Mammalian Bet3 functions as a cytosolic factor participating in transport from the ER to the Golgi apparatus

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Summary

The TRAPP complex identified in yeast regulates vesicular transport in the early secretory pathway. Although some components of the TRAPP complex are structurally conserved in mammalian cells, the function of the mammalian components has not been examined. We describe our biochemical and functional analysis of mammalian Bet3, the most conserved component of the TRAPP complex. Bet3 mRNA is ubiquitously expressed in all tissues. Antibodies raised against recombinant Bet3 specifically recognize a protein of 22 kDa. In contrast to yeast Bet3p, the majority of Bet3 is present in the cytosol. To investigate the possible involvement of Bet3 in transport events in mammalian cells, we utilized a semi-intact cell system that reconstitutes the transport of the envelope glycoprotein of vesicular stomatitis virus (VSV-G) from the ER to the Golgi apparatus. In this system, antibodies against Bet3 inhibit transport in a dose-dependent manner, and cytosol that is immunodepleted of Bet3 is also defective in this transport. This defect can be rescued by supplementing the Bet3-depleted cytosol with recombinant GST-Bet3. We also show that Bet3 acts after COPII but before Rab1, α-SNAP and the EGTA-sensitive stage during ER-Golgi transport. Gel filtration analysis demonstrates that Bet3 exists in two distinct pools in the cytosol, the high-molecular-weight pool may represent the TRAPP complex, whereas the other probably represents the monomeric Bet3.

Key words: Tethering complex, TRAPP, SNARE, Bet3, ER, Golgi, Vesicle, Fusion

Introduction

Intracellular protein transport (in either anterograde or retrograde direction) between different compartments of the secretory and endocytotic pathways is primarily mediated by vesicles that bud from a donor compartment and then fuse with a target compartment (Palade, 1975; Pryer et al., 1992; Ferro-Novick and Jahn, 1994; Rothman, 1994; Pfeffer, 1996; Rothman and Wieland, 1996; Schekman and Orci, 1996; Hong, 1998; Bonifacino and Glick, 2004). Although it is generally believed that SNAREs participate in the late stage of docking and subsequent fusion of vesicles with the target compartment (Sollner et al., 1993; Rothman, 1994; Scheller, 1995; Sudhof, 1995; Pfeffer, 1996; Sutton et al., 1998; Weber et al., 1998; Chen and Scheller, 2001), it is now clear that other proteins (or protein complexes) participate in an earlier event referred to as the tethering process, which serves to deliver vesicles to the proximity of the target membrane and to facilitate the subsequent fusion catalyzed by the SNAREs.

Tethering could be mediated either by multiprotein complexes (Waters and Pfeffer, 1999; Whyte and Munro, 2002; Pfeffer, 2001) or by long, rod-like, coiled-coil proteins (Gillingham and Munro, 2003; Barr, 2000). Several tethering complexes have been identified and include the exocyst (Sec6/Sec8) complex (Grindstaff et al., 1998), the COG (for 'conserved oligomeric Golgi') complex, the VFT/GARP ('Vps fifty-three' or 'Golgi-associated retrograde protein') complex, the HOPS ('homotypic fusion and vacuole protein sorting') complex and the TRAPP ('transport protein particle') complex. The exocyst complex is the best studied and it regulates polarized delivery of vesicles to defined regions of the plasma membrane, both in yeast and mammalian cells (Guo et al., 1997; Guo et al., 1999; TerBush et al., 1996; Kee et al., 1997; Hsu et al., 2004; Yeaman, 2003; Lipschutz and Mostov, 2002). The COG complex serves to regulate traffic at the Golgi apparatus (for both ER-Golgi and intra-Golgi transport) in both yeast and mammalian cells (Krieger et al., 1981; Kingsley et al., 1986a; Kingsley et al., 1986b; Podos et al., 1994; Wuestehube et al., 1996; VanRheenen et al., 1998; VanRheenen et al., 1999; Walter et al., 1998; Chatterton et al., 1999; Spelbrink and Nothwehr, 1999; Kim et al., 1999; Kim et al., 2001; Whyte and Munro, 2001; Ram et al., 2002; Ungar et al., 2002; Suvorova et al., 2001; Suvorova et al., 2002; Loh and Hong, 2002; Farkas et al., 2003). Mammalian proteins homologous to components of the HOPS complex are similarly implicated in endosomal/lysosomal trafficking (Richardson et al., 2004). Although components homologous to yeast TRAPP

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and VFT/GARP complexes have been identified in mammalian cells, their functional importance in mammalian cells has not been established (Sacher and Ferro-Novick, 2001).

During ER-Golgi transport in yeast, the TRAPP complex has been shown to function as a docking site on the early Golgi for COPII-generated vesicles derived from the ER, and the TRAPP complex functions as membrane machinery (Sacher et al., 2001). Components of TRAPP interact genetically with SNAREs and Ypt1p (Morsomme and Riezman, 2002; Sacher et al., 2001). The TRAPP complex consists of ten subunits: Bet3p, Bet5p, Trs20p, Trs23p, Trs31p, Trs33p, Trs65p/Kre11p, Trs85p/Gsg1p, Trs120p and Trs130p. The holo-complex is referred to as TRAPP II, whereas a sub-complex consisting of Bet3p, Bet5p, Trs20p, Trs23p, Trs31p, Trs33p and Trs85p/Gsg1p is referred to as TRAPP I (Sacher et al., 2001). TRAPP I and TRAPP II act in ER-Golgi and intra-Golgi transport, respectively. Biochemically, TRAPP functions as a guanine nucleotide exchange factor (GEF) for the small GTPases Ypt1p, Ypt8p/Ypt31p and Ypt11p/Ypt32p (Jones et al., 2000; Wang et al., 2000). Among the structurally homologous proteins in mammalian cells, Bet3 is the most conserved and therefore it could perform the most important/conserved function compared with the other subunits of the TRAPP complex. Among the ten subunits, Trs33p, Trs65p and Trs85p are dispensable for cell growth, whereas the other seven subunits are all essential for cell growth (Sacher et al., 2000).

Knowledge about protein traffic using the yeast system will enable better understanding of the process in the mammalian system. We have investigated the biochemical and functional aspects of Bet3 so as to establish the functional importance of the TRAPP complex in traffic in mammalian cells. Our results suggest that, although Bet3 is functionally conserved in ER-Golgi transport, its mode of action is significantly different in mammalian cells.

Materials and Methods

Materials

Normal rat kidney (NRK) cells and HeLa cells were obtained from the American Type Culture Collection (ATCC). Synthetic oligonucleotides were purchased from Oligos Etc. Gluthathione-Sepharose 4B was a product of Pharmacia. The pQE-60 HisX6 vector, M15(pREP4) bacterial strain and Ni^{++}-NTA beads were from Qiagen. The pEGFP-C3 vector was from Clontech. Hybond C+ nitrocellulose and Taq DNA polymerase were purchased from Amersham. Pfu DNA polymerase was a product of Stratagene. Supersignal substrate and peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies were products of Pierce. Freunds adjuvants were obtained from Life Technologies-BRL. New Zealand White rabbits were purchased from Sembawang Laboratory Animals Centre. Monoclonal anti-β-COP antibodies (ascites maD), monoclonal anti-β-actin and monoclonal anti-β-tubulin (mouse ascites fluid) were purchased from Sigma.

Northern blot analysis

A cDNA fragment of about 540 bp obtained by the *Bam*HI and *Hin*dIII digestion of mouse EST clone (accession number AA255291) was radiolabeled with ^{32}P using the procedure as described previously (Lowe et al., 1996). A mouse multiple tissue northern blot containing 2 μg of polyA+ mRNA from the indicated tissues (Clontech) was hybridized with the probe and processed as described previously (Lowe et al., 1996).

Expression of recombinant proteins in *Escherichia coli*

Primer 1 (5′ GCC ATG GAA ATG TCG AGG CAG GCG) and primer 2 (5′ CGC GGA TCC CTC TTC TCC AGC TGG) were used to amplify the coding region of mouse Bet3 by polymerase chain reaction (PCR). The PCR product was digested with *Nco*I and *Bam*HI and ligated into the corresponding sites of pQE60 vector. The ligation reaction was transformed into competent M15(pRep4) bacterial cells. Cells harboring properly inserted Bet3 coding region were identified and used for the purification of C-terminally HisX6-tagged Bet3 (Bet3-His6). Bacterial cultures were grown in 2XTY broth containing 100 μg/ml carbenicillin and 25 μg/ml kanamycin at 37°C until an OD_{600} of 0.8 was reached. Expression of the recombinant protein was induced by the addition of isopropylthiogalactoside (IPTG) to 0.5 mM, after which cells were grown at room temperature for a further 12 hours. Bacterial cultures were pelleted and the pellet was resuspended in cracking buffer (100 mM Hepes pH 7.3, 500 mM KCl, 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 mM PMSF and 0.1% Triton X-100) by sonication for 3 minutes. The lysate was centrifuged for 20 minutes at 12,000 ***g*** in a refrigerated SS-34 rotor (Sorvall). The supernatant was passed twice through a column of Ni^{++}-NTA resin (Qiagen) pre-equilibrated with buffer A (20 mM Hepes pH 7.3, 100 mM KCl, 2 mM β-mercaptoethanol, 10% glycerol) containing 25 mM imidazole. The beads were washed with 50 volumes of buffer A containing 25 mM imidazole. Bound protein was eluted in buffer A containing 250 mM imidazole. 1 ml fractions were collected and analyzed by SDS-PAGE. Fractions containing Bet3-His6 were pooled and dialyzed against phosphate-buffered saline (PBS).

To prepare recombinant glutathione sulphotransferase (GST)-fused Bet3 (GST-Bet3), primer 3 (5′ CGC GGA TCC ATG TCG AGG CAG GCG) and primer 4 (5′ CCC AAG CTT CTC TTC TCC AGC TGG) were used to amplify the Bet3 coding sequence by PCR. The resulting product was digested with *Bam*HI and *Hin*dIII, ligated into the corresponding sites of pGEX-KG vector (Guan and Dixon, 1991) and transformed into competent BL21 cells. Cells harboring the recombinant plasmid were identified and used for the purification of GST-Bet3. Bacterial cultures grown at 37°C to an OD_{600} of 0.8 were induced to express GST-Bet3 by the addition of IPTG to a final concentration of 0.5 mM. After a 12 hour induction at room temperature, cells were harvested by centrifugation. The bacterial pellet was resuspended in lysis buffer (PBS containing 50 mM Tris pH 8, 0.5 mM $MgCl_2$, 0.1% Triton X-100) containing 1 mM PMSF and 1 mg/ml lysozyme and lysed by sonication for 3 minutes. Clarified lysates were incubated with glutathione Sepharose 4B (Pharmacia) beads for 1 hour at 4°C. The beads were washed with 50 volumes of lysis buffer and bound protein was eluted with 15 mM reduced glutathione in 20 mM Tris pH 8 with 10% glycerol. Fractions were collected and assayed for GST-Bet3 by SDS-PAGE.

