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The TRAPP Complex Is a Nucleotide Exchanger for Ypt1 and Ypt31/32

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In yeast, the Ypt1 GTPase is required for ER-to-*cis*-Golgi and *cis*-to-medial-Golgi protein transport, while Ypt31/32 are a functional pair of GTPases essential for exit from the trans-Golgi. We have previously identified a Ypt1 guanine nucleotide exchange factor (GEF) activity and characterized it as a large membrane-associated protein complex that localizes to the Golgi and can be extracted from the membrane by salt, but not by detergent. TRAPP is a large protein complex that is required for ER-to-Golgi transport and that has properties similar to those of Ypt1 GEF. Here we show that TRAPP has Ypt1 GEF activity. GST-tagged Bet3p or Bet5p, two of the TRAPP subunits, were expressed in yeast cells and were precipitated by glutathione-agarose (GA) beads. The resulting precipitates can stimulate both GDP release and GTP uptake by Ypt1p. The majority of the Ypt1 GEF activity associated with the GST-Bet3p precipitate has an apparent molecular weight of > 670 kDa, indicating that the GEF activity resides in the TRAPP complex. Surprisingly, TRAPP can also stimulate nucleotide exchange on the Ypt31/32 GTPases, but not on Sec4p, a Ypt-family GTPase required for the last step of the exocytic pathway. Like the previously characterized Ypt1 GEF, the TRAPP Ypt1-GEF activity can be inhibited by the nucleotide-free Ypt1-D124N mutant protein. This mutant protein also inhibits the Ypt32 GEF activity of TRAPP. Coprecipitation and overexpression studies suggest that TRAPP can act as a GEF for Ypt1 and Ypt31/32 in vivo. These data suggest the exciting possibility that a GEF complex common to Ypt1 and Ypt31/32 might coordinate the function of these GTPases in entry into and exit from the Golgi.

INTRODUCTION

Transport of proteins through the secretory pathway involves their orderly progression through a series of membranous compartments. Movement between successive compartments appears to be mediated by vesicles that bud from one compartment and fuse with the next (Jamieson and Palade 1967; Palade 1975). Progress has been made during the last few years in understanding the mechanisms contributing to the directionality and specificity of vesicle formation, targeting, and fusion. GTPases that belong to the Ypt/Rab family are key regulators of vesicular transport in yeast and mammalian cells (Pfeffer, 1992; Ferro-Novick and Novick, 1993; Zerial and Stenmark, 1993). In yeast, four members of this family regulate the different steps of the exocytic pathway. Ypt1 GTPase is essential for the first two steps, ER-to-Golgi and *cis*-to-medial Golgi transport (Segev *et al.*, 1988; Bacon *et al*,. 1989; Baker *et al.*, 1990; Segev, 1991; Jedd *et al.*, 1995). The functional GTPase pair Ypt31/32 is required for exit from the trans-Golgi (Benli *et al.*, 1996; Jedd *et al.*, 1997). Sec4 GTPase is essential for the fusion of trans-Golgi-derived vesicles with the plasma membrane (Novick *et al.*, 1981; Goud *et al.*, 1988).

Like other members of the ras superfamily, the Ypt/Rab GTPases cycle between the GDP- and the GTP-bound forms by exchanging GDP for GTP and hydrolyzing GTP. This cycling is thought to be crucial for the function of GTPases and is regulated by factors that stimulate these reactions. *G*uanine nucleotide *e*xchanger *f*actors (GEFs) stimulate the shift from the GDP to the GTP-bound form, while *G*TPase *a*ctivating *p*roteins (GAPs) stimulate the shift from the GTP- to the GDP-bound form (Bourne *et al.*, 1990). A number of GEFs for the Ypt/Rab family of GTPases have been identified. In general, the known Ypt/Rab GEFs are each part of larger protein complexes, are specific to their single Ypt/Rab target, and do not share homology with one another (Horiuchi *et al.*, 1997; Wada *et al.*, 1997; Walch-Solimena *et al.*, 1997; Hama *et al.*, 1999). However, their precise mechanism of action and the means by which they are regulated is still obscure. In yeast, GEFs have been identified for two Ypt-family GTPases: Sec2p is the GEF for Sec4p, and Vps9p is the

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GEF for Vps21p (Walch-Solimena *et al.*, 1997; Hama *et al.*, 1999). We have previously identified a GEF for Ypt1 GTPase and characterized it as a high molecular weight protein (MW) that resides on the Golgi and is required for Ypt1-mediated protein transport (Jones *et al.*, 1995; Jones *et al.*, 1998). Based on these and our other results (Richardson *et al.*, 1998), we proposed a model for the regulation of Ypt1 GTPase function by its GEF and GAP. In this model, GEF has an essential role at the Golgi in Ypt1 GTPase function in ER-to-Golgi vesicle targeting, while GAP is involved in the process of recycling of Ypt1 GTPase between membranes, which is not required for their function (Jones *et al.*, 1998). A thorough test of this model requires the identification of the genes that encode the GEF and the GAP for Ypt1 GTPase.

A large protein complex that is apparently required for ER-to-Golgi transport and that localizes to the Golgi was identified and given the name TRAPP (*tra*nsport *p*rotein *p*article) (Barrowman *et al.*, 2000; Sacher *et al.*, 1998). Recently, Sacher *et al.* (Sacher *et al.*, 2000) have shown that the extraction properties of this particle from the membrane are remarkably similar to those of the Ypt1 GEF that we previously characterized (Jones *et al.*, 1998). We asked whether TRAPP is the GEF for Ypt1 GTPase. Here, we show that not only can TRAPP stimulate nucleotide exchange by Ypt1, but it also acts as a GEF for the Ypt31/32 GTPases. The significance of this finding might be in the coordination of Ypt1 and Ypt31/32 GTPase functions by a common GEF complex. Since Ypt1 and Ypt31/32 GTPases regulate entry into and exit from the Golgi, respectively, coordination of their functions might play a key role in the steady-state maintenance of Golgi architecture.

MATERIALS AND METHODS

Strains, Plasmids, and Materials

The following yeast strains were used in this study: EJ 758 (Martzen *et al.*, 1999) was used to express GST-tagged proteins in yeast from derivatives of the pYEX 4T-1 plasmid; NSY558 (to express GST from pNS 422); NSY568 (to express GST-Bet3p from pNS423); NSY569 (to express GST-Bet5p from pNS424); and NSY563 (to express GST-Sec2p from pNS425). These strains were isolated from a collection of yeast strains expressing GST-tagged yeast ORFs (Martzen *et al.*, 1999) (Research Genetics). Expression of tagged-ORFs was verified by immunoblot analysis using anti-GST antibodies. In addition, the following yeast strains were used: NSY125 (DBY1034; *MATa his4–539 lys2–801 ura3–52*); NSY222 (*MATα his4 ura3–52 ypt1-A136D*) (Jedd *et al.*, 1995); NSY348 (*MATa his4–539 lys2–801 ura3–52 ypt31::HIS3 ypt32-A141D*) (Jedd *et al.*, 1997), NSY2 (DBY1803, *MATa his4–539 lys2–801 ura3–5 ypt1–1*) (Segev and Botstein 1987). Yeast transformations were performed by the overnight lithium acetate method (Gietz *et al.*, 1992).

