to test the consequences of depletion. We depleted cells of Bet3p (Figure 5D, compare lanes 5–8 with lanes 1–4) by inhibiting its synthesis in a strain (SFNY431) whose sole copy of BET3 was placed under the control of a regulatable promoter, and then prepared fractions for in vitro analysis. Bet3p-depleted cytosol (Figure 5A, lane 2, and 5B, lane 4) or PYCs (not shown) were found to support all stages of ER-to-Golgi transport, budding (Figure 5A, lanes 1 and 2) as well as fusion (Figure 5B, lanes 3 and 4), in an ATP-dependent manner (Figure 5A, lane 4, and B, lane 1). However, Bet3p-depleted Golgi failed to support the consumption of vesicles when assayed in the presence of depleted cytosol (Figure 5B, compare lane 5 with positive control in lane 3 and negative controls in lanes 1 and 2), although vesicle budding occurred normally under these conditions (Figure 5A, lane 3). Activity was partially restored (Figure 5C, compare lane 1 with 2) when wild-type cytosol, which contains small amounts of Bet3p (Figure 5D, compare lane 3 with lanes 2 and 4), was incubated with Bet3p-depleted Golgi. Thus, the depleted acceptor compartment was functional providing it was supplemented with cytosol that contained Bet3p. Formal proof that reconstitution was dependent upon Bet3p will require the addition of purified native TRAPP.

The overexpression of SED5 and the SLY1-20 mutant suppresses bet3-1 at 30°C

Previous studies have shown that the overexpression of v-SNAREs (Bos1p/Sec22p), as well as genes that influence the activity of the v-SNARE (BET1, YPT1), suppresses bet3-1 (Rossi et al., 1995; Stone et al., 1997). In light of the localization data and functional studies presented here, we tested the ability of the t-SNARE gene, SED5, and a gain-of-function mutant (SLY1-20) in the t-SNARE-associated protein gene SLY1 (Dascher et al., 1991) to suppress the growth defect of bet3-1. The ability of SEC17 and SEC18 to act as high-copy suppressors of this mutant was also assessed. This analysis revealed that SED5 and SLY1-20 suppressed bet3-1 at 30°C (Figure 1B and C), but not at higher temperatures. Suppression by SED5 was only observed when this SNARE gene was expressed under the control of the strong promoter, TP1. In contrast, SLY1-20 suppressed bet3-1 when present on a low-copy CEN plasmid, while the overexpression of SEC17 or SEC18 failed to suppress this mutant at any temperature. Thus, BET3 displays genetic interactions with genes that encode SNAREs, or play a role in assembling the SNARE

Fig. 5. Bet3p is required for the transport activity of the acceptor Golgi apparatus. (A) Bet3p is dispensable for vesicle budding. Budding assays were performed in the absence (lanes 1–3) or presence of apyrase (lane 4). The release of vesicles (marked with pro-α-factor that is precipitated with Con A–Sepharose) from wild-type PYCs was assayed in the presence of wild-type cytosol (HSS) and Golgi (HSP) (lanes 1 and 4), Bet3p-depleted cytosol and wild-type Golgi (lane 2), and Bet3p-depleted cytosol with Bet3p-depleted Golgi (lane 3). (B) Transport vesicles fail to be consumed in the absence of Bet3p. This event was assessed by SDS–gel analysis. Wild-type PYCs were assayed with wild-type cytosol (HSS) and Golgi (HSP) in the absence (lane 3) or presence of apyrase (lane 1) or with 7 μg of affinity-purified anti-Bos1p antibody (lane 2). In wild-type, the 26 kDa form of α-factor (three N-linked oligosaccharides) and a minor species (24 kDa) with two N-linked oligosaccharide chains was converted to the high molecular weight product. The remainder of the samples are as follows: lane 4, Bet3p-depleted cytosol (HSS) and wild-type Golgi (HSP); lane 5, Bet3p-depleted cytosol (HSS) and Bet3p-depleted Golgi (HSP). (C) Wild-type cytosol partially restores activity to Bet3p-depleted Golgi. This result is expressed as the percentage maximal anti-outer chain-precipitable counts of wild-type, where wild-type, which is shown in lane 3 of (B), is 100%. Lane 1, wild-type PYCs were assayed with Bet3p-depleted cytosol and Bet3p-depleted Golgi or with wild-type cytosol and Bet3p-depleted Golgi (lane 2). (D) Western blot analysis was used to assess the distribution of Bet3p in fractions assayed for transport. The fractions (200 μg) were as follows: PYCs (lanes 1 and 5); S1 (lanes 2 and 6); HSS (lanes 3 and 7); and HSP (lanes 4 and 8). These fractions were prepared from either wild-type (lanes 1–4) or Bet3p-depleted cells (lanes 5–8) used in the assay. The distribution of the cis-Golgi marker, Sed5p, was found to be unchanged in the Bet3p-depleted cells (not shown).
complex at the target membrane (Lian et al., 1994; Søgaard et al., 1994; Rossi et al., 1995).