Preparation of polyclonal antibodies

250 μg of Bet3-His6 was emulsified with Freund's complete adjuvant and injected subcutaneously into two New Zealand White rabbits. Booster injections with the same amount of antigen in Freund's incomplete adjuvant were administered every two weeks. The rabbits were bled ten days after the second and subsequent boosters. The antiserum was first diluted with an equal volume of PBS and then incubated overnight at 4°C with GST-Bet3 coupled to cyanogen bromide-activated Sepharose-4B. The beads were washed extensively and Bet3-specific antibodies were eluted with low pH elution buffer as described (Lowe et al., 1996).

Immunofluorescence microscopy

NRK cells were grown on coverslips overnight to 50-80% confluence, rinsed twice with PBS with 1 mM $CaCl_2$ and 1 mM

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$MgCl_2$ (PBSCM), and processed as described previously (Lowe et al., 1996). Double labeling of the cells was performed using Bet3 antibodies (5-10 μg/ml) and monoclonal anti-β-COP antibodies or GS28 antibodies in fluorescence dilution buffer (PBSCM with 5% normal goat serum, 5% fetal bovine serum, and 2% bovine serum albumin, pH 7.6). For the antibody neutralization experiment, 0.5 μg of Bet3-His6 was used to neutralize the antibodies. After incubation for 1 hour with the primary antibodies, the cells were washed and then incubated with goat anti-rabbit conjugated to Texas-Red and FITC-conjugated goat anti-mouse secondary antibodies. For NRK cells that were permeabilized prior to fixation, the cells were first incubated with PBSCM containing 0.1% saponin for 30 minutes and then fixed with paraformaldehyde and processed accordingly. To assess the distribution of Bet3 in live cells, N-terminally green fluorescence protein (GFP)-tagged Bet3 was expressed transiently in transfected HeLa cells and visualized with a Zeiss Axiovert 200M fluorescence inverted microscope.

Immunoblotting

Proteins were separated by SDS-PAGE and electro-transferred onto Hybond C+ nitrocellulose. The blots were then incubated for 1 hour at 37°C or overnight at 4°C in blocking buffer (5% non-fat milk, 5% fetal bovine serum in PBS containing 0.05% Tween-20). Subsequently, the blots were incubated in blocking buffer containing primary antibodies for 1 hour at room temperature followed by three washes with PBS containing 0.05% Tween-20 for five minutes each. The blots were then incubated with either goat anti-rabbit or anti-mouse antibody conjugated to horseradish peroxidase (HRP). After three washes in PBS containing 0.05% Tween-20, supersignal substrate (Pierce) was added and the blots were processed according to the manufacturer's protocol.

Cellular fractionation

NRK cells were trypsinized, washed with media containing 10% FCS, and resuspended in 300 μl buffer containing 20 mM Hepes (pH 7.4), 100 mM KCl and 2 mM EDTA. The cells were sonicated twice (15 seconds each) and centrifuged at 600 ***g*** to remove unbroken cells and nuclei. The resulting postnuclear supernatant was centrifuged at 120,000 ***g*** for 1 hour using the TLA 100 rotor (Beckman). The pellet was resuspended in the same volume as the supernatant. 15 μl of the pellet fraction (total membranes, TM) and 15 μl of the supernatant (cytosol) were analyzed and immunoblotted to detect Bet3, GS28, actin and tubulin. The distribution of the proteins between the TM and cytosolic fraction was quantitated using a Biorad GS-800 calibrated densitometer.

Preparation of membrane fractions

Livers from Sprague-Dawley rats (fasted overnight) were rinsed in ice-cold PBS and then minced in homogenization buffer (0.25 M sucrose, 25 mM Hepes pH 7.4, 5 mM $MgCl_2$, 1 mM PMSF). The minced material was homogenized in about three volumes of homogenization buffer and centrifuged at 2000 ***g***. The postnuclear supernatant (PNS) was spun at 150,000 ***g*** for 60 minutes to pellet the TM fraction. The supernatant (cytosol) was also saved. The TM was resuspended in 1.25 M sucrose in centrifuge tubes and then overlaid step-wise with a gradient of 1.1 M and 0.5 M sucrose. After centrifugation at 100,000 ***g*** for 3 hours in a SW28 rotor (Beckman), the Golgi-enriched membrane (G) fraction, which is present at the 0.5/1.1 M interphase, and the microsomal pellet (M), were harvested. All the membrane fractions were diluted fourfold with 25 mM Hepes (pH 7.4), 5 mM $MgCl_2$ and 1 mM PMSF, and pelleted using a Type 45i rotor (Beckman) at 180,000 ***g*** for 1 hour. Membranes were resuspended in homogenization buffer and flash frozen.

Differential extraction of membrane fraction

The Golgi-enriched membrane fraction (25 μg) was extracted in a total volume of 200 μl in PBS, 1 M KCl, 2.5 M Urea, 150 mM Na_2CO_3 (pH 11.5) or 1% deoxycholate (DOC) on ice for 1 hour. After centrifugation at 120,000 ***g*** in a TLA 100 rotor (Beckman) for 1 hour, the resulting supernatant and pellet were separated by SDS-PAGE and processed for immunoblotting to detect Bet3 and GS28.

In vitro ER-Golgi transport assay

The biochemical assay to measure the transport of envelope glycoprotein of vesicular stomatitis virus (VSV-G) from the ER to the cis-Golgi was performed as described (Beckers et al., 1987; Beckers and Balch, 1989; Davidson and Balch, 1993). Briefly, NRK cells were grown on 10 cm petri dishes to form a confluent monolayer and infected with a temperature-sensitive strain of VSV, VSVts045. The cells were pulse labeled with [^{35}S]-methionine at the restrictive temperature (40°C) and then mechanically perforated on ice as described (Beckers et al., 1987; Beckers and Balch, 1989; Davidson and Balch, 1993). These semi-intact cells were then incubated in a complete assay cocktail of 40 μl containing (in final concentrations) 25 mM Hepes-KOH pH 7.2, 90 mM KOAc, 2.5 mM $Mg(OAc)_2$, 5 mM EGTA, 1.8 mM $CaCl_2$, 1 mM ATP, 5 mM creatine phosphate, 0.2 IU of rabbit muscle creatine kinase, 25 μg rat liver cytosol (Davidson and Balch, 1993) and 5 μl (ca. 1-2×10^5 cells) of semi-intact cells. Additional reagents were added as indicated in the Results. Samples were incubated for 60 minutes at 32°C and transport terminated by transfer to ice. The membranes were collected by a brief spin in a microfuge, solubilized in 0.1% SDS and digested overnight with endoglycosidase H (endo-H). Subsequently, 5× SDS sample buffer was added and the samples were separated on 7.5% SDS polyacrylamide gels. Transport was quantified using a Phosphor Imager (Molecular Dynamics).

Immunodepletion of Bet3 from rat liver cytosol

CNBR-activated Sepharose 4B (Pharmacia) was prepared according to the manufacturer's protocol. Saturating amounts of affinity-purified Bet3 antibodies were bound to the beads at 4°C overnight and the beads subsequently blocked with glycine. After extensive washing, the beads were equilibrated with PBS to make a 50% suspension. 100 μl of suspension was incubated with 100 μl (about 2.5 mg) of rat liver cytosol (rlc) for 2 hours at 4°C, and the beads removed by centrifugation. The depletion was checked by western blotting to detect Bet3. The depleted cytosol was flash frozen in liquid nitrogen. As control depletion (mock), the identical procedure was performed using an antibody against haemagglutinin (HA).

Gel filtration

Samples were run in 200 μl aliquots on a FPLC Superdex 75 column (Pharmacia) equilibrated with PBS containing 1% deoxycholate at 4°C. Flow rate was 0.5 ml/min and fractions of 0.7 ml each were collected. The fractions were precipitated with trichloroacetic acid (TCA), separated on SDS-PAGE, transferred to nitrocellulose and blotted with the indicated antibodies.

Results

Summary of mammalian homologs of TRAPP complex and identification of a new homolog of Trs33p

Mammalian proteins homologous to yeast TRAPP were identified either by amino acid homologies or independently by studies of various mammalian proteins (Gavin et al., 2002; Sacher and Ferro-Novick, 2001). Among the ten TRAPP components, seven (Bet3p, Bet5p, Trs20p, Trs23p, Trs31p,

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Trs33p and Trs130p) have structural homologs in humans (Fig. 1A). Despite extensive database searches, mammalian proteins that exhibit significant amino acid relatedness to Trs65p, Trs85p or Trs120p are not found. Although an early proteomic study of immunoprecipitated TRAPP complex (via tagging Bet3) has indicated that KIAA1012 could be a counterpart of Trs85p (Gavin et al., 2002), their amino acid sequences do not show convincing homology. Interestingly, several proteins, including KIAA1012, hypothetical protein AL136752.1, CGI-85 and hypothetical protein FLJ13611, are co-immunoprecipitated with tagged Bet3. Since these proteins do not show amino acid homology with Trs65p, Trs85p or

A

Subunit	Yeast (# of AA)	Human (# of AA)	AA identity	TRAPP I	TRAPP II
Bet5	159	145	30%	+	+
Trs20	175	140(SEDL)	40%	+	+
Bet3	193	180	53%	+	+
Trs23	219	219(Synbindin)	30%	+	+
Trs31	283	188	27%	+	+
Trs33	268	160(Trs33A) 158(Trs33B)	24% 33%	+	+
Trs65	560(Krel1p)	-	-	-	+
Trs85	698(Gsg1p)	-	-	+	+
Trs120	1289	-	-	-	+
Trs130	1102	1259(EHOC-1/GT334)	12%(28%)	-	+

