Identification and characterization of five new subunits of TRAPP

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TRAPP (transport protein particle), a multiprotein complex containing ten subunits, plays a key role in the late stages of endoplasmic reticulum to Golgi traffic in the yeast *Saccharomyces cerevisiae*. We previously described the identification of five TRAPP subunits (Bet5p, Trs20p, Bet3p, Trs23p and Trs33p). Now we report the identification of the remaining five subunits (Trs31p, Trs65p, Trs85p, Trs120p and Trs130p) as well as an initial characterization of the yeast complex and its human homologue. We find that three of the subunits are dispensable for growth and a novel sequence motif is found in Bet3p, Trs31p and Trs33p. Furthermore, biochemical characterization of both yeast and human TRAPP suggests that this complex is anchored to a Triton X-100 resistant fraction of the Golgi. Differences between yeast and human TRAPP as well as the relationship of TRAPP subunits to other docking/tethering factors are discussed.

Introduction

The ability of a cell to maintain the unique identity of its organelles is largely achieved by the flow of membranes and protein both into and out of each compartment. Thus, the specificity of this flow of material must be tightly regulated. In its simplest form, membrane traffic can be divided into three steps: (*i*) the formation of a transport vesicle (budding); (*ii*) vesicle recognition by the appropriate target (targeting/tethering); and (*iii*) the mixing of the contents of the vesicle with the target organelle (fusion). The molecular mechanism of vesicle formation from the endoplasmic reticulum (ER) of the yeast *S. cerevisiae* as well as mammalian cells has been well studied. In addition to the small GTP-binding protein Sar1p, several components that make up the COPII coat are required for vesicle formation in vitro (see Schekman and Orci, 1996, for review).

Contained within the budded vesicle are integral membrane proteins intimately involved in vesicle fusion, namely Bos1p (Lian and Ferro-Novick, 1993), Bet1p (Newman and Ferro-Novick, 1987) and Sec22p (Ossig et al, 1991; Jiang et al, 1995). All three of these proteins interact with the *cis*-Golgi membrane protein Sed5p (Hardwick and Pelham, 1992) both in vitro and in vivo (Søgaard et al, 1994; Sacher et al, 1997; Stone et al, 1997). Collectively, these four proteins are referred to as SNAREs (soluble N-ethyl maleimide sensitive factor attachment protein receptor) and, depending on their homology to one of three neuronal proteins involved in exocytosis, are called v-SNAREs (homologous to synaptobrevin; eg. Bos1p, Sec22p) or t-SNAREs (homologous to either syntaxin or SNAP-25; eg. Sed5p, Bet1p). Although the exact role of SNAREs in membrane fusion is unclear, the molecular details of their interactions have been described. Bos1p and Sec22p form a complex on vesicles (Lian et al, 1994) that binds to the t-SNARE Sed5p, while Bet1p potentiates these interactions (Sacher et al, 1997; Stone et al, 1997). That SNAREs alone can fuse membranes was demonstrated using reconstituted proteoliposomes (Weber et al, 1998), although this proposal has recently been challenged (Ungermann et al, 1998).

Many soluble and peripherally-bound membrane factors also play a role in the overall transport process between the ER and Golgi including Sec18p, Sec17p, Ypt1p, Sec35p and Uso1p (Bacon et al, 1989; Wilson et al, 1989; Nakajima et al, 1991; Griff et al, 1992; VanRheenen et al, 1998). Ypt1p is a small GTP-binding protein required in ER-Golgi traffic at a post-budding step and may be required for intra-Golgi traffic as well (Bacon et al, 1989). While small GTP-binding proteins were once thought to uniquely impart specificity to membrane trafficking steps, the ability of a hybrid protein between Ypt1p and another small GTPase (Sec4p) to function as well as either protein implied that other factor(s) were needed (Brennwald and Novick, 1993; Dunn et al, 1993). Recently, Uso1p was shown to be the only cytosolic protein required for vesicle

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Tab. I. Yeast strains used in this study.

Strain	Genotype	Source
NY13	*MAT***a** *ura3-52* L-A-o	Novick Lab Collection
NY177	*MAT*α *leu2-3,112*	Novick Lab Collection
NY1060	*MAT***a**/α *ura3-52/ura3-52 leu2-3, 112/leu2-3,112* Gal$^+$/ Gal$^+$	Novick Lab Collection
SFNY472	*MAT***a**/α *ura3-52/ura3-52 bet3*Δ*:: URA3/BET3 leu2-3,112/leu2-3,112* Gal$^+$/ Gal$^+$	Ferro-Novick Lab Collection
SFNY26-3a	*MAT***a** *ura3-52*	Ferro-Novick Lab Collection
SFNY583	*MAT*α *ura3-52 bet5*Δ*::URA3 leu2-3,112 BET5-2*X*c-myc::LEU2*	Ferro-Novick Lab Collection
SFNY737	*MAT*α *ura3-52 bet3*Δ*::URA3 leu2-3,112 BET3*-Protein A::*LEU2*	This study
SFNY738	*MAT*α *ura3-52 leu2-3,112 BET3*-Protein A::*LEU2*	This study
SFNY836	*MAT***a**/α *ura3-52/ura3-52 TRS130* Δ*::URA3/ TRS130 leu2-3, 112/leu2-3,112* Gal$^+$/Gal$^+$	This study
SFNY849	*MAT***a**/α *ura3-52/ura3-52 trs120* Δ*::URA3/ TRS120 leu-2-3,112/leu2-3,112* Gal$^+$/Gal$^+$	This study
SFNY792	*MAT***a** *ura3-52 TRS31*-3X*c-myc*	This study
SFNY801	*MAT***a** *ura3-52 TRS65*-3X*c-myc*	This study
SFNY808	*MAT***a** *ura3-52 TRS23*-3X*c-myc*	This study
SFNY845	*MAT***a** *ura3-52 TRS33*-3X*c-myc*	This study
SFNY854	*MAT***a** *ura3-52 TRS120*-3X*c-myc* L-A-o	This study
SFNY857	*MAT***a** *ura3-52 TRS130*-3X*c-myc* L-A-o	This study
SFNY904	*MAT*α *ura3-52 bet3*Δ*::URA3 leu2-3,112 BET3*-Protein A::*LEU2* L-A-o	This study
SFNY929	*MAT***a** *ura3-52 TRS85*-3X*c-myc*	This study

tethering to the Golgi in a reconstituted system (Barlowe, 1997), suggesting that membranes contain additional factors required for this stage of membrane traffic. One candidate is the peripheral membrane protein Sec35p that facilitates vesicle tethering in a Uso1p-dependent fashion (VanRheenen et al, 1998). Sec35p forms a multiprotein complex with Sec34p (Kim et al, 1999), another protein required for this stage of membrane traffic. The role of Sec18p and Sec17p has been the subject of much debate, however recent findings suggest these proteins act in concert to prepare the SNAREs for subsequent fusion events (Mayer et al, 1996).

