Molecular Cell, Vol. 7, 433–442, February, 2001, Copyright ©2001 by Cell Press

TRAPP I Implicated in the Specificity of Tethering in ER-to-Golgi Transport

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Summary

TRAPP is a conserved protein complex required early in the secretory pathway. Here, we report two forms of TRAPP, TRAPP I and TRAPP II, that mediate different transport events. Using chemically pure TRAPP I and COPII vesicles, we have reconstituted vesicle targeting in vitro. The binding of COPII vesicles to TRAPP I is specific, blocked by GTPγS, and, surprisingly, does not require other tethering factors. Our findings imply that TRAPP I is the receptor on the Golgi for COPII vesicles. Once the vesicle binds to TRAPP I, the small GTP binding protein Ypt1p is activated and other tethering factors are recruited.

Introduction

The secretory pathway of the yeast *Saccharomyces cerevisiae* is a well studied model system that is amenable to deciphering the molecular details of membrane traffic. To date, greater than 30 gene products have been shown to function in transport between the endoplasmic reticulum (ER) and Golgi, the first membrane-bound compartments in this pathway. Components of the ER-derived vesicle coat, COPII, aid in cargo selection at the ER (Springer and Schekman, 1998) and in ER membrane deformation (Barlowe et al., 1994; Matsuoka et al., 1998). This coat is distinct from the COPI coat, which acts in membrane traffic within the Golgi (Orci et al., 1993). Once a free-formed vesicle is produced, the concerted action of many different factors, including SNAREs (*s*oluble *N*-ethylmaleimide sensitive factor *a*ttachment protein *re*ceptors; Söllner et al., 1993), Ypt1p (Segev et al., 1988), Uso1p (Nakajima et al., 1991; Barlowe, 1997), TRAPP (Sacher et al., 1998; Barrowman et al., 2000), and the Sec34p/Sec35p complex (Kim et al., 1999; VanRheenen et al., 1999), is required to properly dock a COPII vesicle and fuse it with the *cis*-Golgi. While the SNAREs are believed to mediate membrane fusion, understanding the temporal relationship of the other factors and their interactions is a major focus of current work.

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A number of the factors mentioned above, as well as their mammalian orthologs, are believed to act in several stages of membrane traffic. Ypt1p, the yeast ortholog of the small GTPase Rab1, acts in both ER-to-Golgi and intra-Golgi transport (Bacon et al., 1989; Jedd et al., 1995). Sec34p and Sec35p were originally identified as components required for traffic between the ER and the Golgi (Wuestehube et al., 1996). More recently, however, it has been noted that *SEC34* is identical to *GRD20*, a gene whose product localizes to and acts at the *trans*-Golgi (Spelbrink and Nothwehr, 1999). The closest mammalian ortholog of Uso1p is p115 (Sapperstein et al., 1996). p115 has been reported to have a role in tethering COPI, as well as COPII, vesicles to the Golgi (Sönnichsen et al., 1998; Alvarez et al., 1999; Allan et al., 2000). Collectively, these findings suggest the presence of stage-specific factors that may interact with or recruit multistage transport factors.

We previously identified a multiprotein complex called TRAPP that stably associates with the Golgi under conditions that block ER-to-Golgi traffic (Sacher et al., 1998, 2000; Barrowman et al., 2000). Here, we report that there are two forms of TRAPP (TRAPP I and TRAPP II) that act at different stages of membrane traffic. TRAPP I and TRAPP II cofractionate to an early Golgi compartment that also contains the ER–Golgi SNAREs. Both forms of the complex can exchange nucleotide on Ypt1p, which is consistent with the finding that TRAPP I and TRAPP II act in ER–Golgi and Golgi transport, respectively. Chemically pure TRAPP I binds to COPII vesicles in vitro. This binding reaction occurs on ice in the absence of ATP and other tethering factors. Our findings suggest that the binding of TRAPP I to COPII vesicles is the first of many events in the interaction of a transport vesicle with its target membrane.

Results

TRAPP Subunits Are Found in Two Different Complexes

We previously isolated TRAPP using strains in which Bet3p was fused to either Protein A (Sacher et al., 2000) or c-*myc* (Sacher et al., 1998). Nine polypeptides (Bet5p, Trs20p, Trs23p, Trs31p, Trs33p, Trs65p, Trs85p, Trs120p, and Trs130p) were found to coprecipitate with tagged Bet3p. The overexpression of all but three of these subunits (Trs65p, Trs120p, and Trs130p) suppressed the temperature-sensitive (ts) growth phenotype of the *bet3–1* mutant (Sacher et al., 1998, 2000). These genetic studies suggested that Trs65p, Trs120p, and Trs130p may interact with Bet3p in a different manner than the other subunits. This finding prompted us to look for two forms of TRAPP. TRAPP subunits were monitored using gel filtration chromatography. Radiolabeling and Western blot analysis revealed two peaks of Trs33p, one in fraction 9 and a second in fraction 13 (Figure 1A). These peaks corresponded to molecular sizes of ~1000 kDa and 300 kDa, respectively. The components found in each of these peaks were identified