B

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TRS33B (h)  :                                MADEALFLLLHNEMVSGVYKSAEQGEV-----------------------------------------------ENGR--CITKLENM :  39
TRS33B (m)  :                                MADEALFLLLHNEMVSGVYKSAEQGEV-----------------------------------------------ENGR--CVTKLESM :  39
TRS33B (zf) :                                MADDALFEFLHMEIVAHVYKEQATRDDI-----------------------------------------------DKERVTCVSTLELM :  42
TRS33A (h)  :                                MADTVLFEFLHTEMVAELWAHDPDPG-------------------------------------------------PGGQKMSLSVLEGM :  41
Trs33p      : MSSTHSNNVGHPQSSPQGPLTEQQRAQQQYQIFENSLPKVSQSVYQMLLNEMVPLAMGIERQISGDVISSDSNVTSENGNINNMIKRLKIEEHHTVDIIRSHNLIHELYKADEEEKEKVLARLRNI : 126


TRS33B (h)  : GFRVGQGLIERFT---KDTARFKDE--LDIMKFICKDFWTTVFKKQIDNLRTNHQGIYVLQDNKFRLLTQMS----AGKQYLEHASKYLAFTCGLIRGGLSNLGIKS---IVTAEVSSMPA----- : 148
TRS33B (m)  : GFRVGQGLIERFT---KDTARFKDE--LDIMKFICKDFWTTVFKKQIDNLRTNHQGIYVLQDNKFRLLIQLS----AGKQYLEHASKYLAFTCGLIRGGLSNLGIKS---IVTAEVSSMPA----- : 148
TRS33B (zf) : GFRVGQGLIERFT---KDCPTFKDD--LDIMKFVCKDFWSTIFKKQIDNLRTNHQGTYVLQDNKFALLTQFS----SGKQYLEEAPKYLAFSCGMIRGALSNLGLES---VVTAEVSLMPS----- : 151
TRS33A (h)  : GFRVGQALGERLP---RETLAFREE--LDVLKFLCKDLWVAVFQKQMDSLRTNHQGTYVLQDNSFPLLLPMA----SGLQYLEEAPKFLAFTCGLLRGALYTLGIES---VVTASVAALPV----- : 150
Trs33p      : GFQIGLKLSELLIFSNNPNLKFKEMDLLLIMKFICRDVWKQIFGKQIDNLKTNHRGTFYLLDYDYRPIQSFSLEEDAKNEELKMIEPFLEIPVGIIRGVLSSLGYSSEEVICLASFIDRPTDRPKT : 252


TRS33B (h)  : -----CKFQVMIQKL  : 158
TRS33B (m)  : -----CKFQVMIQKL  : 158
TRS33B (zf) : -----CKFQVVVQKL  : 161
TRS33A (h)  : -----CKFQVVIPKS  : 160
Trs33p      : AFPKGVSFHVQVTMPQ : 268
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C

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BET5  : MT-VHNLYLFDRNGVCLHYSEWHRKKQAGIPKEEEY---------------------------------------------------- :  35
TRS20 : MSGSFYFVIVGHHDNPVFEMEFLPAGKAESKDDH---------------------------------------------------- :  34
TRS23 : MA-IFSVYVVNKAGGLIYQLDSYAPRAEAEKTFSYPLDLLLKLHDERVLVAFGQRDGIRVGHAVLAINGMDVNGRYTADGKEVLEYLGN :  88


BET5  : -------------------KLMYG-MLFSIRSFVSKMSPLDMKDGFLAFQT-SRYKLHYYETPTGIKVVMNTDLGV-GPIRDVLHHIYS : 102
TRS20 : -------------------RHLNQFIAHAALDLVDENMWLSNNMYLKTVDKFNEWFVSAFVTAGHMRFIMLHDIRQEDGIKNFFTDVY- : 103
TRS23 : PANYPVSIRFGRPRLTSNEKLMLASMFHSLFAIGSQLSPEQGSSGIEMLET-DTFKLHCYQTLTGIKFVVLADPRQ-AGIDSLLRKIY- : 174


BET5  : ALYVELVVKNPLCPLGQTVQSELFRSRLDSYVRSLPFFSARAG    : 145
TRS20 : DLYIKFSMN-PFYEPNSPIRSSAFDRKVQFLGKKHLLS         : 140
TRS23 : EIYSDFALKNPFYSLEMPIRCELFDQNLKLALEVAEKAGTFGPGS  : 219
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D

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BET3  :            MSRQANRGTESKKMSSELFTLTYGALVTQLCKD--YENDEDVNKQLDKMGFNIGVRLIEDFLARSNVGRCHDFRETADVIAKVAFKMYLG :  88
TRS31 : MEARFTRGK-SALLERALARPRTEVSLSAFALLFSELVQHCQSR--VFSVAELQSRLAALGRQVGARVLDALVAR--EKGARRETKVLGALLFVKGAVWKA :  96
TRS33A :                       GMADTVLFEFLHTEMVAELWAHDPDPGPGGQKMSLSVL-EGMGFRVGQALGERL-PRETLAFREELDVLKFLCKDLWVA :  77
TRS33B :                        MADEALFLLLHNEMVSGVYKS-AEQGEVENGRCITKL-ENMGFRVGQGLIERF-TKDTARFKDELDIMKFICKDFWTT :  75


BET3  : ITPSITNWSPAGDEFSLILENN---PLVDFVELPDNHSS-LIYSNLLCGVLRGALEMVQMAVEAKFVQDTLKGDGVTEIRMRFIRRIEDNLPAGEE  : 180
TRS31 : LFGKEADKLEQANDDARTFYIIEREPLINTYISVPKENSTLNCASFTAGIVEAVLT-----HSGFPAKVTAHWHKGTTLMIKFEEAVIARDRALEGR : 188
TRS33A : VFQKQMDSLRTNHQGTYVLQDNSFPLLLPMASGLQYLEEAPKFLAFTCGLLRGALY-------TLGIESVVTASVAALPVCKFQVVIPKS       : 160
TRS33B : VFKKQIDNLRTNHQGIYVLQDNKFRLLTQMSAGKQYLEHASKYLAFTCGLIRGGLS-------NLGIKSIVTAEVSSMPACKFQVMIQKL       : 158
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Fig. 1. Summary of mammalian TRAPP subunits. (A) Subunits of the TRAPP complex: the name of the subunit, residue number of yeast or human protein, the amino acid (AA) sequence identity between yeast and human protein, and the presence/absence in TRAPP1 and II are indicated. (B) Amino acid sequence alignment of Trs33B of difference species with human Trs33A and yeast Trs33p (h: human; m: mouse; zf: zebrafish). (C) Bet5, TRS20 and TRS23 are related. The alignment of amino acid sequences of human Bet5, TRS20 and TRS23 is shown. (D) Related sequence homology among human Bet3, TRS31, TRS33A and TRS33B. Identical as well as conserved residues are highlighted in gray.

Trs120p, some of these proteins could be functional counterparts of yeast Trs65p, Trs85p or Trs120p. A similar phenomenon was observed for COG complex in that five of the eight subunits are structurally conserved between yeast and humans, whereas the other three unique proteins could represent functional counterparts (Ungar et al., 2002). Human Trs20 was independently identified as SEDL and has been implicated in ER-Golgi transport (Jang et al., 2002). Trs23 was independently identified as synbindin, which is expressed in neurons in a similar pattern to that of syndecan-2. It binds to the C-terminal cytoplasmic signal Glu-Phe-Tyr-Ala (EFYA) of syndecans and is implicated in the establishment and/or function of dendritic spines synapses (Iryna et al., 2000).

We have searched human genome and other databases with all the yeast and the known mammalian proteins of the TRAPP complex and have identified another novel protein homologous to Trs33p and human Trs33 (Fig. 1B). We have tentatively named the novel protein as Trs33B and the original Trs33 as Trs33A. Trs33B is well conserved among humans, mouse and zebrafish and has a higher amino acid identity (33%) to Trs33p than Trs33A (24%). Trs33A and Trs33B have 56% amino acid sequence identity. Although the biochemical properties of Trs33B and the four candidate functional homologs (KIAA1012, AL136752.1, CGI-85, FLJ1361) will need to be established, these observations strongly suggest that mammalian cells have TRAPP complex with similar compositions. This information is summarized in Fig. 1A. Interestingly, mammalian Bet5, Trs20 and Trs23 showed some sequence relatedness as aligned in Fig. 1C, whereas the sequence homology among mammalian Bet3, Trs31, Trs33A and Trs33B is noticed based on the alignment shown in Fig. 1D. The significance of homology among different subunits of the TRAPP complex remains to be examined. Since Bet3 is the most conserved subunit (53% identity between yeast and human), we decided to focus in more detail on the biochemical, cell biological and functional aspects of Bet3.

Bet3 is expressed ubiquitously

To examine whether Bet3 is involved in a common biological process or in a specific event unique to certain tissues, the expression of Bet3 mRNA was examined in eight different mouse tissues by northern blot analysis (Fig. 2). A mouse multiple tissue northern blot containing 2 μg polyA+ RNA of the indicated tissues was hybridized with radiolabeled Bet3 probe. A single transcript of about 1.6 kb was detected in all tissues at comparable levels except for the skeletal muscle, which expresses a lesser amount of Bet3 mRNA. The ubiquitous expression of Bet3 mRNA suggests that Bet3 might participate in a biological event that occurs in all cells.