We recently identified a novel complex called TRAPP (transport protein particle) that contains 10 subunits, five of which have been described earlier (Rossi et al, 1995; Jiang et al, 1998; Sacher et al, 1998). Bet3p, the best characterized subunit of this complex, localizes to the *cis*-Golgi where it acts prior to SNARE complex formation (Rossi et al, 1995; Sacher et al, 1998) to dock ER-derived vesicles to the Golgi (J. Barrowman, M. Sacher and S. Ferro-Novick, submitted for publication). Here we report the identification of the remaining 5 subunits as well as an initial characterization of all the components. In addition, we provide evidence that an analogous complex exists in mammalian cells.

Materials and methods

Construction of Bet3p-Protein A fusion and TRAPP purification

The Protein A (PrA) coding sequence was inserted before the *BET3* stop codon using several PCR/primer extension reactions. First (reaction 1), the *BET3* promotor and open reading frame (until the stop codon) were amplified with the following primers: primer A: 5′-ATACTAGTCGACTCTAGAAAGAGAAAAACGTATAAAATG-ATTCAAG-3′; primer B: 5′-TTGGATAAAACCATTGCGTTGATC CCAATCTTCGCCGATCGGTATTTCGTC-3′. Primer B contains the first 20 bases of the PrA coding sequence. Next (reaction 2), the PrA coding sequence was amplified from the vector pRIT2T (Pharmacia) using the following primers: primer C: 5′-GACGAAATACCGAT-CGGCGAAGATTGGGATCAACGCAATGGTTTTATCCAA-3′; primer D: 5′-AAAAAGCTTACATGTCCACTAGTCGACGGATC-CCCGGGAATTC-3′. Primer C contained the last 20 bases of *BET3* (before the stop codon), while primer D contained the stop codon and the subsequent 20 bases from the 3′UTR of *BET3*. Finally (reaction 3), the *BET3* 3′UTR was amplified with the following primers: primer E: 5′-GAATTCCCGGGGATCCGTCGACTAGTGGACATGTAAGC-TTTTT-3′; primer F: 5′-ATACTAAGAAGCCGCTCTTCTTCTATA-CATTC-3′. Primer E contained the last 22 bases of Protein A. The products of reactions 2 and 3 were mixed and amplified (reaction 4) using primers C and F above to give PrA fused to the 3′UTR of *BET3*. Subsequently the product of reaction 4 was mixed with reaction 1 and amplified with primers A and F above to give the *BET3*-PrA fusion construct. The 2.3 kb XbaI/NheI fragment from this construct was inserted into the XbaI site of pRS305 (*LEU2* integrating vector), linearized with AflII and integrated at the *LEU2* locus in SFNY472 (see Table I). Transformants were sporulated, dissected and the Ura$^+$ Leu$^+$ colonies were selected. Western blot analysis with anti-Bet3p antiserum confirmed the loss of wild-type Bet3p and the presence of a new immunoreactive band at $\sim$50 kDa, the expected size of the Bet3p-PrA fusion protein. This clone, which was used for the purification of TRAPP, was sequenced along its entire length to verify that no PCR-generated mutations were present.

TRAPP was purified from 15000 OD600 units of cells that were converted to spheroplasts and lysed in buffer A (150 mM KCl, 20 mM HEPES, pH 7.2, 2 mM EDTA, 1% Triton X-100, 0.5 mM DTT, 1 × protease inhibitor cocktail (PIC; Waters and Blobel, 1986)). The lysate was centrifuged at 33000 rpm in a Type 70Ti rotor (Beckman) for 1 h. A total of 3.5 g of lysate was diluted to 10 mg/ml with buffer A and the lysate was incubated with Sepharose CL-4B for 4 h. The beads were removed and the flow through was incubated for 16 h with IgG-Sepharose (Pharmacia). The beads were gently pelleted and loaded into an EconoColumn where they were washed consecutively with 15 ml of buffer B (same as buffer A with 500 mM KCl) and buffer A. The bound protein was eluted from the beads with 0.2 M glycine, pH 2.8. Protein was precipitated from the eluate with 10% trichloroacetic acid (TCA), solubilized in SDS-PAGE sample buffer and fractionated on an SDS-8% polyacrylamide gel. Polypeptides were visualized with Coomassie brilliant blue and those which were specific to the Bet3p-PrA tagged strain were excised and sequenced as described previously (Sacher et al, 1998).

Disruption of the genes encoding TRAPP subunits and epitope tagging

Disruptions of the genes encoding TRAPP subunits were constructed by replacing all or most of the open reading frame (ORF) with a 1.1 kb fragment containing the *URA3* gene. For *TRS20*, *TRS23*, *TRS31*, *TRS33*, *TRS120* and *TRS130* the following fragments were replaced with *URA3*, respectively: XhoI/EcoRV, BsgI/BglII, HindIII/StyI, EcoRI/StyI, AflII/HpaI and HpaI/MscI. All disruptions were transformed into NY1060, sporulated and dissected.

TRAPP subunits were tagged with three *c-myc* epitopes using PCR epitope tagging (Schneider et al, 1995). Since the gag protein encoded by the yeast L-A virus was a common contaminant in our experiments several of the *c-myc*-tagged subunits were constructed in a strain lacking this virus (NY13) as indicated in Table I.

Functional cloning of *TRS31*, *TRS120* and *TRS130*

A functional clone of *TRS31* was obtained by amplifying the ORF (YDR472w) with 400 bases flanking the coding region. The PCR product was cloned into pRS426 (*URA3*, 2 μm) and pBluescriptII. To ensure there were no PCR-generated mutations the clones were sequenced. Functional *TRS120* and *TRS130* clones were prepared by the gap repair method (Rothstein, 1991). The repaired plasmids were rescued from the yeast transformants, amplified in *E. coli* and tested for their ability to restore growth to a *trs120Δ* and *trs130Δ* strain.

Preparation of yeast lysates, immunoprecipitation and Western blot analysis

Radiolabeling experiments were performed as previously described (Sacher et al, 1998). Unlabeled lysates were prepared in a similar way except the cells were grown in YPD medium. Immunoprecipitations were performed with 4×10^7 cpm of radiolabeled lysate or with 1 mg of unlabeled lysate as described in Sacher et al. (1998). For lysates containing Bet3p-PrA samples were precleared with Sepharose CL-4B and then incubated with IgG-Sepharose. Protein was eluted by incubating the beads with 0.2 M glycine, pH 2.8 and neutralized with potassium hydroxide before loading onto the gel. Western blots were processed using the enhanced chemiluminescence system (Amersham) according to the manufacturer's instructions.

HeLa cell lysates and gel filtration chromatography

Lysates of HeLa cells used for immunoprecipitation experiments were made as follows. A total of 2×10^9 cells were resuspended in lysis buffer (20 mM HEPES, pH 7.2, 500 mM NaCl, 1 mM $MgCl_2$, 2% Triton X-100, 1 mM DTT, 1 × PIC), homogenized by 10 strokes with a dounce homogenizer and incubated on ice for 30 min. When samples were denatured, lysates were prepared by homogenizing in 1% SDS and boiled for 3 min The lysates were then centrifuged at 100 000*g* for 1 h. A total of 2 mg of protein was incubated with anti-hbet3 serum for 2.5 h at 4 °C. Immune complexes were collected onto Protein A-Sepharose beads, washed 2 times with buffer B, 6 times with buffer A and once with 20 mM Tris-HCl, pH 7.0. Protein was eluted by boiling samples in SDS-PAGE sample buffer and co-precipitating proteins were detected by Western blot analysis.