Plasmids for the expression in *Escherichia coli* of GST-fused Ypt1 (pNS351), Ypt1-D124N (pNS363), Ypt31 (pNS210), Ypt32 (pNS211), and Sec4 (pNS212) have been described elsewhere (Jones *et al.*, 1995; Jedd *et al.*, 1997; Jones *et al.*, 1998). GST-fused Ypt32-D129N (pNS419) and Ypt31-N126I (pNS417) were constructed in an identical manner.

All chemical reagents were purchased from Sigma (St. Louis, MO), unless otherwise noted. Polyclonal anti-GST antibodies were from Molecular Probes (Eugene, OR). Affinity purified anti-Ypt1 and Ypt31/32 have been described (Segev *et al.*, 1988; Jedd *et al.*, 1997).

Culture Conditions

Yeast strains were grown in synthetic medium lacking leucine and uracil (0.67% yeast nitrogen base without amino acids), supplemented with the appropriate auxotrophic requirements (Rose *et al.*, 1988). Unless otherwise noted, carbon sources were added to 2% (wt/vol).

Purification of GST Fusion Proteins

Ypt1, Ypt32, and Sec4 proteins were expressed in *E. coli* as GST fusion proteins and were purified as previously described; the GST tag was removed by thrombin cleavage (Jones *et al.*, 1995). GST fusion proteins were expressed and purified from yeast cells as described (Martzen *et al.*, 1999), except that the glutathione agarose (GA) chromatography was done with 4 × beads and eluted with 2 × glutathione. After the elution, the preps were concentrated 4-fold, using Centricon 10, then dialyzed into B88 (250 mM sorbitol, 20 mM HEPES pH 6.8, 150 mM KOAc, 5 mM $Mg(OAc)_2$) (Baker *et al.*, 1988) and stored at −80°C. The total protein concentration of the eluted fractions ranged between 0.1–0.2 mg/ml.

Nucleotide Exchange Assays

Nucleotide exchange assays were carried out as previously described (Jones *et al.*, 1995).

GDP Release Assays

Twenty (20) pmol Ypt1p were preloaded by incubating with 40 pmol 5′,8′-^{3}H-GDP (31.7 Ci/mmol; NEN) in preload buffer (20 mM HEPES pH 7.2, 20 mM KOAc, 1 mM DTT, 5 mM EDTA, 1 μg/μl BSA) for 10 min at 30°C. At the end of the incubation, samples were moved to ice, and $MgCl_2$ was added to 10 mM. Reactions were carried out in 50 μl containing 20 mM HEPES pH 7.2, 5 mM $Mg(OAc)_2$, 0.5 mM GTP, 0.5 mM GDP, 1 mM DTT, 0.4 mg/ml BSA, plus GST-Bet3, GST-Bet5 or GST, purified from yeast. Fractions were normalized to have molar concentrations similar to the GST moiety. Exchange reactions were initiated by the addition of 10 pmol Ypt1p-^{3}H-GDP. Incubations were carried out at 30°C for varying periods of time, as noted. When the effects of mutant Ypt proteins were tested, mutant protein was added to the reaction mixture and incubated on ice for 10 min before addition of the substrate. At intervals, 5-μl samples were removed, filtered through nitrocellulose, washed, and counted as described (Jones *et al.*, 1995). In all experiments, initial values were ~ 10–20 × 10^3 dpm per 5 μl.

GTP Uptake Assays

Ypt1p was preloaded as described for the GDP release assay, but with nonradioactive GDP. α-^{32}P-GTP (Amersham, Arlington Heights, IL; 3000 Ci/mmol, diluted to a specific activity of 75 μCi/mmol) was the only nucleotide in the reaction mixture. Exchange reactions were initiated by the addition of 80–100 pmol GTP to a 50-μl reaction mixture containing 10 pmol Ypt1p and GST-Bet3, GST-Bet5 or GST. Samples of 5 μl were removed at intervals, and the amount of α-^{32}P-GTP bound to Ypt1p was determined by filtration as above.

Gel Filtration

Gst-Bet3 (0.75 ml, 0.3 mg protein) was purified from yeast as described above, except that the eluted protein was dialyzed into B88 containing 0.15 M NaCl and applied to a Superdex 200 (Pharmacia, Piscataway, NJ) fast-pressure liquid chromatography column equilibrated in Buffer 88 + 0.2 M NaCl. Flow rate was set at 0.3 ml/min and fractions of 0.3 ml were collected.

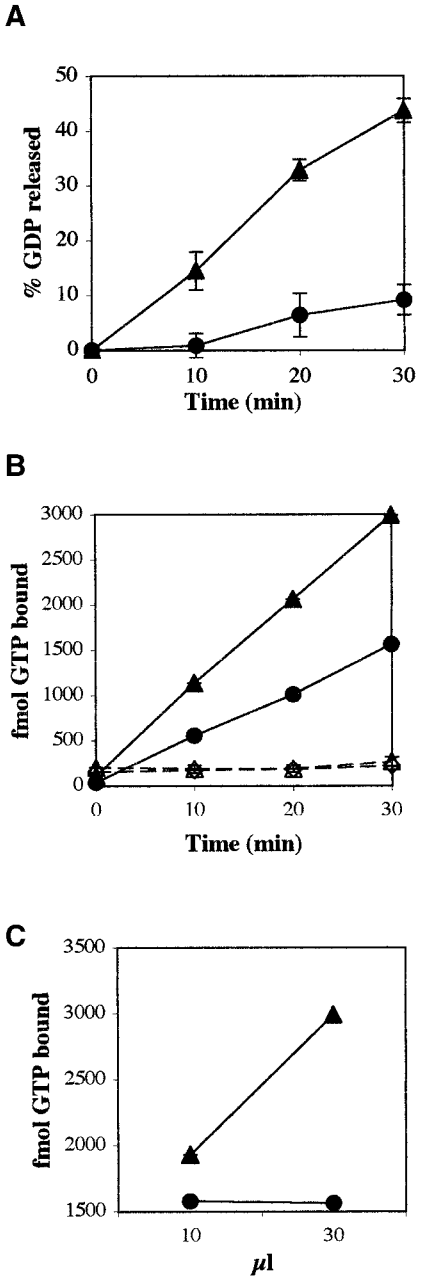

Figure 1. Bet3-associated Ypt1-GEF activity. GST-Bet3 (▲) and GST (●) were expressed in yeast cells and purified using GA. The purified fractions were tested for Ypt1-GEF activity using GDP-release and GTP-uptake filtration assays, with recombinant Ypt1 protein as a substrate. (a) Time course of GDP release. Ypt1p-[^{3}H]GDP was incubated in the presence of 30 μl of GST-Bet3p or GST in a 50 μl reaction. (b) Time course of GTP uptake, in the presence (filled symbols) or absence (open symbols, dashed lines) of 0.2 μM Ypt1p. Reactions were performed in the presence of [α-^{32}P]GTP. (c) GTP uptake by Ypt1p shows a concentration-dependent stimulation by GST-Bet3p, but not by GST. As described in panel b legend, 10 or 30 μl of GST-Bet3 or GST were assayed in a 50-μl reaction; the 30-min time points are shown. Results shown in this figure are the average of duplicate measurements and are representative of 2–6 experiments. Error bars represent range divided by 2.