**The known components of TRAPP are highly conserved**

We reported previously 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 additional homologs of Bet3p, we performed a BLAST search of the expressed sequence tag database (dbEST) at the NCBI (National Center for Biotechnology Information). This search identified a cDNA sequence (343 bp) from a human placental library that encodes a peptide (93 codons) which is 47% identical to a region of Bet3p. The cDNA was amplified by PCR and used to isolate a full-length clone from the same human placental cDNA library. Seven clones were obtained, six of which were ~1.3 kb in length, while the seventh was 2.5 kb. DNA sequence analysis indicated that six of the clones were derived from a common mRNA. The seventh clone was the product of unspliced mRNA that was reverse transcribed during the construction of the library. The 1.3 kb cDNA encoded an ORF of 180 codons (DDBJ/EMBL/GenBank accession No. AJ224335) that was 54% identical and 72% similar in overall sequence to yeast Bet3p (Figure 6C).

A search of dbEST using yeast p20, p23 and p33 revealed multiple sequences from human and murine sources with homologies to the yeast proteins. Contiguous EST sequences were assembled, and full-length human homologs were identified for p20 (Figure 6A), p23 (Figure 6B) and p33 (Figure 6D). Identities between these mammalian and yeast subunits ranged from 31.8 to 41% and similarities ranged from 41.1 to 57% (see legend to Figure 6). A human homolog of *BET5* (p18) which is 29% identical and 53.8% similar to the yeast protein has been reported previously (Jiang et al., 1998). The extremely high evolutionary conservation between the yeast and mammalian subunits indicates that TRAPP plays a critical role in the secretory pathway in both yeast and man.

**Discussion**

Several lines of evidence implicate TRAPP as a key player in the late stages of ER-to-Golgi transport. Fluorescence studies and subcellular fractionation experiments indicate that Bet3p resides on the Golgi and co-localizes with the t-SNARE Sed5p, a *cis*-Golgi marker.
The association of Bet3p with the cis-Golgi is in accord with the notion that TRAPP may target vesicles to this membrane. This proposal is corroborated by in vitro findings demonstrating that Bet3p is required for vesicle targeting and fusion, but not budding. Bet3p appears to function on the Golgi, as the loss of Bet3p activity from otherwise functional Golgi membranes specifically abolishes the transport competence of this compartment in vitro. Finally, the known components of TRAPP are highly conserved, and antibodies to the human homolog of Bet3p have shown that this protein is in a large complex that is similar in size to yeast TRAPP (J.Barrowsman and S.Ferro-Novick, unpublished data).

Previous studies have demonstrated that the overexpression of v-SNAREs (Bos1p/Sec22p), as well as genes that influence the activity of the v-SNARE (BET1, YPT1), suppress bet3-I (Rossi et al., 1995; Stone et al., 1997). Here, we show (Figure 2B and C) that the t-SNARE gene, SED5, and a gain-of-function mutant (SLYI-20) in the t-SNARE-associated protein gene SLY1 suppress the growth defect of bet3-I. Taken together, these genetic findings indicate that Bet3p acts upstream of the SNAREs. Consistent with this proposal is the finding that the SNARE complex fails to form at 37°C in bet3-I (Rossi et al., 1995) and bet5-I (J.Burston and S.Ferro-Novick, unpublished data) mutant cells.