The lysate used for gel filtration was prepared by dounce homogenizing 6×10^9 cells in 2 ml of 20 mM HEPES, pH 7.2., NaCl, EDTA, DTT and PIC were added to final concentrations of 150 mM, 2 mM, 1 mM and 1 × , respectively. The sample was centrifuged at 100 000*g* for 1 h. A total of 8 mg of protein was loaded onto a Superdex-200 gel filtration column (Pharmacia) and 1 ml samples were collected. Samples were boiled in SDS-PAGE sample buffer and proteins were detected by Western blot analysis using anti-hbet3 and anti-htrs20 antiserum.

Extraction studies

Yeast cells (SFNY26–3A) were converted to spheroplasts and lysed in buffer A (20 mM HEPES, pH 7.2) by 10 strokes in a Wheaton A dounce homogenizer. Unbroken cells were removed during a brief centrifugation at 450*g*. Aliquots of the supernatant were mixed with equal volumes of buffer A alone, buffer A containing 1 M NaCl or buffer A containing 4% Triton X-100. Samples were then incubated for 15 min on ice and subsequently centrifuged at 32 700 rpm in an SW50.1 rotor for 1 h. The resulting supernatant fractions were boiled in SDS-PAGE sample buffer while the pellet fractions were resuspended in a volume of buffer A equal to that of the supernatant fraction and boiled in SDS-PAGE sample buffer. Samples were assayed for TRAPP subunits by Western blot analysis as described above.

Results

Purification of TRAPP and identification of five new subunits

Previously we used a *c-myc* epitope-tagged version of Bet3p, one of the components of TRAPP, to purify a denatured form of this complex (Sacher et al, 1998). This purification led to the identification of subunits with apparent molecular sizes of 20 kDa, 23 kDa and 33 kDa. Two other subunits, Bet3p and Bet5p, were identified in genetic screens (Rossi et al, 1995; Jiang et al, 1998). This method of purification did not, however, yield preparations that were sufficiently pure to obtain sequence information for the remaining subunits. In addition, anti-*c-myc* antibody could not quantitatively precipitate TRAPP from a yeast lysate.

To circumvent these problems, we used a different tag to purify this complex. A strain (SFNY737) in which the sole copy of Bet3p was fused at the carboxy-terminus to Protein A (Bet3p-PrA) was constructed for this purpose. This fusion protein was fully functional as it restored growth to *bet3*Δ cells. In addition, precipitation of Bet3p-PrA onto IgG-Sepharose beads yielded the same pattern of subunits as did *c-myc*-tagged Bet3p (Figure 1A; see Sacher et al, 1998), indicating that this fusion protein was incorporated into TRAPP and should allow for purification of this complex. Indeed, incubation of a lysate prepared from SFNY737 with IgG-Sepharose resulted in a quantitative depletion of Bet3p-PrA from the supernatant, while no such effect was seen with untagged Bet3p (Figure 1B). Using this protocol, we purified subunits with apparent molecular sizes of 31 kDa, 65 kDa, 85 kDa, 120 kDa and 130 kDa. Polyacrylamide gel slices containing the purified subunits were digested with trypsin and analyzed by mass spectrometry. Database searches using the identified peptides (Table II) showed that p31 is encoded by the open reading frame (ORF) YDR472w, p65 is encoded by the ORF YGR166w, p85 is encoded by the ORF YDR108w, p120 is encoded by the ORF YDR407c, and p130 is encoded by the ORF YMR218c. The polypeptide with an apparent size of 105 kDa (see starred band in Figure 1A and Sacher et al, 1998) was shown here to be a fragment of p120 (YDR407c). Interestingly, p65 is identical to *KRE11*, a gene previously identified in a screen for cells resistant to K1 killer toxin (Brown et al, 1993).

The protein with an apparent molecular size of 85 kDa (YDR108w) was initially difficult to identify. In earlier purifications of TRAPP, using *c-myc* epitope-tagged Bet3p, a prominent band of 85 kDa was observed. Mass spectrometric analysis identified this as the gag protein encoded by the laboratory yeast virus L-A. This protein was previously shown to be a common contaminant in protein purifications that employ the use of anti-*c-myc* antibody (Terbush et al, 1996). Therefore, to facilitate the identification of this subunit, Bet3p-PrA was introduced into an L-A virus-free strain (SFNY904). As a consequence, the gene encoding p85 was identified as *GSG1* (Table II), a gene of unknown function that indirectly effects sporulation (Kaytor and Livingston, 1995; Engebrecht et al, 1998). Other than Bet3p and Bet5p, the remaining subunits will herein be called *TRS* (TRAPP subunit) followed

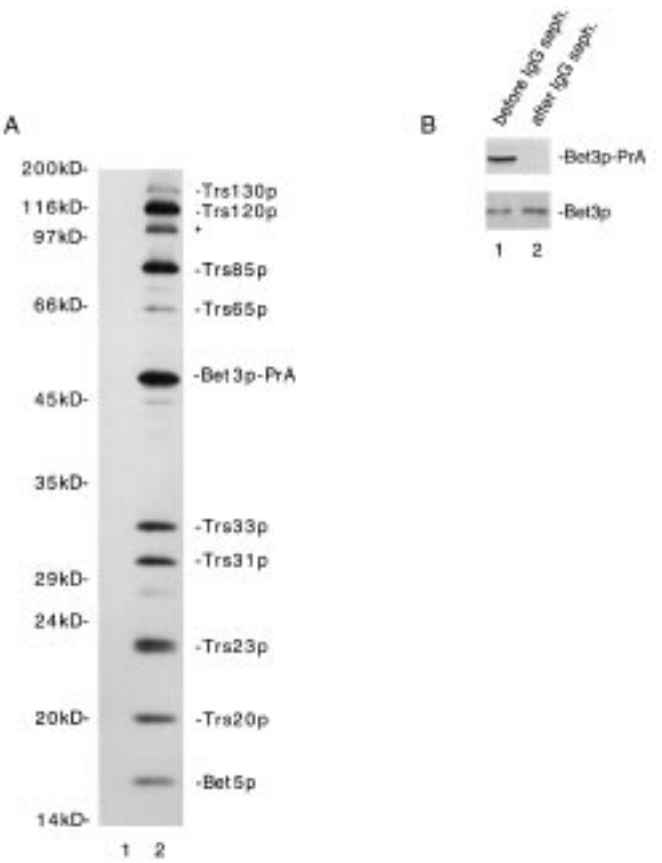

Fig. 1. TRAPP is quantitatively immunoprecipitated from a strain containing the Bet3p-Protein A fusion. (**A**) Radiolabeled lysates (2×10^8 cpm) from SFNY904 (Bet3p-PrA, *lane 2*) and NY13 (wild type, *lane 1*) were prepared as described in the Materials and methods and fractionated on a Superdex-200 gel filtration column. Fractions were collected and precipitated onto IgG-Sepharose beads. Fraction 9 from each strain is shown. TRAPP subunits are indicated on the right of the panel and molecular size markers are indicated on the left. The starred band below Trs120p represents a proteolytic fragment of Trs120p. (**B**) Samples from SFNY737 (*top panel*) and NY13 (*lower panel*) either before (*lane 1*) or after (*lane 2*) treatment with IgG-Sepharose were electrophoresed on an SDS-13% polyacrylamide gel, transferred to nitrocellulose and probed with anti-Bet3p antiserum.

by their apparent molecular sizes (Bet5p, Trs20p, Bet3p, Trs23p, Trs31p, Trs33p, Trs65p, Trs85p, Trs120p, Trs130p). For the purposes of this report, the high molecular weight TRAPP subunits refers to Trs65p, Trs85p, Trs120p and Trs130p, while Bet5p, Trs20p, Bet3p, Trs23p, Trs31p and Trs33p will be referred to as the low molecular weight subunits.