RESULTS

Ypt1 GEF Activity Copurifies with GST-Bet3

The TRAPP complex has localization and extraction characteristics similar to those of the Ypt1 GEF that we previously characterized (Jones *et al.*, 1998; Sacher *et al.*, 2000). Therefore, we wished to test the ability of TRAPP to act as a Ypt1 GEF. To purify the TRAPP complex, we used one of its subunits, Bet3p, tagged with GST at the N-terminus. A GST-Bet3p fusion protein was expressed in yeast cells, purified by GA-beads precipitation, and tested for its ability to stimulate GDP release and GTP uptake by recombinant Ypt1 protein. As a control, we used GST protein expressed and purified in the same way. GST-Bet3p was found to stimulate both GDP release and GTP uptake by Ypt1p above the intrinsic rates, measured either in the presence of GST protein (Figure 1,a and b) or BSA (our unpublished results). The stimulation of GTP uptake by Ypt1 protein was linearly dependent on the amount of the GST-Bet3p purified fraction added to the assay (Figure 1c). These results indicate that Bet3p, or a protein complex that copurifies with it from yeast lysates, has Ypt1 GEF activity.

TRAPP Is a Ypt1-GEF

To determine whether the GST-Bet3p itself or the TRAPP complex possesses Ypt1 GEF activity, we separated free GST-Bet3p from TRAPP on a Superdex 200 gel-filtration column. The expected size of GST-Bet3p is ~ 50 kDa (however, the tendency of GST to dimerize make it likely that the observed apparent MW would be close to 100 kDa). TRAPP was reported to have a molecular weight of ~ 800 kDa on this column (Sacher *et al.*, 1998). As shown by immuno-blot analysis using anti-GST antibodies, GST-Bet3p is found in two peaks on the sizing column: a 50–100 kDa peak, corresponding to the free GST-Bet3p, and a > 670 kDa peak, corresponding to the TRAPP complex. Column fractions were tested for Ypt1-GEF activity using the GTP-uptake assay. We found that the majority of the GEF activity copurifies with the high MW peak containing GST-Bet3 (Figure 2a and b), suggesting that the TRAPP complex has Ypt1 GEF activity. In contrast, only a minor amount of the total GEF activity is associated with the free GST-Bet3 peak seen on the western blot. We conclude that the TRAPP complex is the major contributor of the Bet3-associated Ypt1 GEF activity.

We also used another GST-tagged subunit of TRAPP, Bet5p, to purify the TRAPP complex from yeast cells. We tested the GST-tagged Bet5p purified fraction for stimulation of GDP release and GTP uptake by Ypt1p. The results were qualitatively similar to those obtained with the GST-Bet3p (our unpublished results). Together, these results indicate that TRAPP can act as Ypt1 GEF.

TRAPP Can Act as a GEF for Ypt31/32 GTPases, But Not Sec4

The Ypt/Rab GEFs identified to date are each specific for their GTPase substrate (Horiuchi *et al.*, 1997; Wada *et al.*, 1997; Walch-Solimena *et al.*, 1997; Hama *et al.*, 1999). We wished to determine whether this is true also for TRAPP; therefore, it was tested for its ability to stimulate GDP release and GTP uptake by Ypt31, Ypt32, and Sec4 GTPases. Ypt31p and Ypt32p are functional homologues and behave

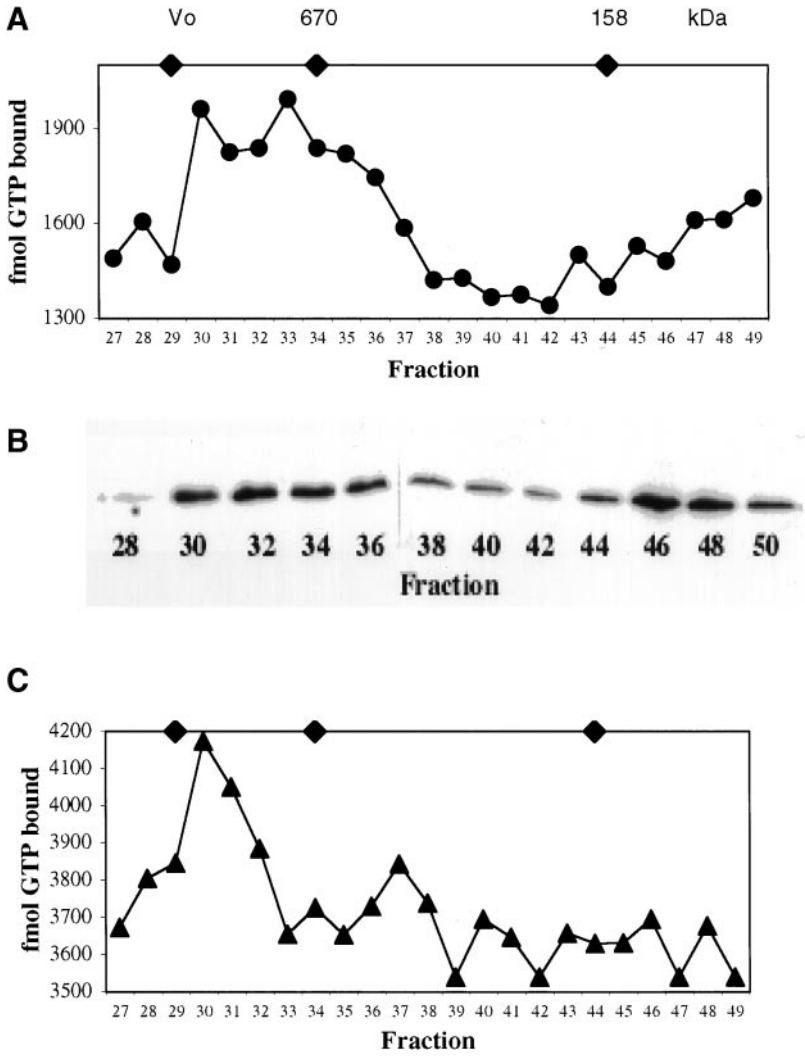

Figure 2. Size fractionation of the purified GST-Bet3p fraction. The purified GST-Bet3 fraction (750 μl, 3 mg protein) was applied to a Superdex 200 gel-filtration column. Fractions (0.3 ml) were collected and assayed as follows: (a) Stimulation of GTP uptake by Ypt1p. As described in Figure 1b, 12.5 μl of column fractions were assayed, but reaction volume was 25 μl; 30-min time points are shown. (b) Western-blot analysis of GST-Bet3p. Analyses by SDS-PAGE of 7.5 μl of column fractions were followed by western blotting, using anti-GST antibody. (b) Stimulation of GTP uptake by Ypt32p. Column fractions were assayed as described in (a) using Ypt32p as a substrate; 60-min time points are shown. The position of the molecular weight markers are shown as diamonds at the top of panel a (from the left: Vo, Blue dextran, 2×10^3 kDa; thyroglobin 670 kDa; aldolase 158 kDa). The results represent two independent experiments.

identically in all of our assays, except that Ypt32p exchanges nucleotides more readily than Ypt31p. This is true of both their intrinsic rates as well as reactions stimulated by crude yeast cell lysates (our unpublished results). The GST-Bet3p precipitate can stimulate both GDP release and GTP uptake by Ypt32p (Figure 3a and b) and, to a lesser extent, by Ypt31p (our unpublished results). Nucleotide exchange on Sec4p can be stimulated by its known GEF, Sec2p (Walch-Solimena *et al.*, 1997), but not by the GST-Bet3 precipitate (Figure 3c).