Many of the genes that suppress bet3-I (Rossi et al., 1995; Figure 2B and C) and bet5-I (Jiang et al., 1998)
also suppress the temperature-sensitive uso1-1 mutant (Sapperstein et al., 1996). Uso1p, which is larger than any of the subunits of TRAPP (206 kDa), is a cytoplasmic factor that may tether transport vesicles to the Golgi apparatus (Nakajima et al., 1991; Barlowe, 1997). Like TRAPP, it acts prior to SNARE complex assembly (Sapperstein et al., 1996). Therefore, TRAPP and Uso1p may function together to mediate a common step in ER-to-Golgi transport. In support of this hypothesis is the recent finding that the overexpression of USO1 suppresses the growth defect of the bet5-1 mutant (Jiang et al., 1998). Since Uso1p is soluble, it may interact with proteins on the transport vesicle as well as a specific factor or complex on the cis-Golgi, such as TRAPP. As a receptor for Uso1p, TRAPP may play a role in directing or trapping transport vesicles to specific sites on the acceptor compartment. Alternatively, Uso1p and TRAPP may each act independently to target vesicles to the cis-Golgi apparatus. Experiments currently in progress should enable us to address these possibilities.

Earlier studies on BET3 suggested a role for its product at multiple stages of the secretory pathway. This hypothesis was based largely on the fact that bet3-1 displays synthetic lethal interactions with certain late-acting sec mutants (i.e. sec2 and sec4) that are blocked in membrane traffic from the Golgi to the cell surface (Rossi et al., 1995). Recent observations, however, have led us to conclude that these synthetic lethal interactions do not reflect an involvement of Bet3p in post-Golgi secretion. Fluorescence studies have shown that membrane-bound Bet3p is associated exclusively with punctate structures and not with the plasma membrane. In addition, the overexpression of SEC2 or SEC4 (as well as of SEC9 and SEC13) cannot suppress the growth defect of the bet3-1 mutant (our unpublished observations), while a number of genes whose products are required for ER-to-Golgi transport can perform this function (Rossi et al., 1995; Figure 2B and C). Thus, as Bet3p acts at the Golgi, the combination of the bet3-1 mutation with certain post-Golgi secretory mutations may aggravate defects in membrane traffic indirectly and lead to cell death. This situation may be comparable with the endocytic/vacuolar transport pathways where genes whose products act in different processes on the same pathway display synthetic lethal interactions with each other (Singer-Krüger and Ferro-Novick, 1997).

While members of the ER-to-Golgi SNARE complex are homologous to their counterparts in other transport events (Ferro-Novick and Jahn, 1994), the five known constituents of TRAPP do not share sequence similarity with other components of the secretory apparatus, including the exocyst, a multiprotein complex thought to be required exclusively for post-Golgi secretion in yeast and mammalian cells (Hsu et al., 1996; TerBush et al., 1996; Guo et al., 1997). The exocyst has been implicated in the targeting of secretory vesicles to the tips of small buds where membrane fusion takes place (TerBush and Novick, 1995). Analogs that function at other stages of the secretory pathway have not been found. We hypothesize that the SNAREs are homologous to each other because the mechanism of endoplasmic membrane fusion is highly conserved at each transport step. However, a putative component of the vesicle targeting machinery that specifically recognizes and binds to only one type of vesicle may not resemble other vesicle targeting receptors that act at a different stage of membrane traffic. Further purification of TRAPP will lead to the identification of the other subunits and, in combination with functional studies, will enable us to probe its proposed role in the targeting of vesicles to the Golgi apparatus.

Materials and methods

Construction of a yeast strain containing epitope-tagged BET3

To construct triple c-myc-tagged Bet3p, two c-myc epitopes were inserted by site-directed mutagenesis into plasmid pGR15 (Rossi et al., 1995) to yield a protein with the following C-terminus: IGEDAEQKLISEEDLA-EQKLISEEDLAEQKLISEEDLA. After confirming that the mutagenesis was correct by DNA sequence analysis, this construct was used to replace the wild-type BET3 gene in SFNY26-3A by the pop-in/pop-out method (Guthrie and Fink, 1991). Yeast containing triple-tagged Bet3p as the sole copy of BET3 (SFNY656) showed the same growth properties as wild-type.