Bet3p co-immunoprecipitates with the Trs subunits

To directly confirm the presence of the Trs proteins in TRAPP, we tagged each of the putative subunits with three *c-myc* epitopes. If the tagged proteins are components of TRAPP, we would expect that anti-*c-myc* ascites fluid (9E10) should also precipitate Bet3p from lysates containing the tagged protein. As shown in Figure 2, this was indeed the case. Note that, although *c-myc*-tagged Trs20p was functional, the *c-myc* epitope was not accessible to the antibody under native conditions. This subunit, which was precipitated with a polyclonal antibody directed against Trs20p, also precipitated with Bet3p.

Tab. II. Peptides identified by mass spectrometry of TRAPP subunits.

Subunit	Sequence name	Peptides identified
Trs31p	YDR472w	(R)ASVSPSSLPR (R)IYSESLLFK
Trs65p	*KRE11*	(R)FSLGAASTTSLVNSK (K)LQYPIFSLLNMR (K)SVDANFNNQDSLQDPQAK (K)YTLDKYSSEEILPSFEPVYSWSSAATK
Trs85p	*GSG1*	(R)DLLVNFK (R)DSQGNEYFASSSSEFLMR (R)DSTQSQYIR (K)FLHGNGLILSK (R)FTRPLGDLIETR (K)YSTDHFTDEDILSEGLTR
Trs120p	YDR407c	(R)ILDSDPTSNEIPLESDVSSLESLENR (R)LFYGVQLLLDEPK (R)SITLPLIIK (K)VSEINLEIIPIEK (K)EYIQPSITNESITSYWDPFILR (K)ILGNFQLLAGR (K)INVDDPSQSTWLILQK (R)LFYGVQLLLDEPK
Trs130p	YMR218c	(K)ASVLSIFQK (K)ETFVDETVFQENFLTLTK (K)FGKDFPHVQTLEVR (K)GNNFEEQLLTR (K)LEDQTLTAFVGAFQLIK (K)NMGGDVIVFGASDFLLK (K)ASVLSIFQK (K)NLPVSFSMDEIQAR

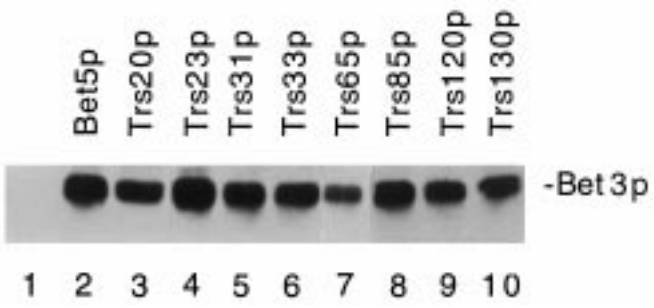

Fig. 2. TRAPP subunits co-precipitate with Bet3p. Lysates from strains containing a *myc*-tagged subunit (*lanes 2*, and *4–10*) and an untagged strain (*lanes 1* and *3*) were prepared and immunoprecipitated with 9E10 (anti-*c-myc*) ascites fluid (*lanes 1, 2, 4–10*) or anti-Trs20p antibody (*lane 3*). Immune complexes were collected onto Protein A-Sepharose beads, fractionated by SDS-PAGE and transferred to nitrocellulose. The membrane was then probed with anti-Bet3p antiserum. The co-precipitating subunit (i.e., target of the primary antibody) is indicated above each lane.

It was previously shown that overexpression of *BET5*, as well as *TRS20*, *TRS23* and *TRS33*, suppress the growth defect of *bet3-1* at 30 °C. *BET5* and *TRS23* also suppressed *bet3-1* at 34 °C (Jiang et al, 1998; Sacher et al, 1998). Interestingly, the growth defect of other secretory mutants that block transport from the ER to the Golgi complex was not suppressed by the overexpression of these genes. Thus, the specific suppression displayed by these genes appears to be indicative of the fact that they encode components of a complex that includes Bet3p. We tested the ability of overexpressed *TRS31*, *TRS65*, *TRS85*, *TRS120* and *TRS130* to suppress *bet3-1*. As shown in

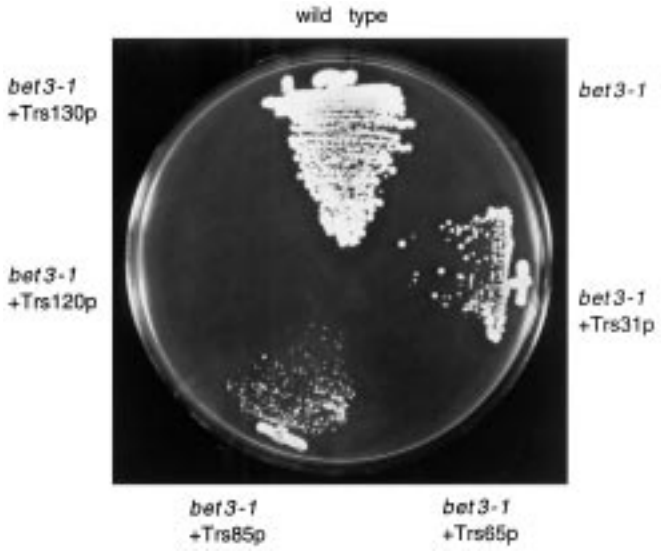

Fig. 3. The overexpression of *TRS31* and *TRS85* suppresses the *bet3-1* mutant. SFNY596 (see Table I) was transformed with either *TRS31*, *TRS65*, *TRS85*, *TRS120* or *TRS130* in a 2 μm (pRS426) vector as indicated. Transformants were grown for three days on YPD plates at 30°C.

Figure 3, overexpression of *TRS31* and *TRS85* suppressed the temperature-sensitive growth defect of *bet3-1* at 30°C, but not 34°C. However, overexpression of *TRS65*, *TRS120* and *TRS130* failed to suppress *bet3-1* at any temperature. Thus, there appears to be a delineation in the ability of Trs65p, Trs120p and Trs130p versus Trs85p and the low molecular weight subunits to suppress *bet3-1*.