To determine whether the Ypt32 GEF activity is due to Bet3p itself or to the high MW complex, we tested the Ypt32 GEF activity of the Superdex 200 column fractions of GST-Bet3p. As described above for the Ypt1 GEF activity, only the high MW peak containing GST-Bet3p has a Ypt32 GEF activity (Figure 2c). In fact, the peak of Ypt32 GEF activity is narrower than that of the Ypt1 GEF, suggesting that the broad peak of Ypt1 GEF activity may represent more than one species of the complex. The Ypt1 GEF that we characterized previously did not stimulate nucleotide exchange by Ypt31/32 or Sec4 GTPases (Jones *et al.*, 1998). Ypt31/32 GEF activity was present in the P100 (100,000 $\times$ *g* pellet) fraction that was used for the Ypt1 GEF purification; however, this activity was lost after a detergent extraction step used for the Ypt1 GEF purification (Jones and Segev, unpublished data). The GST-Bet3p purification does not include a detergent extraction step. One possibility is that a detergent-sensitive factor might be important for TRAPP to function as a Ypt31/32 GEF but not as a Ypt1 GEF. This effect may contribute to the apparent heterogeneity of the Ypt1-GEF peak (Figure 2a) and to the lower apparent MW of the previously identified Ypt1 GEF (Jones *et al.*, 1998). Together, these results indicate that TRAPP can act as a GEF for Ypt1 and Ypt31/32 but not for Sec4.

Nucleotide-free Ypt Mutant Proteins Inhibit the Ypt GEF Activity of TRAPP

Mutant forms of GTPases that cannot bind nucleotides frequently exhibit dominant phenotypes due to inhibition of their nucleotide exchangers. These nucleotide-free mutant proteins have higher affinity for the GEF than do the wild-

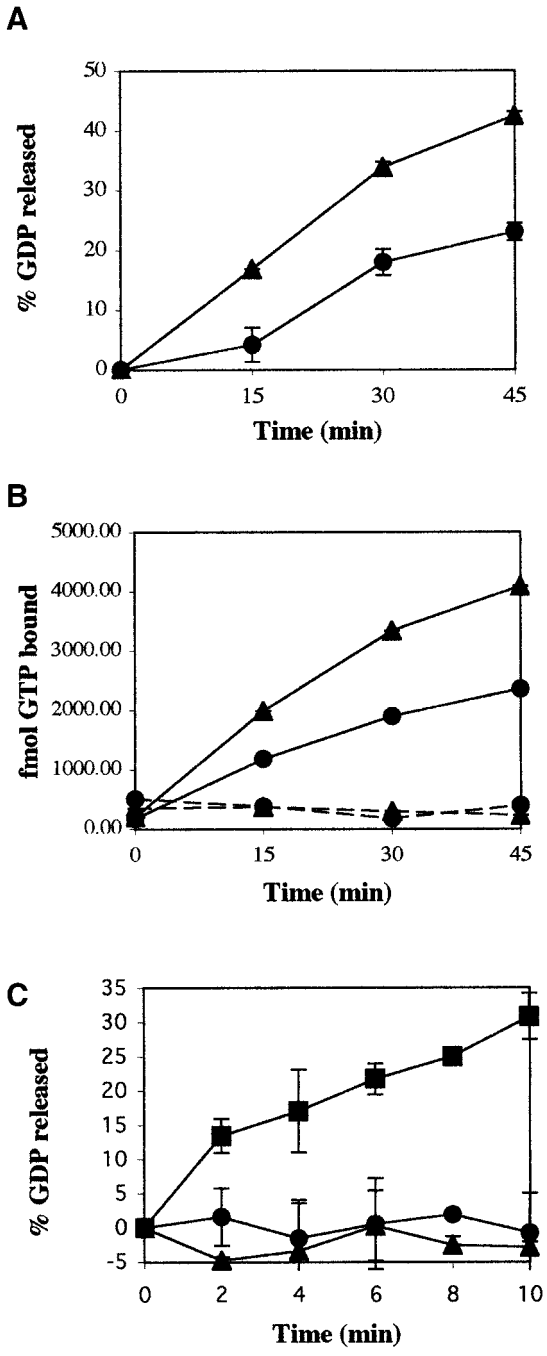

Figure 3. Bet3-associated GEF activity for Ypt32, but not Sec4. GST-Bet3 (▲) or GST (●) were purified from yeast cells and tested for Ypt32 and Sec4 GEF activity using GDP-release and GTP-uptake filtration assays and recombinant Ypt32 or Sec4 protein (as described in Figure 1 legend). (a) Time course of GDP release from Ypt32p. (b) Time course of GTP uptake by Ypt32p, in the presence (solid lines) or absence (dashed lines) of Ypt32p. (c) Time course of GDP release from Sec4p. GST-Sec2 (squares) was used as a positive control for a Sec4-GEF. The data is expressed as percent GDP bound to Sec4p during the reaction. Results shown in this figure are the average of duplicate measurements and are representative of 2–4 experiments. Error bars represent range divided by 2.

type proteins and therefore sequester the GEF from the wild-type substrate (Hwang *et al.*, 1989; Powers *et al.*, 1989; Powers *et al.*, 1991; Hwang *et al.*, 1993; Lai *et al.*, 1993; Haney and Broach 1994). We showed previously that Ypt1-GEF activity can be inhibited by the Ypt1-D124N mutant protein, which cannot bind GDP or GTP (Jones *et al.*, 1995; Jones *et al.*, 1998). The ability of this mutant protein to inhibit the Ypt1 GEF activity of TRAPP was tested. Bet3-associated Ypt1-GEF activity is completely inhibited by the addition of a twofold excess of the Ypt1-D124N mutant protein, but not by a twofold excess of the wild-type Ypt1p (Figure 4a). The wild-type protein can also compete for the GEF activity, but at a much higher concentration; addition of wild-type protein in an 80-fold excess results in 30% inhibition of measurable GDP release. This result, together with the fact that the localization and extraction and properties of the previously characterized Ypt1 GEF and TRAPP are similar, suggests strongly that TRAPP is basically the Ypt1 GEF that we have previously identified.

The *YPT31* and *YPT32* genes were originally isolated by us as high-copy suppressors of the *YPT1-D124N* dominant mutation (S. Jones, H. Smiley, and N. Segev, unpublished, and Jedd *et al.*, 1997). Based on this result and the fact that TRAPP can act as a Ypt31/32 GEF, we expected that the nucleotide-free Ypt1-D124N mutant protein would inhibit not only the Ypt1 GEF activity of TRAPP, but also its Ypt31/32 GEF activity. We tested the ability of this mutant protein to inhibit the Bet3-associated Ypt32 GEF activity in vitro. The GDP release assay indicates that Ypt1-D124N mutant protein, but not wild-type Ypt1p, inhibits more than 50% of the Ypt32 GEF activity (Figure 4b). Together, these results suggest that TRAPP can act as Ypt1 and Ypt31/32 GEF in vivo, and that the nucleotide-free Ypt1 mutant protein inhibits both its Ypt1 and Ypt31/32 GEF activities in vivo.