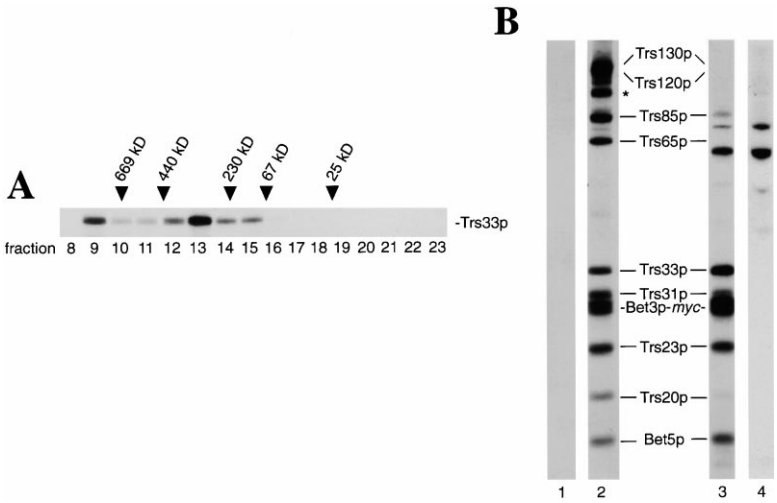

Figure 1. TRAPP Subunits Are Found in Two Different Complexes

(A) Trs33p is found in two high-molecular-weight fractions. A radiolabeled lysate was prepared from a strain containing Bet3p-*myc* as described before (Sacher et al., 1998; Sacher and Ferro-Novick, 2001). An aliquot of 200 $\times$ 10^6 cpm was applied to a Superdex-200 gel filtration column and 25 fractions of 1 ml each were collected. Each fraction was immunoprecipitated with 2 μl of anti-c-*myc* antibody and analyzed on a 13% SDS polyacrylamide gel. The fractionation of Trs33p is shown. Molecular weight standards are thyroglobulin (669 kDa), ferritin (440 kDa), catalase (230 kDa), bovine serum albumin (67 kDa), and chymotrypsinogen A (25 kDa).

(B) Trs65p, Trs120p and Trs130p are only found in TRAPP II. Radiolabeled lysates were prepared from an untagged strain (lanes 1 and 4) and a Bet3p-*myc* tagged strain (lanes 2 and 3), fractionated by gel filtration, and immunoprecipitated as described in (A). Fractions 9 (lanes 1 and 2) and 13 (lanes 3 and 4) are shown. TRAPP subunits are indicated between lanes 2 and 3. The band marked by an asterisk represents a proteolytic breakdown product of Trs120p (see Sacher et al., 2000).

by fractionating a radiolabeled lysate prepared from the Bet3p-*myc* tagged strain, and an untagged strain as a control. Each fraction was precipitated with anti-c-*myc* antibody. As shown in Figure 1B, the material in fraction 9 contained all ten previously identified TRAPP subunits (compare lane 2 with untagged control in lane 1), while fraction 13 contained seven of the ten subunits of TRAPP (lane 3) as well as contaminating bands that were also present in the untagged control (lane 4). Interestingly, the three subunits missing in fraction 13 (Trs65p, Trs120p, and Trs130p) were the products of the genes that did not suppress *bet3–1*. The same result was obtained with other tagged subunits (data not shown). While the subunits in fraction 9 were found in equimolar amounts, some of the subunits in fraction 13, notably Trs85p and Trs20p, were clearly substoichiometric compared to the other subunits (Figure 1B, compare lanes 2 and 3). We have named the smaller complex found in fraction 13 TRAPP I, and the larger complex found in fraction 9 TRAPP II.

TRAPP I, but Not TRAPP II, Functions in ER–Golgi Transport In Vitro

We previously reported that the depletion of Bet3p from fractions used in an in vitro transport assay, which measures ER–Golgi membrane traffic (Ruohola et al., 1988), blocks the binding of vesicles to the Golgi. Reconstitution of transport was dependent upon the addition of cytosol that contains Bet3p (Sacher et al. 1998; Barrowman et al., 2000). In these earlier studies, the integrity of the complex was not assessed. When this experiment was performed, both TRAPP I and TRAPP II were found to be unstable in the absence of Bet3p (data not shown).