Sequence analysis of the TRAPP subunits

We previously reported human homologues of Trs20p, Trs23p, Trs33p, Bet3p, and Bet5p (Jiang et al, 1998; Sacher et al, 1998). Using Trs31p as a query we searched dbEST and identified potential homologues from several eukaryotic sources (Figure 4A). All putative homologues were shorter than the yeast protein due to one large gap. While it is unclear why this region of Trs31p was lost during evolution it is interesting to note that it is highly serine rich (30%). The human homologue of Trs31p is 32% identical and 60% similar at the amino acid level to the yeast protein. Alignment of Trs33p, Trs31p and Bet3p from yeast and humans revealed several conserved features (Figure 4B). Two shared motifs as well as several conserved bulky,

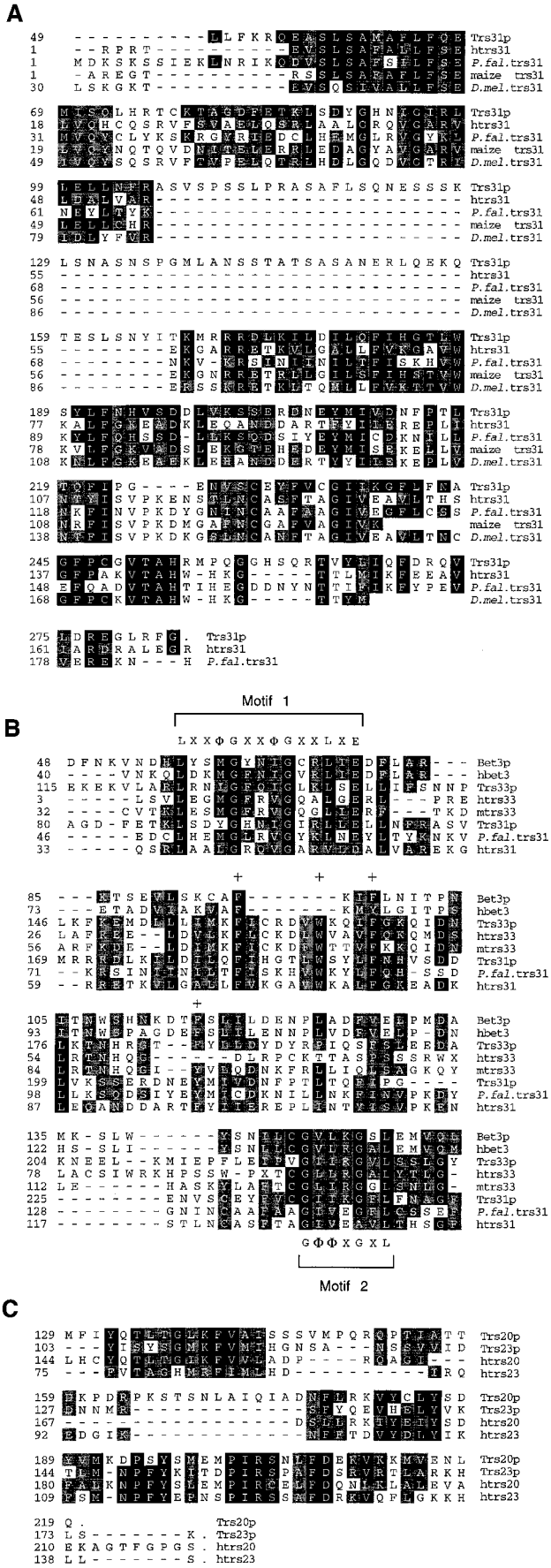

Fig. 4. Sequence analysis of the TRAPP subunits. (**A**) Trs31p was used as a query to search dbEST. Contiguous sequences were built using SeqMan and aligned using MegAlign. Residues identical or similar to Trs31p are shaded in *black* and *gray*, respectively. Homologues of Trs31p in human, *Plasmodium falciparum*, maize, and *Drosophila melanogaster* are reported. (**B**) Yeast sequences for Bet3p, Trs31p and Trs33p, as well as their mammalian homologues, were aligned. Identical residues (minimum of 5/7) are shaded in *black* and similarities are shaded in *gray*. The motifs were built when identities were found in 7 of the 8 sequences. The plus (+) symbols indicate conserved, bulky hydrophobic residues. In the motifs, X indicates any amino acid and Φ indicates a hydrophobic amino acid. (**C**) Trs20p was used as a query in a PSI-BLAST search of the nonredundant GenBank database revealing similarity to Trs23p. Yeast and human subunits are aligned with identities and similarities shaded in *black* and *gray*, respectively.

hydrophobic residues were detected. While human trs31 is slightly more divergent in these motifs, it is noteworthy that this homologue was identified by a search of dbEST and that single nucleotide changes can account for the divergence. Cloning of the human homologue should help clarify these discrepancies.

A BLAST search of the non-redundant GenBank database using Trs130p as a query revealed significant homology (E value of 7×10^{-8}) between this subunit and a protein of similar size called GT334 (Lafrenière et al, 1997). GT334 maps to a region of human chromosome 21 where at least 6 inherited disorders also map. Some of the genes in this region of the chromosome encode proteins of known function but several, including GT334, are of unknown function. The amino terminal 230 amino acids of Trs130p are most homologous to GT334. GT334 may be a relative or homologue of Trs130p. Presently, its subcellular distribution is unknown. Interestingly, of the ten subunits of TRAPP, only Trs130p contains a region which shows significant potential to be involved in a coiled-coil interaction. This region (amino acids 184–211) overlaps with the region of strongest homology between Trs130p and GT334.

Position-specific iterated (PSI)-BLAST is a more sensitive means than BLAST to detect weak but biologically relevant sequence similarities between proteins (Altschul et al, 1997). Using the yeast Trs20p sequence as a query in PSI-BLAST under stringent conditions (threshold value of 0.001), yeast Trs23p gave a statistically significant alignment (E value of 2×10^{-30}). While these proteins showed alignment along their entire lengths, the homology between their carboxy terminal halves was strongest (Figure 4C). The aligned residues were also well conserved in the human homologues. Although the biological significance of this alignment is unknown, we speculate that these two TRAPP subunits may be ancesterally related.

TRS33, TRS65 and *TRS85* are dispensable for growth

To determine if the *TRS* genes encode proteins essential for the vegetative growth of yeast, we examined the consequences of disrupting each of the identified ORFs on cell viability. All *TRS* genes were disrupted by replacing all or most of each ORF with *URA3*. To replace the wild-type copy with the disrupted *TRS* gene, a linear fragment of DNA that contained the disruption was transformed into a diploid strain (NY1060). The diploid was sporulated and then 20–33 tetrads were analyzed. As shown in Table III, *TRS33*, *TRS65* and *TRS85* were found to be dispensable, while the remaining *TRS* genes were not. Disruptions of *TRS20*, *TRS23*, *TRS31*, *TRS120* and *TRS130* displayed 2:2 segregation for viability and the viable spores were always Ura-. Microscopic examination of the arrested colonies revealed that cells containing the disruptions were capable of undergoing 2–3 rounds of cell division before growth ceased. The one exception was *TRS130* which divided 4–5 times before growth was arrested.