The *YPT31-N126I* and *YPT32-D129N* mutations in the guanine binding loop are expected to result in mutant proteins that are defective in nucleotide binding. In contrast to the analogous *YPT1* mutations, these mutations do not exert dominant inhibiting phenotypes in vivo (Yoo *et al.*, 1999; and our unpublished results). We tested whether the Ypt31/32 mutant proteins can inhibit the Ypt1 GEF activity of TRAPP. Both Ypt31-N126I and Ypt32-D129N mutant proteins have only a very mild inhibitory effect on the Bet3-associated Ypt1 GEF activity (Figure 5). Despite the fact that higher concentrations were used, the observed level of inhibition was far less than that seen with Ypt1-D124N (see Figure 4a). These mutant proteins also have a very mild inhibitory effect on the Bet3-associated Ypt31/32 GEF activity (our unpublished results). The weak inhibitory effect of the nucleotide-free Ypt31/32 mutant proteins on the TRAPP GEF activity might explain their lack of effect in vivo. The correlation between the in vivo and the in vitro phenotypes also supports the widely-held notion that the mechanism by which nucleotide-free mutant proteins inhibit secretion and cell growth is through their inhibitory effect on GEFs.

Ypt1 and Ypt31/32 GTPases Coprecipitate with TRAPP

The results described above indicate that the TRAPP complex can act as a GEF for Ypt1 and Ypt31/32 GTPases in vitro. To assess whether Ypt1 and Ypt31/32 interact with

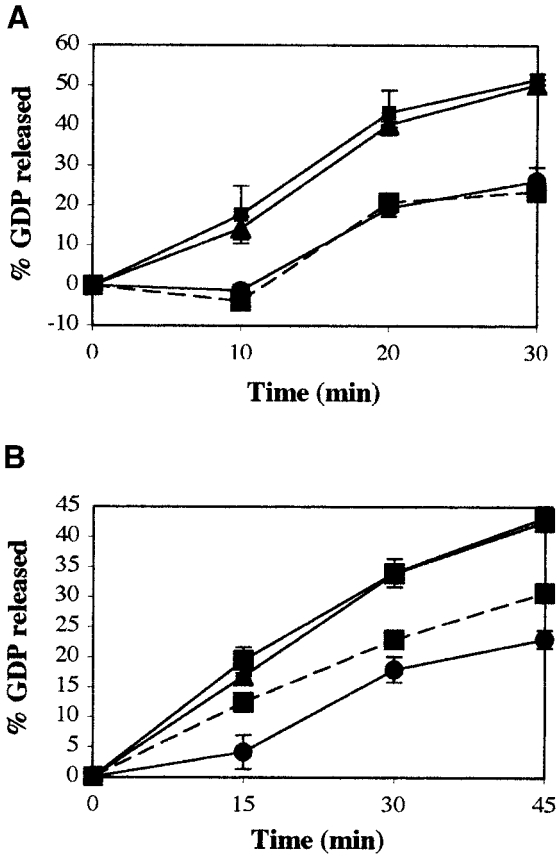

Figure 4. The nucleotide-free mutant Ypt1p-D124N protein is a potent inhibitor of Bet3-associated Ypt1 and Ypt32 GEF activities. a. GST-Bet3p Ypt1-GEF activity can be inhibited by the Ypt1p-D124N mutant protein, but not by wild-type Ypt1p. GST-Bet3 or GST (circles) purified from yeast cells were tested for Ypt1-GEF activity using GDP-release filtration assay and recombinant Ypt1 protein as described in Figure 1a legend. The GST-Bet3p reactions were done in the absence (triangles) or presence of 0.4 μM Ypt1-D124N protein (squares, dashed line), or wild-type Ypt1p (squares, solid line). Results shown in this figure are the average of duplicate measurements and are representative of 3 experiments. Error bars represent range divided by 2. b. GST-Bet3p Ypt32-GEF activity can be inhibited by the Ypt1p-D124N mutant protein, but not by wild-type Ypt1p. Purified GST-Bet3- or GST- (circles) -containing fractions were tested for Ypt32-GEF activity using GDP-release filtration assay and recombinant Ypt32 protein as described in Figure 1a legend. The GST-Bet3p reactions were done in the absence (triangles) or presence of 0.4 μM Ypt1-D124N protein (squares, dashed line), or wild-type Ypt1p (squares, solid line). Results shown in this figure are the average of duplicate measurements and are representative of 2 experiments. Error bars represent range divided by 2.

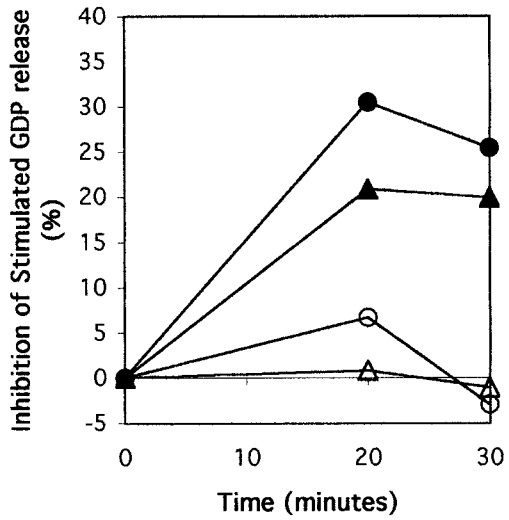

Figure 5. Ypt31-N126I and Ypt32-D129N inhibition of Ypt1 GEF. Purified GST-Bet3- or GST-containing fractions were tested for Ypt1-GEF activity using the GDP-release filtration assay with recombinant Ypt1 protein as described in the legend for Figure 1a. The GST-Bet3p reactions were carried out in the absence or presence of 1.6 μM Ypt31-N126I (●), 1.0 μM Ypt32-D129N (▲), or 1.6 μM Ypt31 wild-type (○) and 1.0 μM Ypt32 wild-type (△) proteins. The results are expressed as percent inhibition by mutant or wild-type proteins of GEF-stimulated GDP-release. Intrinsic exchange, measured in the GST control, was subtracted from the values. Results shown in this figure are the average of duplicate measurements and are representative of two experiments. Error, calculated as average deviation (range divided by 2) was +/− 1% for all the data points.

TRAPP in yeast cells, we tested whether they coprecipitate from yeast cell lysates. The GA-purified GST-Bet3 fraction was tested for the presence of Ypt1 and Ypt31/32 proteins using immuno-blot analysis. The GST-Bet3 fraction, but not the GST fraction, contains both Ypt1 and Ypt31/32 proteins (Figure 6a). The fraction of the total cellular Ypt1 and Ypt31/32 proteins that coprecipitate with GST-Bet3 is ~8%. To determine whether Ypt1 and Ypt31/32 coprecipitate with Bet3p itself or with the TRAPP complex, fractions from the GST-Bet3 Superdex 200 column were tested for the presence of these proteins using immunoblot analysis. Both Ypt1 and Ypt31/32 proteins are present in the high-molecular weight peak, which corresponds to the TRAPP complex, but not in the low-MW peak of free GST-Bet3 subunit (Figure 6b). Together, these results suggest that Ypt1 and Ypt31/32 GTPases interact with the TRAPP complex in yeast cells.

Genetic Interactions between YPT1, YPT31/32 and BET3

Overexpression of *YPT1* was previously shown to suppress the *bet3–1* mutation (Rossi *et al.*, 1995). We examined the effect of overexpression of *BET3* on *ypt1* and *ypt31/32* recessive mutations. Overexpression of GST-Bet3p has no effect on wild-type cells, but it exacerbates *ypt1* and *ypt31/32* mutant phenotypes. Specifically, cells carrying a recessive temperature-sensitive allele of *YPT1*, *ypt1*-A136D, or *ypt1*-T40K (*ypt1–1*), and that overexpress GST-Bet3p grow more slowly than cells expressing GST alone even at their permissive temperatures. *Ypt31Δ/ypt32A141D* mutant cells that express GST-Bet3p grow more slowly than cells expressing GST alone, only at semipermissive temperature (35°C) but not at permissive temperature (26°C) (Figure 7). These genetic interactions of *YPT1* and *YPT31/32* with *BET3* support the idea that TRAPP acts as a Ypt GEF in vivo.