Bet3 is a 22 kDa protein present in both membrane-bound and cytosolic forms

The entire coding region of mouse Bet3 was expressed as a C-terminally HisX6-tagged recombinant protein (Bet3-His6) as well as an N-terminal GST fusion (GST-Bet3) in *E. coli*. Purified Bet3-His6 was used to immunize rabbits, and antibodies specific for Bet3 were isolated by affinity purification using the recombinant GST-Bet3 crosslinked to

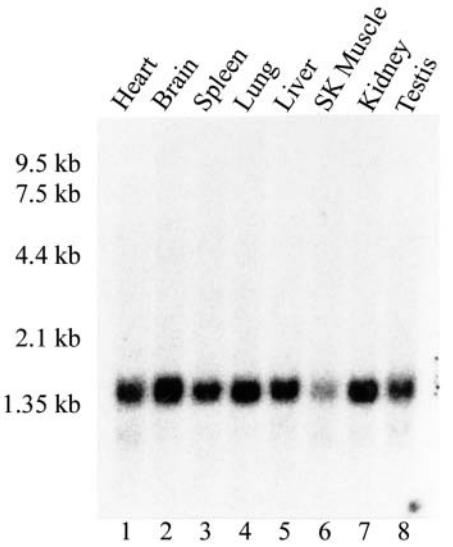

Fig. 2. Northern blot analysis of Bet3 mRNA in eight mouse tissues. A multiple tissue blot (Clontech) containing 2 μg of polyA+ RNA from the indicated tissues was hybridized with ^{32}P-labeled Bet3 probes and processed for autoradiography.

glutathione-sepharose beads. The antibodies were used to determine the distribution of Bet3 in subcellular fractions obtained from rat liver. A post-nuclear supernatant (PNS) obtained after a low-speed spin (2000 ***g***), which removes unbroken cells, cell debris and nuclei, was centrifuged at 150,000 ***g*** to yield a total membrane (TM) pellet and a supernatant that comprises the cytosol (C). The TM pellet was then floated onto sucrose gradients to give a microsomal (M) pellet and the lighter Golgi-enriched membrane (G) fraction. 25 μg of proteins from each fraction was separated by SDS-PAGE and immunoblotted to detect Bet3 and the Golgi SNARE, GS28 (Subramaniam et al., 1995; Subramaniam et al., 1996) or stained with Coomassie Blue R-250 (Fig. 3A). As shown by the protein profiles (Fig. 3A, right panel), comparable amounts of proteins from these fractions were analyzed. GS28 is detected predominantly in the G fraction, as expected for an integral membrane protein of the Golgi apparatus (Fig. 3A, center panel). A single polypeptide with an apparent molecular weight of 22 kDa was detected by Bet3 antibodies (Fig. 3A, left panel). Detection of this 22 kDa polypeptide by Bet3 antibodies is specific because pre-incubation of the antibodies with recombinant GST-Bet3 but not with GST or other GST fusion proteins abolished its detection (data not shown). Bet3 is significantly enriched in the G fraction but is also present in the cytosolic fraction (Fig. 3A, left panel). These results indicate that Bet3 exists both in a soluble form in the cytosol and in a membrane-bound form that fractionates with the G fraction.

The majority of Bet3 is present in the cytosol

Although Bet3 is present both in cytosolic and membrane-bound fractions, it is apparent that more Bet3 is associated with the cytosol because much more cytosol was obtained as compared with the amount of Golgi-enriched membranes during the subcellular fractionation of rat liver. In order to gain

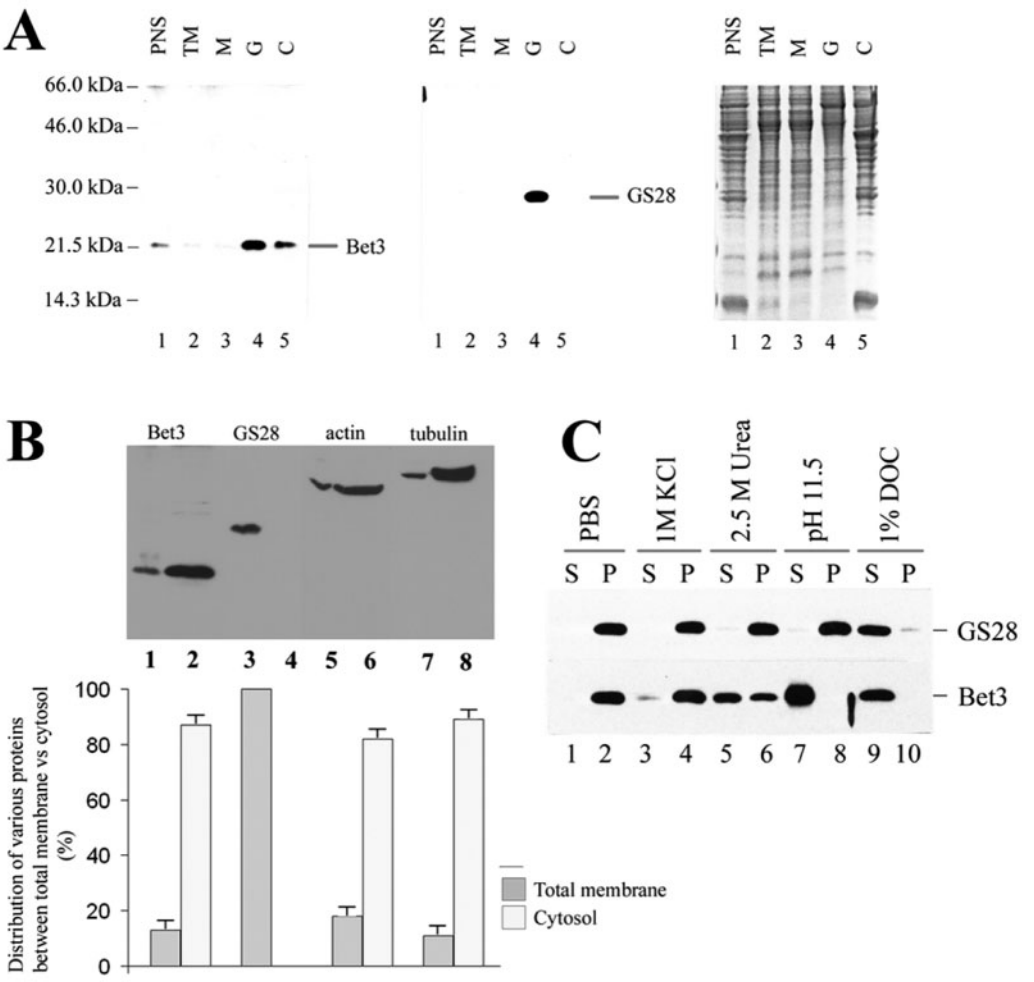

Fig. 3. (A) Distribution of Bet3 in various subcellular fractions derived from rat liver. 25 μg of protein from the indicated fractions (PNS, postnuclear supernatant; TM, total membrane; M, microsome-enriched membrane; G, Golgi-enriched membranes; C, cytosol) was separated by SDS-PAGE and stained with Coomassie Blue R-250 (right panel) or transferred to filters and immunoblotted with antibodies against Bet3 (left panel) or GS28 (centre panel). (B) Bet3 is present predominantly in the cytosol. NRK cells were fractionated into total membrane (TM; odd lanes) and cytosol (C; even lanes) fractions. Equivalent fractions of TM and C were separated by SDS-PAGE, transferred to a filter and immunoblotted to detect Bet3 (lanes 1 and 2), GS28 (lanes 3 and 4), β-actin (lanes 5 and 6) or β-tubulin (lanes 7 and 8). Their distributions between TM and C fractions were quantitated as shown below the blot. (C) Bet3 is a peripheral membrane protein. Golgi-enriched membranes were extracted with PBS, 1 M KCl, 2.5 M urea, 150 mM sodium carbonate (pH 11.5), or 1% sodium deoxycholate (1% DOC), respectively. The resulting supernatants (S) and pellets (P) were separated by SDS-PAGE, transferred to a filter and immunoblotted to detect GS28 (upper panel) or Bet3 (lower panel).

more insight into this, the distribution of Bet3 in cultured cells was analyzed.

Normal rat kidney (NRK) cells were processed to yield TM pellet and the C fraction. The TM pellet was resuspended in a volume of buffer equivalent to the C fraction. Equal volumes of the TM and C fractions were resolved by SDS-PAGE, and transferred onto nitrocellulose membranes and immunoblotted with antibodies against Bet3, GS28, β-actin and β-tubulin (Fig. 3B). As shown, GS28 is found exclusively in the TM fraction (lanes 3 and 4). The distribution of Bet3 (lanes 1 and 2) is similar to that of actin and tubulin (lanes 5-8), with more being detected in the C fraction than the TM. The ratio of the abundance of these cytosolic proteins between the TM and C fractions is approximately 15% versus 85%. These results suggest that, although a significant amount of Bet3 is membrane bound, the majority of Bet3 is present in the cytosol.

The membrane pool of Bet3 is tightly associated with the membrane

The presence of a significant amount of Bet3 in the G fraction led us to examine the nature of this association. Golgi-enriched membranes isolated from rat liver were extracted with PBS, 1 M KCl, 2.5 M urea, 0.1 M sodium carbonate (pH 11.5) and 1% sodium deoxycholate (DOC). The extracts were centrifuged at 100,000 ***g***. The resulting supernatants and pellets were analyzed by immunoblot to detect Bet3 and GS28 (Fig. 3C). As shown previously (Subramaniam et al., 1995), GS28 is not extracted by PBS (lanes 1 and 2), 1 M KCl (lanes 3 and 4), 2.5 M urea (lanes 5 and 6) or alkaline pH (lanes 7 and 8), but is efficiently extracted by detergent (lanes 9 and 10). Interestingly, Bet3 is not extracted by PBS (lanes 1 and 2) or 1 M KCl (lanes 3 and 4), suggesting that Bet3 is tightly associated with the membrane. However, the majority of Bet3 could be extracted by 2.5 M urea (lanes 5 and 6), whereas complete extraction of Bet3 from the membrane was achieved by pH 11.5 (lanes 7 and 8) or the detergent (lanes 9 and 10), suggesting that the membrane pool of Bet3 behaves like a tightly-associated peripheral membrane protein.