TRAPP contains multiple copies of Bet3p

During the construction of SFNY737, a Leu^+Ura^- spore (SFNY738) that contained one copy of Bet3p and one copy of Bet3p-PrA was identified (see Materials and methods). This strain was used to determine whether TRAPP contains more than one copy of Bet3p. If TRAPP contains multiple copies of this subunit then Bet3p and Bet3p-PrA should be in the same complex in these cells. Treatment of a lysate prepared from SFNY738 with IgG-Sepharose should therefore precipitate Bet3p as well as Bet3p-PrA. As shown in Figure 5, IgG-Sepharose only precipitated Bet3p when it was tagged with Protein A (Figure 5, compare lanes 1 and 5 with 2 and 6; see also Figure 1B). When a lysate prepared from SFNY738 was treated with IgG-Sepharose, we quantitatively precipitated

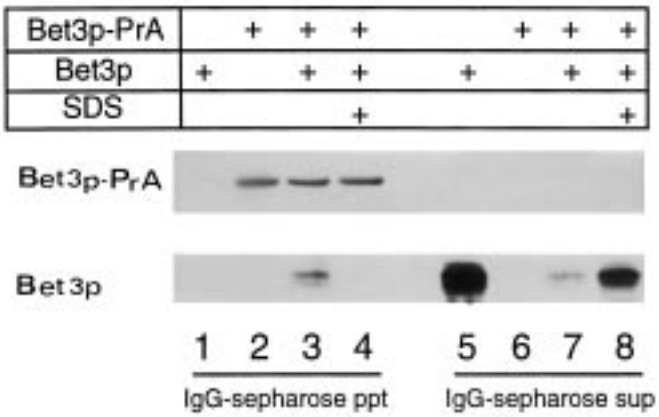

Fig. 5. TRAPP contains multiple copies of Bet3p. Lysates were prepared from SFNY26–3A (wild type, *lanes 1* and *5*), SFNY737 (Bet3p-PrA, *lanes 2* and *6*) and SFNY738 (Bet3p/Bet3p-PrA, *lanes 3*, *4*, *7* and *8*) as described in the Materials and methods. Samples were left untreated (*lanes 1–3* and *5–7*) or boiled in 2% SDS (*lanes 4* and *8*) prior to treatment with IgG-Sepharose. The beads were washed and the supernatants were removed and precipitated with TCA. Protein bound to the beads was eluted with 0.2 M glycine, pH 2.8. Precipitates (*lanes 1–4*) and supernatant fractions (*lanes 5–8*) were fractionated by SDS-PAGE, transferred to nitrocellulose and probed with anti-Bet3p antiserum.

Tab. III. Summary of TRAPP subunit disruptions.

subunit	gene/sequence	null phenotype	No. of tetrads dissected	No. Ura^+ colonies	No. of cells in arrested colony
Bet5p	*BET5*	lethal[a]	n.d.[c]	n.d.[c]	8–12
Trs20p	YBR254c	lethal	23	0	4–8
Bet3p	*BET3*	lethal[d]	n.d.[d]	n.d.[d]	n.d.
Trs23p	YDR246w	lethal	20	0	4–8
Trs31p	YDR472w	lethal	24	0	4–6
Trs33p	YOR115c	viable[b]	23	46	–
Trs65p	*KRE11*	viable[e]	n.d.[e]	n.d.[e]	–
Trs85p	*GSG1*	viable	n.d.[f]	n.d.[f]	–
Trs120p	YDR407c	lethal	24	0	6–10
Trs130p	YMR218c	lethal	33	0	12–35

n.d. = not done in this study; a = lethal genes show 2 : 2 viability for tetrads; b = viable phenotype shows 4 : 0 viability for tetrads; c = refer to Jiang et al., 1998; d = refer to Rossi et al., 1995; e = refer to Brown et al., 1993; f = refer to Kaytor and Livingston, 1995.

Bet3p-PrA with a portion of the Bet3p (Figure 5, lanes 3 and 7). The Bet3p remaining in the supernatant presumably arose from non-precipitated TRAPP that only contained Bet3p. Treatment of this lysate with IgG-Sepharose after denaturation with SDS confirmed that Bet3p was precipitated by virtue of its association with Bet3p-PrA (Figure 5, lanes 4 and 8). These results indicate that TRAPP contains at least two copies of Bet3p.

We next addressed the stoichiometry of the subunits. Based on the known cysteine and methionine content of the TRAPP subunits, we determined that all identified components are present in the fully assembled complex in approximately equimolar ratios (data not shown). Although Trs130p does not appear to be present in equimolar amounts in Figure 1A, we have observed that this subunit is more stable when it is tagged with *c-myc*. Indeed, a proteolytic product of Trs130p can be seen as a faint band below Trs31p (Figure 1A). Therefore, the stoichiometries were determined from a strain in which Trs130p was *myc*-tagged. Since there are at least two copies of Bet3p in TRAPP, this would argue that at least two copies of the other subunits are needed to maintain this stoichiometry. The sum of the molecular masses gives a protein complex with a predicted size of 1094 kDa, in close agreement to the previously reported size (Sacher et al, 1998). Assuming two copies of each subunit in the complex, quantitative immunoblotting suggests that TRAPP comprises $\sim$0.01% of the total cellular protein.

TRAPP subunits are resistant to extraction by Triton X-100

The association of TRAPP with the *cis*-Golgi was examined by performing extraction studies. This was done by incubating lysates with buffer alone, buffer containing 0.5 M salt or buffer containing 2% Triton X-100 and analyzing the 100 000*g* supernatant and pellet fractions for the presence of various protein markers. In addition to TRAPP subunits Trs33p and Bet3p, we followed Sec35p, a peripheral membrane protein, and Bos1p, an integral membrane protein of the ER. In buffer alone, Trs33p (Figure 6A, lanes 1 and 2) and Bet3p (data not shown) distributed mostly with the pellet fraction, Sec35p was largely found in the soluble fraction and Bos1p was exclusively in the pellet (Figure 6A, lanes 1 and 2). In the presence of 0.5 M salt, Sec35p and Trs33p were efficiently extracted, while Bos1p remained with the pellet fraction (Figure 6A, lanes 3 and 4). The integrity of the TRAPP complex was maintained under these conditions, as all subunits could be precipitated with *myc*-tagged Bet3p following an incubation in the presence of high salt (0.5 M), and the immunoprecipitation pattern was identical to that seen with low salt (Figure 6C). In the presence of 2% Triton X-100, Trs33p was resistant to extraction, while Bos1p and Sec35p were both solubilized under these conditions (Figure 6A, lanes 5 and 6).

HeLa cell lysates contain a complex that is analogous to TRAPP

As mentioned above, mammalian homologues of the low molecular weight Trs proteins have now been identified. Since the mouse and human homologues of bet5 were found to be 98% identical (Jiang et al, 1998), we reasoned that other TRAPP subunits may show a strong degree of homology as well. We therefore raised an antibody to human bet3 (hbet3) and probed lysates from a variety of cell lines. As shown in Figure 7A, in addition to human (HeLa) cells, an immunoreactive band of the appropriate molecular size was detected in cell lines from monkey (COS7), rat (PC12) and hamster (CHO). These results indicate that (*i*) TRAPP subunits exist in many different mammalian organisms and (*ii*) the bet3 subunit is likely to be highly conserved in amino acid sequence in different mammalian species. Indeed, the mouse bet3 homologue (GenBank accession number AAB96937) is 100% identical to hbet3.