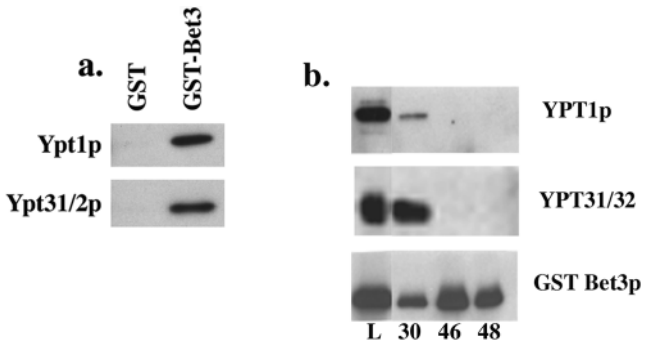

Figure 6. Coprecipitation of Ypt1 and Ypt31/32 With GST-Bet3 and TRAPP. (a) GST-Bet3p or GST were expressed in yeast cells and purified using GA. Equal volumes of eluted preps were analyzed by SDS-PAGE followed by western blotting using anti-Ypt1 (top panel) or Ypt31/32 (lower panel) antibodies. Ypt1 and Ypt31/32 proteins coprecipitate only with GST-Bet3p but not with GST alone. (b) Superdex 200 column fractions containing GST-Bet3, high MW (fraction 30), low MW (fractions 46–48), and the GST-Bet3 fraction that was loaded on the column (L), were analyzed by SDS-PAGE followed by western blotting using anti-Ypt1 (top panel), Ypt31/32 (middle panel), or anti-GST (lower panel) antibodies. Ypt1 and Ypt31/32 proteins coprecipitate only with the High MW but not with the Low MW GST-Bet3p.

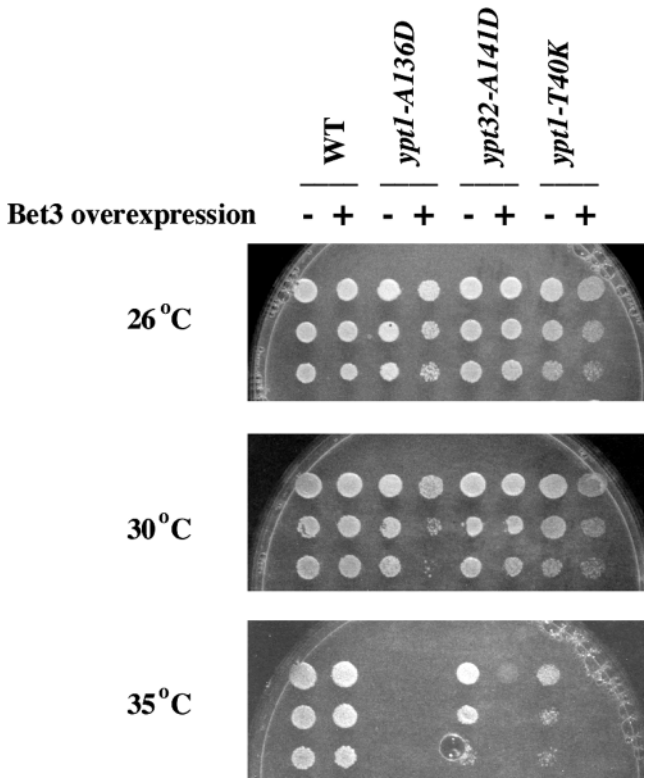

Figure 7. Overexpression of Bet3p exacerbates *ypt1* and *ypt31/32* mutant phenotypes. Effect of *BET3* overexpression on wild-type (NSY125), *ypt1-A136D* (NSY222), *ypt1-T40K* (NSY2), and *ypt31*Δ/*ypt32-A141D* (NSY348) cells. Yeast strains were transformed with plasmids expressing GST-Bet3p (+), or GST alone (−). Shown are 10-fold serial dilutions of transformed cells plated on SD-Ura plates and grown at indicated temperatures. Wild-type cells are not affected by expression of the GST-Bet3p while *ypt* mutant cells are affected by expression of GST-Bet3p but not GST alone. Results shown in this figure are representative of two independent transformants for each strain.

DISCUSSION

In this study, we show data suggesting that the TRAPP complex can act as a GEF for the Ypt1 and Ypt31/32 GTPases, both in vitro and in vivo. Since Ypt1 GTPase is required for ER-to-Golgi transport, while the Ypt31/32 GTPase pair is essential for exit from the trans-Golgi (Segev *et al.*, 1988; Baker *et al.*, 1990; Jedd *et al.*, 1995; Jedd *et al.*, 1997), these findings raise the intriguing possibility that a common GEF complex for the Ypt1 and Ypt31/32 GTPases might coordinate entry into and exit from the Golgi apparatus. Such coordination would clearly be important in maintaining the integrity and the morphology of the Golgi (see Figure 8 for a model). In addition, coordination of Ypt/Rab GTPases by their GEFs might be a general mechanism for the steady-state maintenance of compartment morphology.

The idea that TRAPP can act as a GEF for Ypt1 is supported by the fact that the GEF activity could be purified using two different tagged subunits of this complex, Bet3p and Bet5p. In addition, the Ypt1 and Ypt32 GEF activities associated with Bet3p fractionated as a large complex on a sizing column. The idea that TRAPP functions as a GEF for Ypt1 and Ypt31/32 GTPases in vivo as well is supported by three lines of evidence. First, genes encoding TRAPP components interact genetically with *YPT1* and *YPT31/32*. Specifically, overexpression of *BET3* exacerbates the growth phenotypes of both *ypt1* and *ypt31/32* mutations (this study). In addition, overexpression of *YPT1* was previously shown to suppress the growth phenotype of *bet3* and *bet5* mutations (Rossi *et al.*, 1995; Jiang *et al.*, 1998). These results suggest that the protein products of these genes interact (but not necessarily directly) and are consistent with a role for TRAPP in activation of the Ypt1 and Ypt31/32 GTPases in the cell. Second, Bet3p coprecipitates with both Ypt1 and Ypt31/32 proteins, and these Ypt proteins are present only in the high-molecular weight peak of the GST-Bet3 precipitate, indicating that they interact with TRAPP in yeast cell lysates. Third, *YPT1* and *YPT31/32* interact genetically. Specifically, *YPT31* and *YPT32* were originally identified by us as high-copy suppressors of a dominant negative *YPT1* mutation (S. Jones, H. Smiley, and N. Segev, unpublished data; and Jedd *et al.*, 1997). This interaction, together with our findings that this dominant negative Ypt1 mutant protein inhibits TRAPP's GEF activity for Ypt1 and Ypt32 GTPases, suggests that these two GTPases share a common GEF.