Analysis of the localization of Bet3 by immunofluorescence microscopy

Using Bet3 antibodies in immunofluorescence microscopy, Bet3 was also seen to be present in the cytosol (Fig. 4A, a); by contrast, β-COP antibodies label the Golgi apparatus (Fig. 4A,

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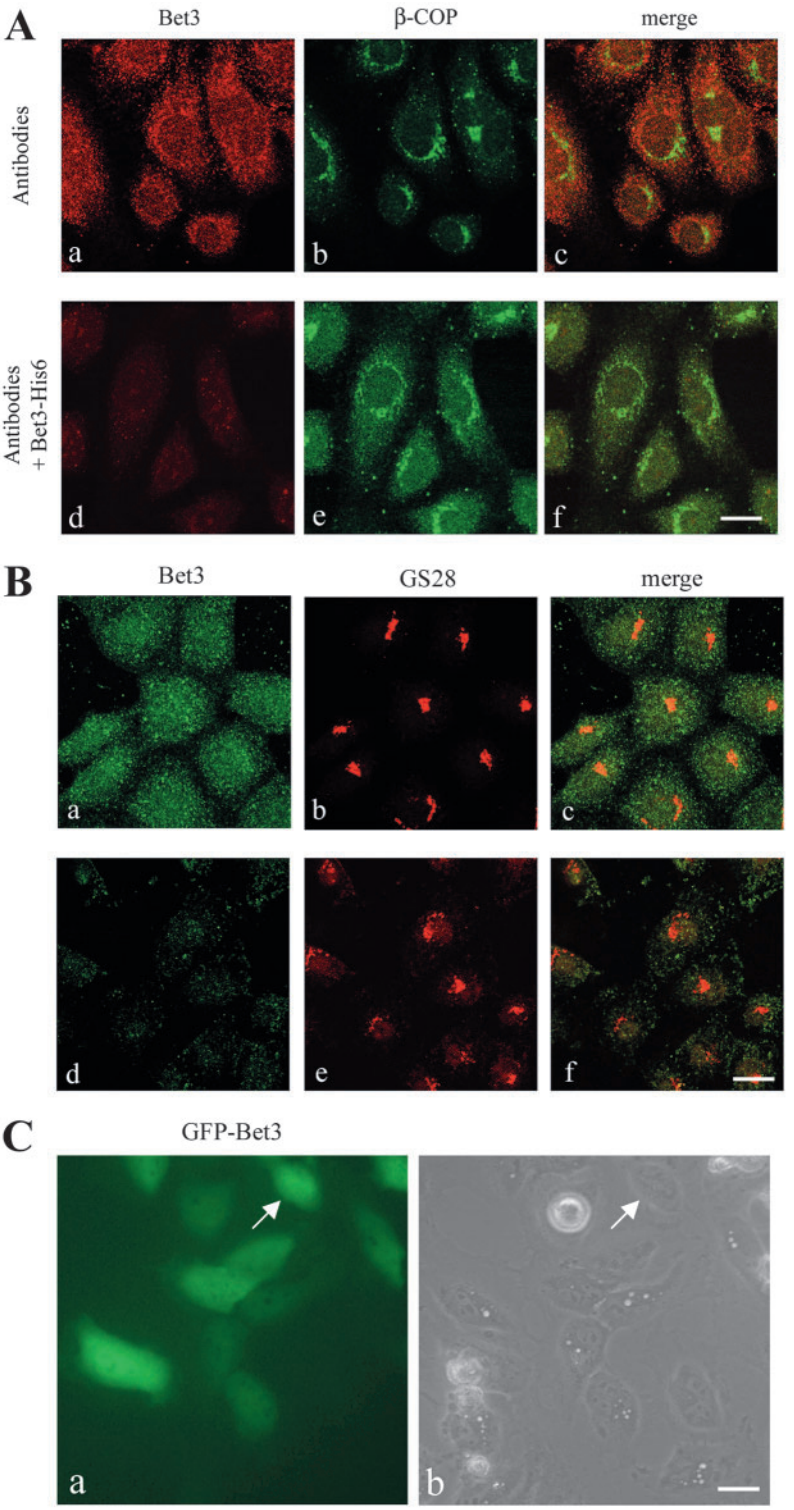

Fig. 4. Bet3 is distributed in the cytosol. (A) NRK cells were fixed, permeabilized and double labeled with Bet3 antibodies (a and d) and a monoclonal antibody against β-COP (b and e). The cytosolic labeling of Bet3 (d) but not β-COP (e) was abolished by prior incubation of the antibodies with Bet3-His6. The merged images are shown in c and f. Bar, 10 μm. (B) Double labeling of Bet3 antibodies (a and d) and GS28 antibodies (b and e). NRK cells were permeabilized with saponin after (a-c) or before (d-f) fixation with paraformaldehyde. The merged images are shown in c and f. Bar, 10 μm. (C) HeLa cells were transiently transfected to express GFP-Bet3, which displayed cytosolic distribution in live cells expressing low-to-medial levels of GFP-Bet3. Some nuclear accumulation was detected in cells expressing very high levels (marked by white arrow).

b) in NRK cells. Furthermore, the cytosolic labeling of Bet3 (Fig. 4A, d) but not β-COP (Fig. 4A, e) was abolished by pre-incubation of the antibodies with Bet3-His6, further confirming the specificity of the antibodies. In contrast to cells that were fixed before permeabilization (Fig. 4B, a), the majority of Bet3 had leaked out if NRK cells were first permeabilized before fixation (Fig. 4B, d). Whatever is left in the cell is not enriched in any particular structure in a way that is noticeable by immunofluorescence. This behavior is different from that of GS28, in which its Golgi labeling is undisturbed whether the permeabilization with saponin was performed before (Fig. 4B, e) or after (Fig. 4B, b) fixation. The cytosolic distribution of Bet3 is further confirmed by assessing its distribution in live cells when N-terminally GFP-tagged Bet3 was expressed transiently in transfected HeLa cells. As GST-Bet3 can functionally replace depleted cytosolic Bet3 in ER-Golgi transport (see below), we believe that N-terminally GFP-tagged Bet3 is functionally intact. We have monitored the distribution of GFP-Bet3 in cells with different levels of expression. As seen in Fig. 4C, GFP-Bet3 displayed cytosolic distribution in live cells expressing low-to-medial levels of GFP-Bet3. Some nuclear accumulation was detected in cells expressing very high levels of GFP-Bet3 (e.g. Fig. 4C, cell marked by white arrow).

Antibodies against Bet3 inhibit in vitro ER-Golgi transport

To investigate the possible involvement of Bet3 in transport events in mammalian cells, we utilized a semi-intact cell system that reconstitutes the transport of the envelope glycoprotein of vesicular stomatitis virus (VSV-G) from the ER to the Golgi apparatus (Beckers et al., 1987; Davidson and Balch, 1993; Balch et al., 1994). The transport of VSV-G is monitored by following the extent of conversion of the endo-H-sensitive ER form to the endo-H-resistant Golgi form. In a standard assay in the presence of rat liver cytosol (rlc) and an ATP-regenerating system, about 70-80% of VSV-G was transported (Fig. 5, lane 2), whereas only background levels (around 10%) of transport were detected when the assay was performed on ice (lane 1). As shown previously (Subramaniam et al., 1996), in vitro ER-Golgi transport was potently inhibited by antibodies against GS28 (lane 3). Significantly, transport was also inhibited by Bet3 antibodies in a dose-dependent manner (lanes 4 to 9). Half-maximal inhibition was observed with approximately 1.5 μg of Bet3 antibodies (lane 6), whereas 2 μg or more of Bet3 antibodies inhibited the transport to background levels (lanes 7-9). To establish that the inhibition

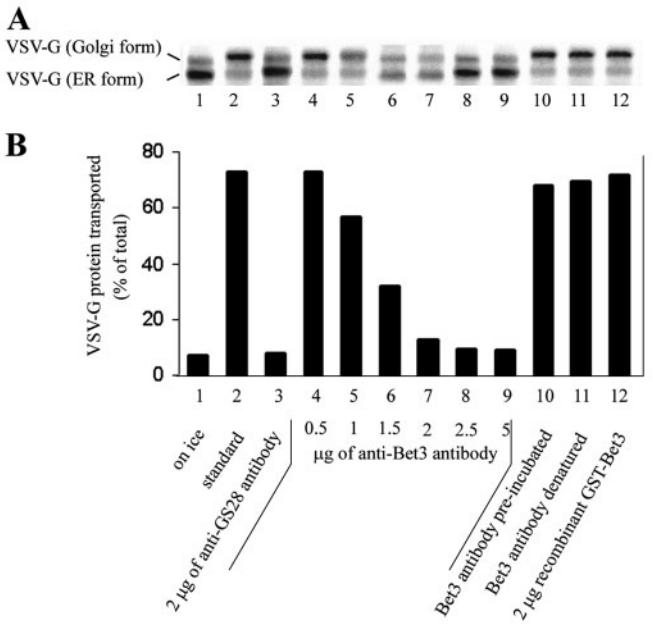

Fig. 5. Antibodies against Bet3 inhibit in vitro transport of VSV-G from the ER to the Golgi apparatus. Semi-intact NRK cells were incubated in the presence of cytosol and ATP on ice (lane 1) or at 32°C for 60 minutes (lanes 2-12). Increasing amounts of anti-Bet3 antibodies were added as indicated (lanes 4-9). Control assays were supplemented with 2 μg of antibody against GS28 (lane 3), 2 μg anti-Bet3 antibodies pre-incubated with 2 μg of GST-Bet3 (lane 10), 2 μg heat-inactivated anti-Bet3 antibodies (lane 11), or 2 μg GST-Bet3 alone (lane 12), respectively. Transport was measured by monitoring the percentage of conversion of the total pool of VSV-G from the endo-H-sensitive to the endo-H-resistant Golgi form (A). The transport was quantified (B).

was a result of antibody binding to endogenous Bet3, the antibodies were boiled for 5 minutes, cooled on ice and then added into the reaction. As seen (lane 11), boiled antibodies had no inhibitory effect on the transport. Furthermore, pre-incubation of 2 μg of antibodies with 2 μg of GST-Bet3 did not have any significant effect on the transport (lane 10), whereas 2 μg of GST-Bet3 itself had no inhibitory effect (lane 12). These results suggest that specific binding of antibodies to the endogenous Bet3 is the basis for the observed inhibitory effect of Bet3 antibodies and that Bet3 plays a role in ER-Golgi transport in mammalian cells.