Since homologues of the low molecular weight subunits of TRAPP can be found in a variety of mammalian species, we analyzed human cells for the presence of an analogous complex. Antibodies were raised to human Trs20p (htrs20) and the ability of anti-hbet3 antibody to co-precipitate htrs20 was tested. We detected co-precipitation of htrs20 with hbet3 under native but not denaturing conditions (Figure 7B, compare lanes 2 and 3). In addition, preimmune serum failed to co-precipitate htrs20 with hbet3 (Figure 7B, lane 1). The size of the hbet3 complex was estimated by gel filtration of a HeLa cell lysate on a Superdex-200 column and the fractions were assayed for the presence of hbet3 (data not shown) and htrs20 by Western blot analysis. These proteins co-eluted in a peak with an estimated size of $\sim$670 kDa (Figure 7C). In addition, a second, more abundant peak of material was found. While the exact size of this peak is hard to estimate, it likely represents monomeric hbet3 and htrs20 since the denatured subunits also fractionated in this region (data not shown). This finding is unlike what we have observed in yeast, where no free pool of any of the subunits has been detected (data not shown).

Since yeast Bet3p and Trs33p were shown to be resistant to extraction by 2% Triton X-100, we examined the extraction profile of hbet3 and htrs20 from the 100 000*g* particulate fraction. HeLa cells were lysed in the buffer described above for the extraction studies in yeast. The lysate was then incubated in the absence or presence of either 0.5 M NaCl or 2% Triton X-100 (final concentration). In contrast to what was observed in yeast (see lanes 1 and 2 in Figure 6A), hbet3 and htrs20 were largely soluble under the conditions used to prepare the lysate (Figure 6B, lanes 1 and 2). In the presence of 0.5 M NaCl, htrs20 was efficiently extracted from the particulate fraction (Figure 6B, lanes 3 and 4). Like in yeast, the integrity of the human complex is maintained under these conditions (data not shown). However, a significant fraction of the trs20 that pelleted was resistant to extraction by Triton X-100 while the transferrin receptor, an integral membrane protein, was completely solubilized (Figure 6B, lanes 5 and 6). These results indicate that HeLa cells contain a complex analogous to yeast TRAPP that, like the yeast complex, is anchored to a Triton X-100 resistant matrix in the cell.

Discussion

TRAPP, a multiprotein complex containing ten subunits, mediates the docking of ER-derived transport vesicles to the *cis*-Golgi. In earlier studies (Rossi et al, 1995; Jiang et al, 1998; Sacher et al, 1998), we reported the identification of five of these subunits. Here we report the identification of the five remaining subunits. *TRS65* was initially identified as *KRE11* in a screen for cells resistant to K1 killer toxin (Brown et al, 1993). This toxin, secreted by cells containing the yeast viruses M1 and L-A, binds to $\beta 1 \rightarrow 6$ glucans at the cell surface where it kills K1-sensitive strains (Hutchins and Bussey, 1983). The

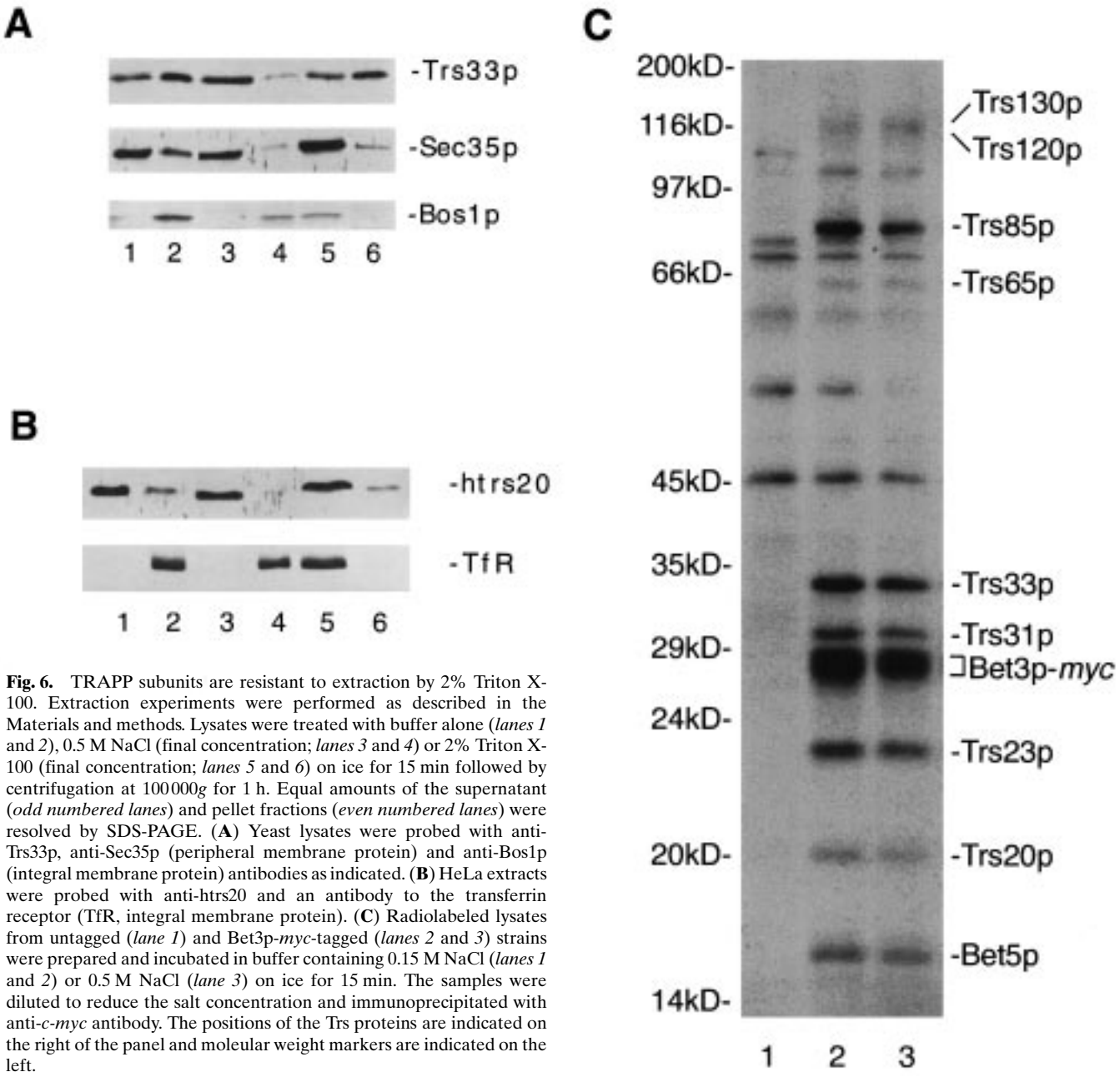

Fig. 6. TRAPP subunits are resistant to extraction by 2% Triton X-100. Extraction experiments were performed as described in the Materials and methods. Lysates were treated with buffer alone (*lanes 1* and *2*), 0.5 M NaCl (final concentration; *lanes 3* and *4*) or 2% Triton X-100 (final concentration; *lanes 5* and *6*) on ice for 15 min followed by centrifugation at 100000*g* for 1 h. Equal amounts of the supernatant (*odd numbered lanes*) and pellet fractions (*even numbered lanes*) were resolved by SDS-PAGE. (**A**) Yeast lysates were probed with anti-Trs33p, anti-Sec35p (peripheral membrane protein) and anti-Bos1p (integral membrane protein) antibodies as indicated. (**B**) HeLa extracts were probed with anti-htrs20 and an antibody to the transferrin receptor (TfR, integral membrane protein). (**C**) Radiolabeled lysates from untagged (*lane 1*) and Bet3p-*myc*-tagged (*lanes 2* and *3*) strains were prepared and incubated in buffer containing 0.15 M NaCl (*lanes 1* and *2*) or 0.5 M NaCl (*lane 3*) on ice for 15 min. The samples were diluted to reduce the salt concentration and immunoprecipitated with anti-*c-myc* antibody. The positions of the Trs proteins are indicated on the right of the panel and moleular weight markers are indicated on the left.