TRAPP has some attributes that are similar to the previously characterized Ypt1 GEF. Both reside on the Golgi, have high molecular weight and similar extraction profiles, and are inhibited by nucleotide-free dominant mutant Ypt1 proteins. However, the two GEFs are probably not identical complexes, since the previously characterized Ypt1-GEF has a smaller MW and does not act as a GEF for Ypt31/32 (Jones *et al.*, 1998). This is most likely attributable to the inclusion of a detergent extraction step in the purification of the previously characterized Ypt1-GEF. Thus, the simplest explanation for the distinction between the two Ypt1-GEFs is that

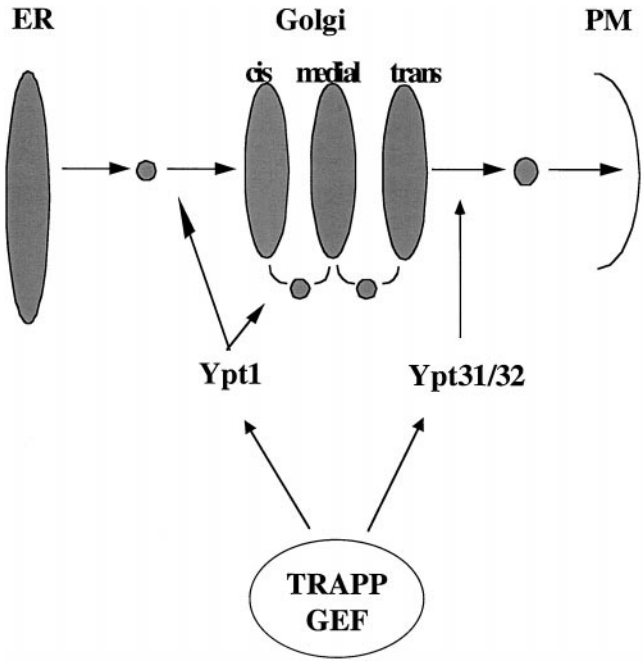

Figure 8. A model for the coordination of entry into and exit from the yeast Golgi by a GEF complex common to Ypt1 and Ypt31/32 GTPases. Ypt1 GTPase is required for ER-to-Golgi transport Golgi (Segev *et al.,* 1988; Baker *et al.,* 1990; Jedd *et al.,* 1995), while the Ypt31/32 functional pair is essential for exit from the trans-Golgi (Jedd *et al.,* 1997). A common GEF complex, TRAPP, might coordinate these steps through the regulation of the Ypt GTPases activation. The importance of such a coordination is discussed in the text.

the previously characterized Ypt1-GEF lacks one or more of the TRAPP subunits that are important for its activity on Ypt31/32p, but not its activity on Ypt1p. However, it is still a formal possibility that the two Ypt1 GEFs are distinct and that there is more than one Ypt1 GEF in yeast cells.

We have previously proposed a model for the role of the regulation of nucleotide cycling in Ypt1-mediated vesicle targeting. In this model, GEF is important for vesicle targeting, and nucleotide exchange occurs at the acceptor compartment, while GTP hydrolysis by GAP is not important for this process but might be only involved in recycling of Ypt proteins between membranes (Jones *et al.,* 1998). The idea that the GEF function is required for Ypt1-mediated ER-to-Golgi transport was based on the finding that the nucleotide-free Ypt1 mutant proteins are potent inhibitors of the Ypt1 GEF activity and of ER-to-Golgi transport in vivo and in vitro (Jones *et al.,* 1995). The idea that GEF acts at the acceptor compartment was based on the localization of the Ypt1 GEF activity to the Golgi (Jones *et al.,* 1998). The identification of TRAPP as the GEF for Ypt1 supports the first part of this model, since TRAPP is essential for ER-to-Golgi vesicle targeting and localizes to the yeast Golgi (Barrowman *et al.,* 2000; Sacher *et al.,* 2000; Sacher *et al.,* 1998), which serves as the acceptor compartment for Ypt1p in ER-to-Golgi transport. Together, these data support the model in which the Ypt1 GEF is essential for Ypt1 function, and their interaction occurs at the acceptor membrane, which, for Ypt1 GTPase, is the Golgi.

TRAPP, a GEF for Ypt1 and Ypt31/32, is a large protein complex. Other Ypt/Rab GEFs have also been shown to be parts of large protein complexes, e.g., Sec2p (Nair *et al.,* 1990), Rab3A-GRF (Burstein and Macara 1992), and Rabex-5 (Horiuchi *et al.,* 1997). However, to date, the known Ypt/Rab GEFs were reported to be specific for a single Ypt/Rab target (Horiuchi *et al.,* 1997; Wada *et al.,* 1997; Walch-Solimena *et al.,* 1997; Hama *et al.,* 1999). The fact that TRAPP acts as a GEF for Ypt1 and Ypt31/32 GTPases is, therefore, surprising. It is possible that different TRAPP subunits act as GEFs for the Ypt1 and Ypt31/32 GTPases. The localization of TRAPP to the Golgi apparatus (Barrowman *et al.,* 2000) is consistent with its role as a GEF for both Ypt1 and Ypt31/32 GTPases. We propose that the GEF resides where the function of its Ypt substrate is required, and the functions of Ypt1 and Ypt31/32 GTPases are required at the two ends of the Golgi. 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), while the function of Ypt31/32 GTPases is essential for the formation of trans-Golgi vesicles (Jedd *et al.,* 1997). To date, TRAPP has been shown to be required for ER-to Golgi transport (Barrowman *et al.,* 2000; Sacher *et al.,* 1998). Our current findings predict that TRAPP would also have a role in later steps of the yeast secretory pathway.

There are several open questions regarding the function of TRAPP as the GEF for Ypt1 and Ypt31/32 GTPases, and regarding the function of Ypt/Rab GEFs in general. It remains to be determined which subunits of TRAPP have the Ypt1 and Ypt31/32 binding and GEF activities. Identification of TRAPP as a Ypt1 and Ypt31/32 GEF is an important first step toward resolving the mechanism by which Ypt/Rab GEFs act in protein transport.

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REFERENCES

Bacon, R.A., Salminen, A., Ruohola, H., Novick, P., and Ferro-Novick, S. (1989). The GTP-binding protein Ypt1 is required for transport in vitro: The Golgi apparatus is defective in *ypt1* mutants. J. Cell Biol. *109,* 1015–1022.

Baker, D., Hicke, L., Rexach, M., Schleyer, M., and Schekman, R. (1988). Reconstitution of *SEC* gene product-dependent intercompartmental protein transport. Cell *54,* 335–344.

Baker, D., Wuestehube, L., Schekman, R., Botstein, D., and Segev, N. (1990). GTP-binding Ypt1 protein and Ca2+ function independently in a cell-free transport reaction.Proc Natl. Acad. Sci. USA *87,* 355–359.

Barrowman, J., Sacher, M., and Ferro-Novick, S. (2000). TRAPP stably associates with the Golgi and is required for vesicle docking. EMBO J. *19,* 862–869.

Benli, M., Doring, F., Robinson, D.G., Yang, X., and Gallwitz, D. (1996). Two GTPase isoforms, Ypt31p, and Ypt32p, are essential for Golgi function in yeast. EMBO J. *15,* 6460–6475.

Bourne, H.R., Sanders, D.A., and McCormick, F. (1990). The GTPase superfamily: a conserved switch for diverse cell functions. Nature *348*, 125–132.

Burstein, E.S., and Macara, I.G. (1992). Characterization of a guanine nucleotide-releasing factor and a GTPase-activating protein that are specific for the *ras*-related protein $p25^{rab3A}$. Proc. Natl. Acad. Sci. USA *89*, 1154–1158.

Cao, X., and Barlowe, C. (2000). Asymmetric requirements for a Rab GTPase and SNARE proteins in fusion of COPII vesicles with acceptor membranes. J. Cell. Biol. *149*, 55–66.