In vivo labeling and immunoprecipitations

For c-myc precipitations, 30 μl of cells at OD600, radiolabeled in 15 μl with 100 μCi of ProMix/ml for 2 h at 25°C, were converted to spheroplasts and lysed in 0.6 ml of lysis buffer [20 mM HEPES (pH 7.2), 150 mM KCl, 0.5 mM dithiothreitol (DTT), 2 mM EDTA, 1× protein inhibitor cocktail (PIC)]. The cell lysate was centrifuged at 100,000 g for 1 h, and the radiolabeled supernatant (A) (20 mM HEPES pH 7.4, 150 mM KCl, 0.5 mM DTT, 2 mM EDTA, 0.5% Triton X-100 and 1× PIC; Ruohola et al., 1988) to 50×10^5 c.p.m./ml. The supernatant was pre-cleared during a 1 h incubation with protein A-Sepharose beads and then the sample was transferred to a new tube containing 2 μl of 9E10 (anti-c-myc epitope) ascites fluid. The antigen–antibody complexes that formed during a 2 h incubation (or overnight) at 4°C were precipitated onto protein A-Sepharose beads (1 h at 4°C). The beads were washed three times with buffer B (20 mM HEPES pH 7.4, 500 mM KCl, 0.5 mM DTT, 2 mM EDTA, 0.5% Triton X-100, 1× PIC) and three times with buffer A. After the final wash, the samples were heated in sample buffer and analyzed on a 15% SDS–polyacrylamide gel. To immunoprecipitate the membrane-bound form of the complex, the 100 000 g pellet (described above) was extracted with 2% Triton X-100 and then centrifuged at 100 000 g for 30 min. The resulting supernatant was immunoprecipitated as above. The soluble and membrane-bound forms of the complex appeared to contain the same constituents.

Purification of TRAPP

SFNY656 (myc Bet3p) and SFNY26-3A (wild-type) were converted to spheroplasts, lysed in buffer C (20 mM HEPES pH 7.4, 150 mM KCl, 0.5 mM DTT, 2 mM EDTA, 2% Triton X-100 and 1× PIC) and centrifuged at 100,000 g for 1 h. The high-speed supernatant (1.2 g) was diluted with buffer D (20 mM HEPES pH 7.4, 500 mM KCl, 0.5 mM DTT, 2 mM EDTA, 0.5% Triton X-100, 1× PIC) and three times with buffer A. After the final wash, the samples were heated in sample buffer and analyzed on a 13% SDS–polyacrylamide gel. To immunoprecipitate the membrane-bound form of the complex, the 100 000 g pellet (described above) was extracted with 2% Triton X-100 and then centrifuged at 100 000 g for 30 min. The resulting supernatant was immunoprecipitated as above. The soluble and membrane-bound forms of the complex appeared to contain the same constituents.

Microelectrospray high performance liquid chromatography

Microelectrospray columns were constructed from 360 μm o.d.×100 μm i.d. fused silica capillary with the column tip tapered to a 5–10 μm opening. The columns were packed with Perseptive Biosystems (Framingham, MA) POROS 10 R2, a 10 mm reversed-phase packing material, to a length of 10–12 cm. The flow through the column was split pre-column to achieve a flow rate of 150 nl/min. Typically, the flow from the HPLC pumps was 150 nl/min. The mobile phase used for
gradient elution consisted of (A) 0.5% acetic acid and (B) acetonitrile/water 80:20 (v/v) containing 0.5% acetic acid. The gradient was linear from 0 to 40% B in 50 min followed by 40–80% B in 10 min or 0–60% B in 30 min. Mass spectra were recorded on a TSQ700 (Finnigan, San Jose, CA) equipped with an electrospray ionization source. Electrospray was performed by setting the needle voltage at 1.6 kV. Tandem mass spectra were acquired using an Instrument Control Language (ICL) as described previously (McCormack et al., 1997).

Database searching
Amino acid sequence databases were searched directly with tandem mass spectra using the computer algorithm, SEQUEST, described previously (Eng et al., 1994; Yates et al., 1995). The S.cerevisiae sequence database (7499 entries) was obtained from the Stanford yeast sequencing project (http://genome-stanford.edu/).