screen for *KRE* genes was used to identify genes whose products were required for cell wall biosynthesis (Brown et al, 1994). Our finding that *KRE11* is identical to *TRS65* suggests that some *KRE* gene products may be required for the transport of β1 → 6 glucans to their extracellular location. Interestingly, all of the subunits of TRAPP, when over-expressed, suppress the temperature-sensitive growth defect of *bet3-1* except for Trs65p, Trs120p and Trs 130p. One interpretation of the data is that these subunits may modulate the activity of the other subunits or may play a different role from the other subunits. We are currently addressing this possibility by making conditional mutants in these *TRS* genes and studying the resulting phenotypes.

Sequence analysis of the TRAPP subunits has revealed several interesting features. The identification of two conserved motifs and several bulky hydrophobic residues in three of the TRAPP subunits was unexpected. While the function(s) of these motifs is unclear they may be required for the assembly of the complex or its localization to the Golgi. Interestingly, the sequence of the *bet3-1* mutation demonstrated that the second conserved glycine in the first motif was altered (J. Barrowman and S. Ferro-Novick, unpublished observation), underscoring the importance of this motif in Bet3p function. Another interesting feature was the absence of a serine-rich region from all Trs31p homologues. This region may be dispensable in yeast or its function may be compensated for in higher eukaryotes by another subunit(s). Given the large amount of serine residues we are presently examining the phosphorylation state of Trs31p.

Since there are multiple copies of Bet3p, and by inference all other nine subunits, there are at least 20 polypeptides in the fully assembled TRAPP complex. Why are there so many

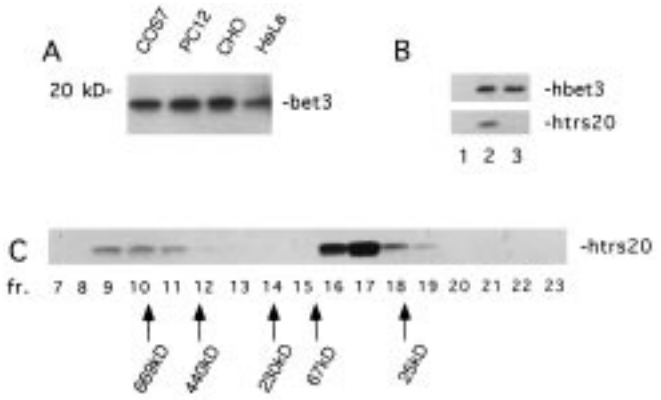

Fig. 7. HeLa cells contain hbet3 in a high molecular weight complex. (**A**) Lysates from COS7, PC12, CHO and HeLa cells (indicated above each lane) were prepared and 200 mg of total protein were electrophoresed on an SDS-15% polyacrylamide gel, transferred to nitrocellulose and probed with anti-hbet3 antiserum. (**B**) HeLa extracts were prepared under native (*lanes 1* and *2*) or denaturing (*lane 3*) conditions as described in Materials and methods and immunoprecipitated with pre-immune serum (*lane 1*) or anti-hbet3 antibody (*lanes 2* and *3*). Samples were fractionated by SDS-PAGE, transferred to nitrocellulose and probed with anti-hbet3 serum (*upper panel*) or anti-htrs20 serum (*lower panel*). (**C**) Lysate from HeLa cells was fractionated on a Superdex-200 gel filtration column. Fractions (1 ml) were probed with anti-htrs20 serum. Molecular size standards used were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (230 kDa), bovine serum albumin (67 kDa) and chymotrypsinogen A (25 kDa).

subunits? TRAPP subunits may interact with many different factors. For example, to maintain its proper localization one or more of the subunits must interact with a receptor on the Golgi matrix and possibly on membranes as well. In addition, as a factor mediating vesicle docking, Bet3p and, perhaps, other subunits of this complex may interact with one or more components on ER-derived COPII vesicles. Finally, TRAPP may relay a signal to recruit other tethering factors such as Uso1p and the Sec34p/Sec35p complex. The refinement of vesicle docking assays should ultimately help to elucidate the temporal relationship of all these docking factors.

Vesicle docking involves at least two steps (Pfeffer, 1999). There is an initial recognition step between components on the vesicle and target membrane followed by the binding of the vesicle to its acceptor compartment. Once the initial recognition step takes place, (general) tethering factors may be recruited. In this respect, it is noteworthy that *bet3-1* is suppressed by SNAREs specifically implicated in ER to Golgi traffic (*BET1*, *BOS1*, *SEC22*, *SED5*) (Rossi et al, 1995; Sacher et al, 1998), but not by SNAREs that act at later stages of the exocytic pathway (*GOS1*, *YKT6*, *SFT1*, *VTI1*, *SNC1*, *SNC2* (data not shown). By contrast, *uso1* and *sec35* display genetic interactions with the former as well as some of the latter SNAREs (Sapperstein et al, 1996; VanRheenen et al, 1998; Kim et al, 1999). Of the factors required for ER to Golgi docking, only Bet3p localizes at steady state to the Golgi (Sacher et al, 1998), while Uso1p, Sec34p and Sec35p are largely soluble proteins (Barlowe, 1997; VanRheenen et al, 1998; Kim et al, 1999), suggesting that they are recruited to membranes in response to membrane traffic.

Although the subunits are well conserved, there is a striking difference between yeast and human TRAPP. HeLa lysates contain an assembled complex of ~670 kDa and a substantial pool of unassembled subunits while yeast lysates contain a 1094 kDa complex and show no free pool of any TRAPP subunits. The 670 kDa complex in HeLa cells may be equivalent to the 1094 kDa complex in yeast. The difference in size may be accounted for by the fact that most of the human homologues are smaller (see Sacher et al, 1998 and Figure 4A). HeLa cells also have a larger soluble pool of TRAPP subunits hbet3 and htrs20 than yeast which may be a consequence of the larger pool of unassembled subunits in human cells. Consistent with this notion, the Triton X-100 insoluble fraction in HeLa cells is predominantly composed of the 670 kDa complex (data not shown). The small amount of soluble TRAPP in yeast may represent assembled complex that has sheared from the Golgi matrix and/or membranes during lysis. The fact that mammalian cells contain a large pool of unassembled TRAPP subunits may reflect differences in how Golgi dynamics are regulated in mammalian cells versus yeast. For example, while disassembly of the Golgi during mitosis in mammalian cells is well documented (Lucocq et al, 1987; Warren, 1993), this phenomenon has not been reported in yeast. A large soluble, unassembled pool of TRAPP subunits in mammalian cells could allow for rapid assembly/disassembly of the complex in response to various cell cycle cues.

Now that the subunits of TRAPP have been identified, we can focus our attention on understanding the molecular details of how this complex functions. The elucidation of proteins which interact with TRAPP will be an important first step in unraveling its role in membrane traffic.

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