Cytosolic pool of Bet3 is required for ER-Golgi transport

Yeast Bet3p is primarily associated with the membrane and only the membrane-bound Bet3p is functionally important for ER-Golgi transport (Sacher et al., 1998). Although the majority of Bet3 is present in the cytosol, a significant amount of Bet3 is membrane bound and it is interesting to assess whether the membrane-bound Bet3 is sufficient for ER-Golgi transport or whether the cytosolic pool of Bet3 is also necessary for the transport. We have therefore investigated whether the inhibition by Bet3 antibodies is mediated by their binding to the cytosolic Bet3, membrane-bound Bet3, or both. As shown in Fig. 6, when Bet3 antibodies were added to a standard transport reaction (Fig. 6, lane 3, membrane plus cytosol), inhibition of ER-Golgi transport was observed. When semi-intact cells were first incubated with Bet3 antibodies for 1 hour on ice followed by a washing step to remove unbound antibodies and then used in the transport assay, normal transport was observed (lane 4, membrane only). This suggests that, when the membrane pool of Bet3 is bound by antibodies, normal transport could still occur in the presence of cytosol that contains functional Bet3. When cytosol was pre-incubated with Bet3 antibodies for 1 hour on ice and then immediately used in the transport reaction, ER-Golgi transport was inhibited (lane 5, cytosol only). These results suggest that inhibiting membrane-bound Bet3 is not sufficient to inhibit ER-Golgi transport and that cytosolic Bet3 plays an essential role in ER-Golgi transport. By contrast, anti-GS28 potently inhibits ER-Golgi transport when only semi-intact cells were incubated with the antibody (lane 7). Similar to Bet3, antibodies against α-SNAP inhibited ER-Golgi transport when added to the standard reaction (lane 9) or the cytosol (lane 11), but not the semi-intact cells (lane 10), consistent with the previous observation that cytosolic α-SNAP is necessary for ER-Golgi transport (Peter et al., 1998). The above observation could be explained alternatively by proposing that cytosolic Bet3 could compete for the antibodies bound to the membrane Bet3 and functional Bet3 is then restored on the membrane. To examine directly the role of cytosolic Bet3, rat liver cytosol was immunodepleted of endogenous

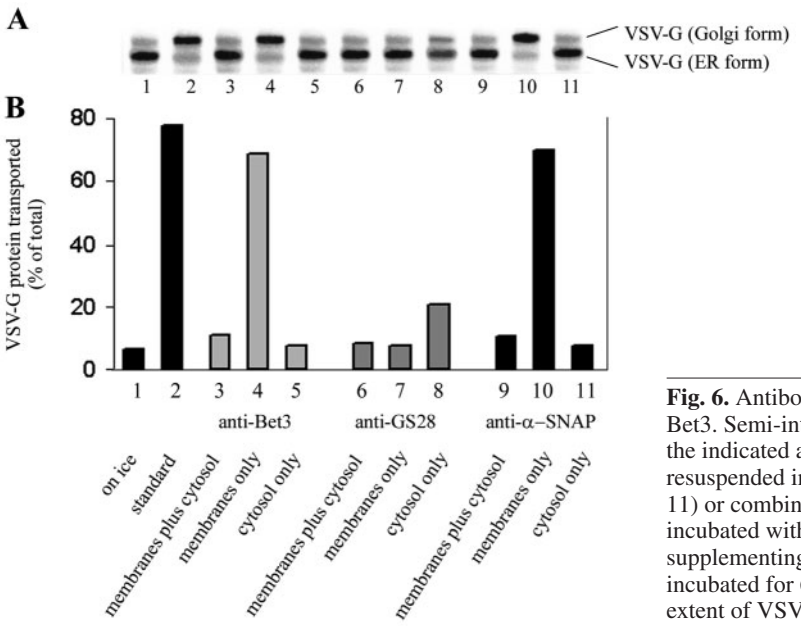

Fig. 6. Antibodies against Bet3 functionally inhibit the cytosolic pool of Bet3. Semi-intact NRK cells (lanes 4, 7, 10) were incubated with 2 μg of the indicated antibodies for 1 hour on ice, washed by centrifugation and resuspended in standard transport cocktail. Likewise, cytosol (lanes 5, 8, 11) or combinations of semi-intact cells and cytosol (lanes 3, 6, 9) were incubated with 2 μg of the indicated antibodies for 1 hour on ice. After supplementing with complete transport cocktail, assays were then incubated for 60 minutes on ice (lane 1) or at 32°C (lanes 2 to 11). The extent of VSV-G transport was monitored (A) and quantified (B).

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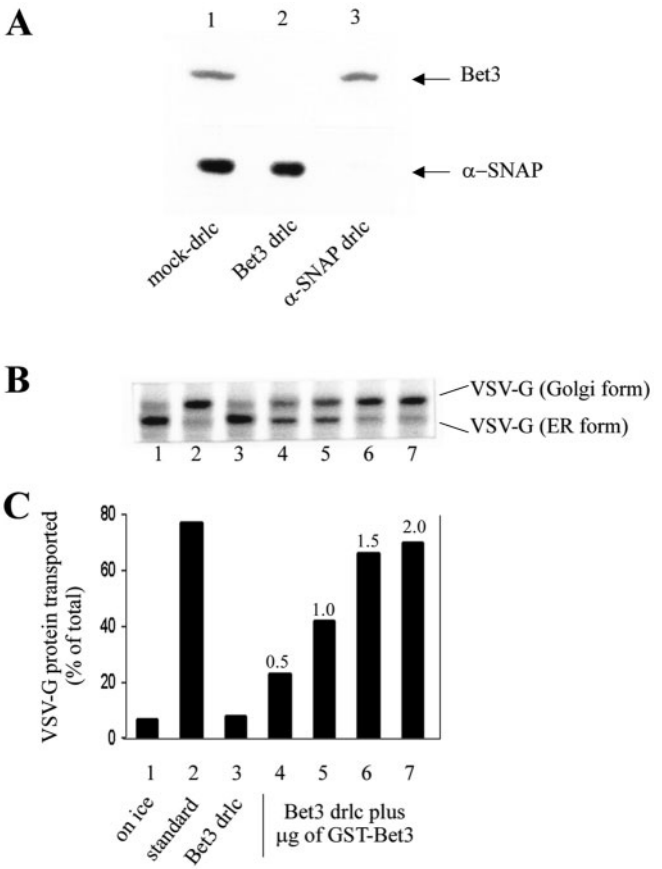

Fig. 7. Cytosolic Bet3 is necessary for ER-Golgi transport. (A) Rat liver cytosol (rlc) was immunodepleted with anti-HA antibodies (lane 1, mock-drlc), anti-Bet3 antibodies (lane 2, Bet3-drlc), or anti-α-SNAP antibodies (lane 3, α-SNAP drlc). The immunodepleted cytosols were analyzed by immunoblot to detect Bet3 (upper panel) or α-SNAP (lower panel). (B and C) Semi-intact NRK cells were incubated in transport cocktail with mock-drlc (lanes 1 and 2) or Bet3-drlc (lanes 3-7) supplemented with the indicated amounts (μg) of GST-Bet3 (lanes 4-7). Transport reactions were incubated for 60 minutes on ice (lane 1) or at 32°C (lanes 2-7). The extent of VSV-G transport was monitored (B) and quantified (C).

Bet3 by using anti-Bet3 antibodies coupled to Sepharose. As revealed by immunoblot analysis (Fig. 7A), Bet3 is essentially undetectable in the Bet3-depleted cytosol (lane 2), whereas Bet3 was detected in the mock-depleted (lane 1) and α-SNAP-depleted (lane 3) cytosol. Normal levels of α-SNAP were detected in mock-depleted (lane 1) and Bet3-depleted (lane 2) cytosol, but undetectable in α-SNAP-depleted cytosol (lane 3). Mock-depleted cytosol (Fig. 7B, lane 2) supported ER-Golgi transport, whereas Bet3-depleted cytosol failed to support ER-Golgi transport (lane 3), suggesting that cytosolic Bet3 is indeed necessary for ER-Golgi transport. More importantly, the defect of Bet3-depleted cytosol could be rescued by relatively high concentrations of recombinant GST-Bet3 (lanes 4-7).

Bet3 functions prior to the EGTA-sensitive stage during ER-Golgi transport

To gain mechanistic insight into the function of Bet3 in ER-Golgi transport, we have performed several experiments to define the stage at which Bet3 functions in ER-Golgi transport. We have first established that antibodies against Bet3 must be added before the EGTA-sensitive stage during in vitro ER-Golgi transport. EGTA has been shown to inhibit reversibly the in vitro ER-Golgi transport at a stage after docking of transport intermediates but before the actual fusion (Rexach and Schekman, 1991; Balch et al., 1994; Pind et al., 1994; Aridor et al., 1995; Lupashin et al., 1996). In a typical experiment, ER-Golgi transport is initially performed in the presence of EGTA to arrest transport at the EGTA-sensitive step (stage I). The semi-intact cells are then washed to remove the inhibitor and resuspended in fresh transport cocktail containing fresh cytosol and ATP (stage II) (Fig. 8A). In the presence of EGTA, VSV-G was arrested in the endo-H-sensitive ER form (Fig. 8B and C, lane 3). However, a second stage of incubation in fresh transport cocktail allowed VSV-G to be converted to the endo-H-resistant Golgi form (lane 4). As shown previously (Subramaniam et al., 1996), antibodies against GS28 inhibited ER-Golgi transport when they were added to the reaction either in the first stage (lane 5) or the second stage (lane 6). This is consistent with the notion that antibodies against GS28 can still inhibit ER-Golgi transport after the EGTA-sensitive stage (Subramaniam et al., 1996). Bet1 has previously been shown to be present in the pre-Golgi intermediate compartment and it might participate in the ER-Golgi transport before the EGTA-sensitive stage (Zhang et al., 1997), because antibodies against Bet1 potently inhibited ER-Golgi transport when they were added in the stage I (lane 7) but not in the stage II (lane 8) of the reaction. Similarly to Bet1, antibodies against Bet3 inhibited ER-Golgi transport only when they were added at stage I (lane 9) but not stage II (lane 10), suggesting that Bet3 antibodies can no longer block the function of Bet3 when they are added after the EGTA-sensitive stage. Furthermore, like normal rlc (lane 11), Bet3-depleted rlc (lane 12) can support stage II transport, suggesting that cytosolic Bet3 is no longer required after the EGTA-sensitive stage.