Fluorescence
A Bet3p–GFP fusion was constructed in several steps. First, using PCR, the first 10 bases from the 3'-untranslated region of BET3 were fused behind the stop codon of GFP. Second, using PCR extension, fusion was inserted into plasmid pSFN515 (SEN, LEU2) subsequent to the removal of a 0.63 kb HinIII fragment containing the BET3–GFP fusion was inserted into plasmid pSFN515 (SEN, LEU2) subsequent to the removal of a 0.63 kb HinIII–HindIII fragment that contains the BET3 gene. The resulting plasmid (pSFN516) was then transformed into a diploid strain in which one copy of BET3 was disrupted by URA3. The diploid strain was sporulated and tetrad analysis was performed. A Leu^+ Ura^- haploid colony (SFNY696) was used in the fluorescence studies. A sec7 strain containing the fusion protein was constructed by crossing SFNY696 to the sec7-1 mutant and then performing tetrad analysis. A Leu^- Ura^- colony that was temperature-sensitive for growth was selected for the experiment shown in Figure 3. For fluorescence studies, yeast cells were grown to early log phase and then directly examined on a Bio-Rad confocal microscope.

Subcellular fractionation and enzyme assays
Subcellular fractionation was performed as before (Newman et al., 1992) with 600 units of cells (SFNY726-3A) at OD_{690}, which were converted to spheroplasts and lysed in 8 ml of lysis buffer (20 mM triethanolamine pH 7.2, 1 mM EDTA, 0.8 M sorbitol). The unbroken cells (P1) were separated from the lysate during centrifugation at 450 g for 3 min. The resulting SI fraction was then centrifuged at 10 000 g for 10 min to generate pellet (P2) and supernatant (S2) fractions. The P3 (pellet) and S3 (supernatant) fractions were prepared by centrifuging the S2 fraction for 1 h at 100 000 g; and then sucrose density centrifugation was performed on the P2 and P3 fractions. Each fraction was homogenized in 55% (w/w) sucrose that was buffered with 20 mM HEPES (pH 7.2) and then placed at the bottom of a 12 ml Beckman pollyallomer tube (14×89 mm). Sucrose solutions (w/w) buffered with 20 mM HEPES (pH 7.2) were layered as follows: 1 ml 50%, 1 ml 47.5%, 1.5 ml 45%, 1.5 ml 42%, 1.5 ml 40%, 1 ml 37.5%, 1 ml 35% and 1 ml 30%. The samples were centrifuged in an SW41 rotor for 16 h at 170 000 g and 0.5 ml fractions were collected from the top of the gradient. GDPase (Abeijon et al., 1989) and Kex2p assays (Cunningham and Wickner, 1989) were performed as described previously, and Sed5p as well as Bet3p were localized by Western blot analysis using the ECL method (Amersham).

In vitro transport assay and preparation of fractions
To deplete cells of Bet3p, SFNY431 [MATa, Gal^+, ura3-52, leu2-3, 112, bet3A::URA3, pGR10 (GAL1-BET3, LEU2)] was grown to stationary phase in YP medium with 2% raffinose and 0.5% galactose and then diluted to an OD_{690} = 0.01 into YP medium containing 2% glucose. After 11 h, the cells were harvested and fractions were prepared for the transport assay. The assay, and the preparation of fractions, was performed as described previously (Ruohola et al., 1988; Groesch et al., 1990; Lian and Ferro-Novick, 1993).

Cloning the human homolog of BET3
Using the Bet3p sequence, a BLAST search of the dbEST database (Altschul et al., 1990) yielded a match with an EST from a human placental cDNA library (Strategene). This partial cDNA was amplified by PCR using the following primer set (5' to 3'): sense primer GGC ACC GAG AGC AAG AAA ATG AGC, antisense primer CCG AAC TAC TTC ATG ACC ACC. The PCR products were then used as a probe to screen ~450 000 bacteriophage plaques from a Clontech human placental library (catalog # HL0514) by the protocol supplied by the manufacturer. Through three or four rounds of subsequent purification, seven clones were purified until all plaques on the plate were positive. Restriction enzyme digestion indicated that six of the clones contained inserts of 1.3 kb, while the seventh had a 2.5 kb insert that was found to be the product of reverse-transcribed unspliced mRNA. Plasmid clones were sequenced at the Keck Foundation at Yale University. Sequence analysis and the database searches were performed using the Wisconsin Genetics Computer Group (GCG) software version 8.1.

Accession number
The nucleotide sequence data for human bet3 will appear in the DDBJ/EMBL/GenBank and nucleotide sequence database under the accession No. AJ224335.

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