Ferro-Novick, S., and Novick, P. (1993). The Role of GTP-binding proteins in transport along the exocytic pathway. Annu. Rev. Cell. Biol. *9*, 575–599.

Gietz, D., St. Jean, A., Woods, R., and Schiestl, R. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucl. Acids. Res. *20*, 1425.

Goud, B., Salminen, A., Walworth, N.C., and Novick, P. (1988). A GTP-binding protein required for secretion rapidly associates with secretory vesicles and the plasma membrane in yeast. Cell *53*, 753–768.

Hama, H., Tall, G.G., and Horazdovsky, B. (1999). Vps9p is a guanine nucleotide exchange factor involved in vesicle-mediated vacuolar protein transport. J. Biol. Chem. *274*, 15284–15291.

Haney, S.A., and Broach, J.R. (1994). Cdc25p, the guanine nucelotide exchange factor for the Ras proteins of *Saccharomyces cerevisiae*, promotes exchange by stabilizing ras in a nucleotide-free state. J. Biol. Chem. *269,* 16541–16548.

Horiuchi, H., Lippe, R., McBride, H.M., Rubino, M., Woodman, P., Stenmark, H., Rybin, V., Wilm, M., Ashman, K., Mann, M., and Zerial, M. (1997). A novel Rab5 GDP/GTP exchange factor complexed to Rabaptin-5 links nucleotide exchange to effector recruitment and function. Cell *90*, 1149–1159.

Hwang, Y.-W., Sanchez, A., and Miller, D., L. (1989). Mutagenesis of bacterial elongatin factor tu at lysine 136, J. Biol. Chem. *264,* 8304–8309.

Hwang, Y.W., Zhong, J.M., Poullet, P., and Parmeggiani, A. (1993). Inhibition of SDC25C-domain-induced guanine-nucleotide exchange by guanine ring binding domain mutants of v-H-ras. J. Biol. Chem. *268*, 24692–24698.

Jamieson, J.D., and Palade, G.E. (1967). Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of peripheral elements of the Golgi complex. J. Cell. Biol. *34*, 577–596.

Jedd, G., Mulholland, J., and Segev, N. (1997). Two new Ypt GTPases are required for exit from the yeast trans-Golgi compartment J. Cell. Biol.*137*, 563–580.

Jedd, G., Richardson, C.J., Litt, R.J., and Segev, N. (1995). The Ypt1 GTPase is essential for the first two steps of the yeast secretory pathway. J. Cell. Biol. *131*, 583–590.

Jiang, Y., Scarpa, A., Zhang, L., Stone, S., Feliciano, E., and Ferro-Novick, S. (1998). A high copy suppressor screen reveals genetic interactions between BET3 and a new gene. Evidence for a novel complex in ER-to-Golgi transport. Genetics *149*, 833–841.

Jones, S., Litt, R.J., Richardson, C.J., and Segev, N. (1995). Requirement of nucleotide exchange for Ypt1 GTPase mediated protein transport. J. Cell. Biol. *130*, 1051–1061.

Jones, S., Richardson, C.J., Litt, R.J., and Segev, N. (1998). Identification of regulators for Ypt1 GTPase nucleotide cycling. Mol. Biol. Cell. *9*, 2819–2837.

Lai, C.-C., Boguski, M., Broek, D., and Powers, S. (1993). Influence of guanine nucleotides on complex formation between ras and CDC25 proteins. Mol. Cell. Biol. *13*, 1345–1352.

Martzen, M.R., McCraith, S.M., Spinelli, S.L., Torres, F.M., Fields, S., Grayhack, E.J., and Phizicky, E.M. (1999). A biochemical genomics approach for identifying genes by the activity of their products. Science *286*, 1153–1155.

Nair, J., Muller, H., Peterson, M., and Novick, P. (1990). Sec2 protein contains a coiled-coil domain essential for vesicular transport and a dispensable carboxy terminal domain. J. Cell. Biol. *110*, 1897–1909.

Novick, P., Ferro, S., and Schekman, R. (1981). Order of events in the yeast secretory pathway. Cell *25*, 461–469.

Palade, G. (1975). Intracellular aspects of the process of protein synthesis. Science *189,* 347–358.

Pfeffer, S. (1992). GTP-Binding proteins in intracellular transport. Trends Cell. Biol. *2*, 41–46.

Powers, S., Gonzales, E., Christensen, T., Cubert, J., and Broek, D. (1991). Functional cloning of BUD5, a CDC25-related gene from *S. cerevisiae* that can suppress a dominant-negative *RAS2 mutant*. Cell *65*, 1225–1231.

Powers, S., O'Neill, K., and Wigler, M. (1989). Dominant yeast and mammalian RAS mutants that interfere with the CDC25-dependent activation of wild-type RAS in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *9,* 390–395.

Richardson, C.J., Jones, S., Litt, R.J., and Segev, N. (1998). GTP hydrolysis is not important for Ypt1 GTPase function in vesicular transport. Mol. Cell. Biol. 1998 Feb; *18*, 827–838.

Rose, M., Winston, F., and Heiter, P. (1988). Methods in Yeast Genetics, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Rossi, G., Kolstad, K., Stone, S., Palluault, F., and Ferro-Novick, S. (1995). BET3 encodes a novel hydrophilic protein that acts in conjunction with yeast SNAREs. Mol. Biol. Cell. *6*, 1769–1780.

Sacher, M., Barrowman, J., Schielz, D., Yates, J., and Ferro-Novick, S. (2000). Identification and characterization of five new subunits of TRAPP. Eur. J. Cell. Biol. *79*, 71–80.

Sacher, M., Jiang, Y., Barrowman, J., Scarpa, A., Burston, J., Zhang, L., Schieltz, D., Yates, J.R., 3rd,, Abeliovich, H., and Ferro-Novick, S. (1998). TRAPP, a highly conserved novel complex on the cis-Golgi that mediates vesicle docking and fusion. EMBO J. *17,* 2494–2503.

Segev, N. (1991). Mediation of the attachment or fusion step in vesicular transport by the GTP-binding Ypt1 protein. Science *252*, 1553–1556.

Segev, N., and Botstein, D. (1987). The ras-like yeast YPT1 gene is itself essential for growth, sporulation, and starvation response. Mol. Cell. Biol. *7*, 2367–2377.

Segev, N., Mulholland, J., and Botstein, D. (1988). The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. Cell *52*, 915–924.

Wada, M., Nakanishi, H., Satoh, A., Hirano, H., Obaishi, H., Matsuura, Y., and Takai, Y. (1997). Isolation and characterization of a GDP/GTP exchange protein specific for the Rab3 subfamily small G proteins. J. Biol. Chem. *272*, 3875–3878.

Walch-Solimena, C., Collins, R., and Novick, P. (1997). Sec2 mediates nucleotide exchange on Sec4p and is involved in polarized delivery of post-Golgi vesicles. J. Cell. Biol. *137*, 1495–1509.

Yoo, J.S., Grabowski, R., Xing, L., Trepte, H.H., Schmitt, H.D., and Gallwitz, D. (1999). Functional implications of genetic interactions between genes encoding small GTPases involved in vesicular transport in yeast. Mol. Genet. *261*, 80–91.

Zerial, M., and Stenmark, H. (1993). Rab GTPases in vesicular transport. Curr. Opin. Cell. Biol. *5*, 613–620.