Bet3 acts after COPII but before Rab1 and α-SNAP during ER-Golgi transport

To define more precisely the stage at which Bet3 acts during ER-Golgi transport, we have used combinations of Bet3-depleted cytosol and cytosols depleted of various essential components using a modified two-stage assay (Fig. 9). Transport assay of semi-intact cells was first performed in the presence of cytosol depleted of an essential component (for example X) at 32°C for 90 minutes so that the transport is arrested at the stage that requires X component. After a brief spin and a washing step, a second stage of incubation was performed in the presence of cytosol depleted of another essential component (such as Y). Under such a setting, we are comparing the temporal relationship of factor Y (in the X-depleted cytosol) versus factor X (in the Y-depleted cytosol). If component Y acts before X, we should detect normal transport if factor Y (using X-depleted cytosol) is present in the first stage; and X (using Y-depleted cytosol) is present in the second stage. Consistently, transport should be inhibited/reduced if factor X is present in the first stage and Y is present in the second stage. Sec13 is a component of COPII involved in ER export (Schekman and Orci, 1996; Aridor et al., 1998; Tang et al., 1997). When Bet3-depleted cytosol was used in

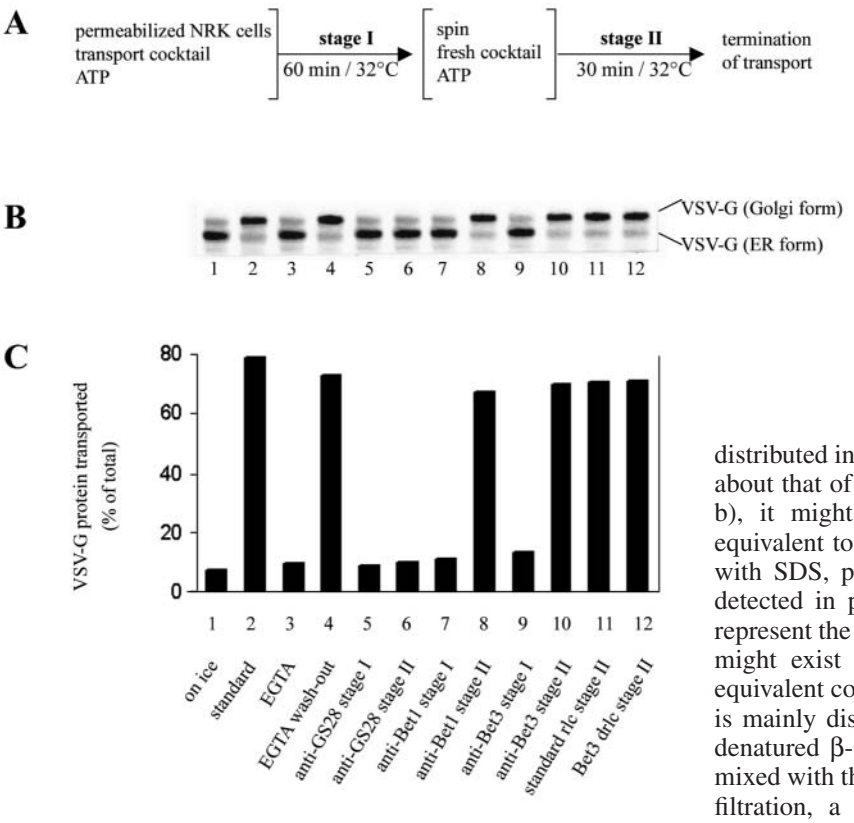

Fig. 8. Bet3 acts before the EGTA-sensitive step in ER-Golgi transport in vitro. As outlined in (A), permeabilized NRK cells, cytosol and ATP were incubated in the absence (lanes 1 and 2) or presence of 8 mM EGTA (lanes 3-12) on ice (lane 1) or at 32°C (lanes 2-12) for 60 minutes (stage I). The membranes were then collected by a brief spin and resuspended in fresh transport cocktail including fresh ATP and cytosol (lanes 2-11) or Bet3 immunodepleted cytosol (Bet3 drlc, lane 12). Reagents were added at stage I or stage II as indicated in (C). Transport was measured by the conversion of the VSV-G protein to the endo-H-resistant form (B) and quantified (C).

stage I (Sec13 in stage I) and Sec13-depleted cytosol used in stage II (Bet3 in the second stage), normal transport was observed (Fig. 9B,I), suggesting that Bet3 participates in a stage after the action of COPII. Consistent with this notion, when Bet3 (Sec13-depleted) and Sec13 (Bet3-depleted) in the cytosol were used in stage I and II, respectively, only a background level of transport was observed (II). When α-SNAP (Bet3-depleted) and Bet3 (α-SNAP-depleted) in the cytosol were used in the first and second stage, respectively, only background levels of transport were observed (III), suggesting that α-SNAP cannot act before Bet3. Furthermore, when Bet3 (α-SNAP-depleted) and α-SNAP (Bet3-depleted) in the cytosol were used for the first and second stages, respectively, normal transport was achieved (IV), suggesting that Bet3 acts indeed before α-SNAP. Similar experiments using combinations of Bet3-depleted cytosol with Rab1-depleted cytosol (V and VI) suggest that Bet3 also acts before Rab1. We have previously established that both Rab1 and α-SNAP act before the EGTA-sensitive stage and that Rab1 acts before α-SNAP (Peter et al., 1998). On the basis of these results, sequential action of COPII → Bet3 → Rab1 → α-SNAP → EGTA-sensitive step → GS28 during ER-Golgi transport could be envisioned in mammalian cells.

Cytosolic Bet3 exists in two distinct pools

In yeast, Bet3p exists in a large protein complex termed TRAPP (Sacher et al., 1998). To see whether a similar protein complex also exists in mammalian cells, we have analyzed the distribution of Bet3 by gel filtration (Fig. 10). Two pools of Bet3 were observed, one high-molecular-weight pool (pool I) is distributed in fractions 2-4, whereas the other (pool II) is distributed in fractions 8-10 (A, panel a). Since pool I has sizes about that of coatomer, which is found in fractions 2-5 (panel b), it might represent a Bet3-containing protein complex equivalent to yeast TRAPP. When cytosol was first denatured with SDS, pool I was no longer detected and Bet3 is only detected in pool II (panel c), suggesting that pool II might represent the free form of Bet3. These results suggest that Bet3 might exist in a dynamic equilibrium between a TRAPP-equivalent complex and the free form. Recombinant GST-Bet3 is mainly distributed in fractions 6-8 (B, panel a), similar to denatured β-COP (A, panel d). However, when GST-Bet3 is mixed with the Bet3-depleted cytosol and then analyzed by gel filtration, a significant amount of GST-Bet3 is shifted to fractions 2-4, suggesting that GST-Bet3 could be incorporated into a TRAPP-equivalent protein complex.

Discussion

The TRAPP complex containing ten subunits has been well characterized in yeast. In the mammalian context, although only seven homologs of the yeast TRAPP complex have been structurally identified, the remaining three (Trs65p, Trs85p and Trs120p) might have functional counterparts that are not structurally related to the yeast ones.

Since Bet3 is the most conserved subunit, we have generated antibodies against it to initiate our study of its role in mammalian cells. The first conclusion is that Bet3 plays an important role in ER-Golgi transport in mammalian cells. However, the mode of action is significantly different in that the membrane-bound Bet3p is sufficient for ER-Golgi transport in yeast, whereas the cytosolic Bet3 in mammalian cells is necessary for ER-Golgi transport. This could be a reflection of the observation that the majority of Bet3 is present in the cytosol in mammalian cells. The second important conclusion is that Bet3 acts after COPII but before Rab1 and α-SNAP in a stage before the EGTA-sensitive step during ER-Golgi transport. This is consistent with the observation that yeast TRAPP complex acts as a GEF for Ypt1p in yeast (Jones et al., 2000; Wang et al., 2000). One of the possible ways for this to occur is for mammalian TRAPP complex to regulate similarly the activation step of Rab1. The third interesting point is that there exists equilibrium between free subunits and TRAPP complex in mammalian cells and this could be another reason that cytosolic Bet3 is necessary for ER-Golgi transport.

The antibodies raised against recombinant Bet3 recognize

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A

B

	stage I	stage II	VSV-G protein transported (%)
I	Bet3 drlc	mSEC13 drlc	71
II	mSEC13 drlc	Bet3 drlc	11
III	Bet3 drlc	α-SNAP drlc	12
IV	α-SNAP drlc	Bet3 drlc	64
V	Bet3 drlc	Rab1 drlc	10
VI	Rab1 drlc	Bet3 drlc	67

Fig. 9. Bet3 is functionally required after Sec13 but before Rab1 and α-SNAP. Semi-intact NRK cells were incubated in transport cocktail with the indicated immunodepleted cytosols for 90 minutes at 32°C (stage I). The cells were subsequently washed by centrifugation and resuspended in fresh transport cocktail supplemented with the indicated immunodepleted cytosols for an additional 45 minutes (stage II). The extent of VSV-G transported to the Golgi was quantified (B).

specifically a 22 kDa protein that is present in both cytosol and in Golgi-enriched membranes derived from rat liver. Since detection of this 22 kDa protein is totally abolished by pre-incubation of antibodies with recombinant Bet3 but not with GST or other recombinant proteins, we conclude that the 22 kDa protein represents the endogenous Bet3. When NRK cells were fractionated into total membrane and cytosol, it was found that the majority (about 86%) of Bet3 is present in the cytosol when equal fractions of total membrane and cytosol were used for the immunoblot analysis. The presence of a significant amount (about 15%) of Bet3 in the membrane led us to test out the extent of its association with the membranes. The membrane-associated fraction of Bet3 is tightly associated with the membrane because it is not extracted by 1 M KCl. These results suggest that Bet3 exists primarily in the cytosol and is recruited to the membrane in a dynamic manner. This is in contrast to what was observed in yeast Bet3p, where the majority of the protein is membrane-associated (Barrowman et al., 2000; Sacher et al., 1998). Consistent with our results, human Bet3 and Trs20 were largely soluble in an earlier study (Sacher et al., 2000).

We have searched several genomes and other databases with the known yeast and mammalian proteins of the TRAPP complex and have identified a novel protein (Trs33B) homologous to Trs33p and Trs33 (referred to as Trs33A). This will certainly introduce additional complexity in terms of the function and regulation of mammalian TRAPP complex, particularly as three of the ten subunits do not have clear structural counterparts between yeast and mammalian cells. Future studies of Trs33A and Trs33B, as well as the four potential candidates (KIAA1012, hypothetical protein AL136752.1, CGI-85, and hypothetical protein FLJ13611) of the functional counterparts (Gavin et al., 2002), will definitely be needed before we can fully appreciate the conservation and diversification of the role and mode of action of the TRAPP complex in mammalian cells. Interestingly, Trs20 was independently identified as SEDL, the three-dimensional structure of which has an unexpected similarity to the N-terminal regulatory domain of two SNAREs, Ykt6p and mammalian Sec22b (Gonzalez et al., 2001; Jang et al., 2002;

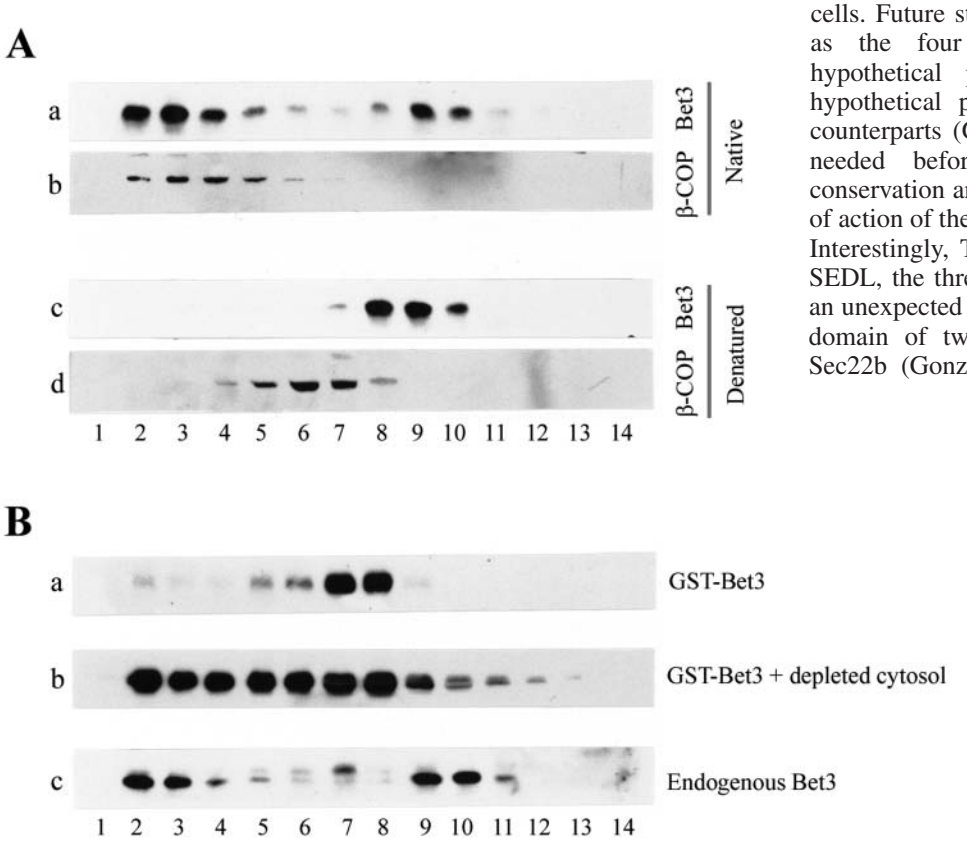

Fig. 10. Two pools of Bet3 in the cytosol as revealed by gel filtration analysis. (A) Rat liver cytosol (panels a and b) or denatured cytosol (panels c and d) was resolved by gel filtration and the resulting fractions were analyzed by immunoblotting to detect Bet3 (panels a and c) or β-COP (panels b and d). (B) GST-Bet3 alone (panel a) or GST-Bet3 pre-incubated with Bet3-depleted rlc (panel b) were resolved by gel filtration and the resulting fractions were analyzed by immunoblotting to detect GST-Bet3. Also shown is the distribution of Bet3 in control cytosol (panel c).

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Tochio et al., 2001), although the primary amino acid sequence does not show any homology to Ykt6p or Sec22b. The N-terminal domain of Ykt6p influences the kinetics and proper assembly of SNARE complexes (Tochio et al., 2001). The N-terminal domain of mSec22b is also likely to provide some important regulatory functions, because expression of the N-terminal deletion mutant of VAMP-7, a homolog of Sec22b, was found to increase SNARE complex formation and strongly stimulate neurite outgrowth of PC12 cells (Martinez-Arca et al., 2000). The structural similarity of Trs20/SEDL to the N-terminal domain of these SNAREs suggests a regulatory function of TRAPP complex in the assembly and/or function of SNARE complexes involving Ykt6 and Sec22b, which have been shown to be important in ER-Golgi transport (Zhang et al., 1999; Zhang and Hong, 2001). Interestingly, Trs23 was independently identified as Synbindin, which forms clusters in dendritic spines when syndecan-2 is coexpressed in neurons. Immunoelectron microscopy suggests that synbindin is present on postsynaptic membranes and intracellular vesicles within dendrites, suggesting a role in postsynaptic membrane trafficking (Iryna et al., 2000). Although a role of Trs23/synbindin remains to be examined in this particular context, its clear homology with Trs23p indicates that it could function as part of the TRAPP complex in ER-Golgi transport, in addition to its role in synapses. The amino acid sequence homology among Bet5, TRS20/SEDL and Trs23/synbindin suggest that they might all adopt a similar structure and participate in regulating the action of the SNARE complex. The genetic interaction of the TRAPP complex and SNAREs does support such a possibility (Sacher et al., 1998). The participation of Trs23/synbindin in post-Golgi traffic (synaptic spines) indicates that TRAPP complex and/or its subunits could act at several transport events, which will be an interesting issue to be addressed by future studies.

All the six low-molecular-weight TRAPP subunits (Bet3p, Bet5p, Trs20p, Trs23p, Trs31p and Trs33p) are structurally conserved between yeast and mammals, whereas only one of the four high-molecular-weight subunits (Trs130p) is structurally conserved. This indicates that the large subunits might evolve more rapidly to accommodate the needs of different architectures and mechanisms in different organisms. Trs130p shares significant homology to a protein called GT334 (Lafreniere et al., 1997). Trs130p contains a region that has potential to be involved in a coiled-coil interaction, and overlaps with the region of highest homology to GT334. In addition to the homology among Bet5, TRS20/SEDL and Trs23/synbindin, the three other low-molecular-weight TRAPP complex subunits (Bet3, Trs31 and Trs33A/B) also share some amino acid sequence relatedness (Fig. 1D) (Sacher et al., 2000), although the significance remains to be explored.

A role of Bet3 in ER-Golgi transport was established by several lines of evidence. First, VSV-G transport in semi-intact cells could be inhibited by Bet3 antibodies in a dose-dependent manner, and this inhibition is specific because denatured antibodies had no effect on VSV-G transport and the inhibitory effect of the antibodies could be neutralized by pre-incubation with recombinant Bet3. The importance of cytosolic Bet3 is established by the observation that cytosol could support ER-Golgi transport when a membrane pool of Bet3 in semi-intact cells was decorated with the antibodies, suggesting that the membrane pool of Bet3 is not necessary for ER-Golgi transport. When cytosol was pre-incubated with Bet3 antibodies, inhibition of ER-Golgi transport of VSV-G was observed. In addition, the membrane pool is not sufficient to support ER-Golgi transport, because Bet3-depleted cytosol could not support this transport, establishing an essential role of cytosolic Bet3 in ER-Golgi transport. Furthermore, Bet3-depleted cytosol could be rendered competent in supporting ER-Golgi transport when supplemented with recombinant GST-Bet3. However, relatively high concentrations of recombinant GST-Bet3 are required to rescue transport competence of the Bet3-depleted cytosol. Several possibilities could explain this point. One possibility is that only a fraction of GST-Bet3 is folded properly and exists in a functional conformation. The second possibility relates to the observation that cytosolic Bet3 exists in two distinct pools. One high-molecular-weight pool has a size comparable with that of coatomer, whereas the other reflects the free form of Bet3. Since Bet3p in yeast is found in the TRAPP protein complex (Sacher et al., 1998), the high-molecular-weight complex containing Bet3 might be equivalent to TRAPP complex and is the functional form of Bet3 associated with other components of this complex. As tagged Bet3 in mammalian cells can bring down other components of this complex (Gavin et al., 2002), Bet3-depleted cytosol might contain less amounts of other subunits in such a way that high concentration of GST-Bet3 is necessary to drive the formation of functional TRAPP complex. Our observation that a significant amount of GST-Bet3 was incorporated into the high-molecular-weight form when incubated with Bet3-depleted cytosol suggests that only a fraction of GST-Bet3 could associate with other subunits of the complex. This is in marked contrast to TRAPP complex in yeast, in which only large complexes but not free pools of any subunits were detected (Sacher et al., 2000; Sacher and Ferro-Novick, 2001).

Mechanistic understanding of the participation of Bet3 in ER-Golgi transport came from the observation that it functions after COPII but before Rab1, α-SNAP and the EGTA-sensitive stage during ER-Golgi transport. EGTA has been shown previously to inhibit in vitro ER-Golgi transport after docking of transport intermediates but before actual fusion (Rexach and Schekman, 1991; Balch et al., 1994; Pind et al., 1994; Aridor et al., 1995; Lupashin et al., 1996). Several lines of evidence suggest that Bet3 functions before the EGTA-sensitive stage. Although Bet3 antibodies potently inhibited ER-Golgi transport when added before the EGTA-sensitive stage, addition of Bet3 antibodies to the in vitro transport reaction after the EGTA-sensitive stage had no effect on ER-Golgi transport. Second, Bet3-depleted cytosol can support events downstream of the EGTA-sensitive stage, suggesting that cytosolic Bet3 is no longer required after the EGTA-sensitive stage. In addition, Rab1 and α-SNAP have both been shown to participate in ER-Golgi transport before the EGTA-sensitive stage but downstream of the action of Sec13 (Peter et al., 1998). The demonstration that Bet3 acts before Rab1 and α-SNAP further supports this conclusion. On the basis of these results and the observation that Trs20 (and probably Bet5 and Trs23) has a three-dimensional structure similar to Ykt6 and Sec22b, it is plausible to propose that Bet3/TRAPP might act together with Ykt6 and Sec22b during ER-Golgi transport, in addition to the possibility that the TRAPP complex activates Rab1 by guanine nucleotide exchange.

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The TRAPP complex was shown to act as the GEF for Ypt1 and Ypt31/32 GTPases both in vitro and in vivo (Jones et al., 2000). The function of Ypt1 GTPase is required at the cis-Golgi for the targeting and fusion of ER-derived vesicles (Cao and Barlowe, 2000; Segev, 1991), whereas the function of Ypt31/32 GTPases is essential for the formation of trans-Golgi vesicles (Jedd et al., 1997). It has also been shown that increasing gene dosage of YPT31/32 suppressed the lethality resulting from deletion of the essential TRS130 gene and also partially suppresses the deletion of GCS1, an Arf GAP (Zhang et al., 2002). TRAPP has also been shown to be required for ER-Golgi transport (Barrowman et al., 2000; Sacher et al., 1998) (and this study). Future studies examining the role of the TRAPP complex in regulating the function of Rab1 or other Rab proteins will provide additional insight about the exact role and mechanism of action of the TRAPP complex.

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