Since Bet3p is a component of both TRAPP I and TRAPP II, we wanted to determine which complex was required for reconstitution. Cytosols used to reconstitute transport activity in vitro are prepared by centrifugation of a yeast lysate at a speed that may pellet the larger TRAPP II complex. This was in fact the case (Figure 2A, compare lanes 1 and 2), and therefore, we deduced that TRAPP I is the active component in our assay. To directly address this, the reconstitution assay was performed using fractions from gel-filtered lysates enriched in either TRAPP I or TRAPP II. As expected, the fraction containing TRAPP I showed a dose-dependent increase in the reconstitution of transport activity (Figure 2B, lanes 1 and 2), while TRAPP II showed no activity (lanes 3 and 4). These results directly demonstrate that TRAPP I, but not TRAPP II, is required for ER–Golgi traffic in vitro.

A Temperature-Sensitive *trs130* Mutant Accumulates Aberrant Golgi Structures

To address the role of TRAPP II in membrane traffic, we constructed a temperature-sensitive mutant in *TRS130*, a gene whose product is unique to TRAPP II. The *trs130* mutant was constructed by truncating the last 33 amino acids of *TRS130* with (*trs130*^{ts1}) or without (*trs130*^{ts2}) the aid of a transposable element. The phenotype of both mutants was examined morphologically by electron microscopy (EM) and compared to other mutants. Mutants that block vesicle targeting between the ER and Golgi characteristically accumulate dilated ER as well as small, ~50 nm diameter vesicles. This morphology was previously noted for *bet3–1* (Rossi et al., 1995), and the same phenotype was observed for a mutant with a defect in another TRAPP subunit, *bet5* (compare wild-type cells in Figure 3A to *bet5–1* in Figure 3D). The morphology of *trs130*^{ts2} (Figure 3C) and *trs130*^{ts1} (data not shown) at 37°C was strikingly different from ER–Golgi blocked mutants. Instead of dilated ER and small vesicles, an abundance of toroid-shaped structures accumulated, similar to the aberrant Golgi reported in mutants such as *sec7*, *sec14*, *sft1,* and *pik1,* whose products act at the level of the Golgi (Novick et al., 1980; Banfield et al., 1995; Walch-Solimena and Novick, 1999). The morphology of the *trs130* mutants was dependent on the temperature shift, as cells grown at the permis-

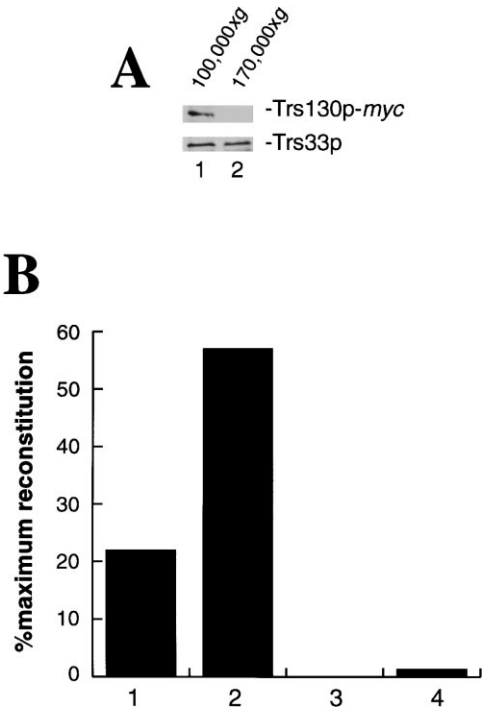

Figure 2. TRAPP I Reconstitutes ER-to-Golgi Transport In Vitro to Bet3p-Depleted Fractions

(A) TRAPP II is pelleted from cytosols used to reconstitute transport in vitro. A lysate was prepared from a strain containing Trs130p-*myc* as described in Experimental Procedures and centrifuged for 1 hr at 100,000 × *g* (lane 1) or 4 hr at 170,000 × *g* (lane 2). Aliquots containing 300 μg of protein were analyzed by Western blot analysis (ECL) using anti-c-*myc* antibody (top panel; 1:5000) and anti-Trs33p (bottom panel; 1:2500).

(B) TRAPP I reconstitutes transport. A lysate prepared from wild-type cells was fractionated by gel filtration chromatography, and fractions containing TRAPP I and TRAPP II were prepared as described in the Experimental Procedures. Increasing amounts of the fraction containing TRAPP I (lane 1, 40 μl; lane 2, 80 μl), but not TRAPP II (lane 3, 40 μl; lane 4, 80 μl), reconstituted transport activity. Reconstitution is expressed as the percent of maximum reconstitution obtained with a wild-type cytosol. The experiment was performed three times and a representative result is shown.

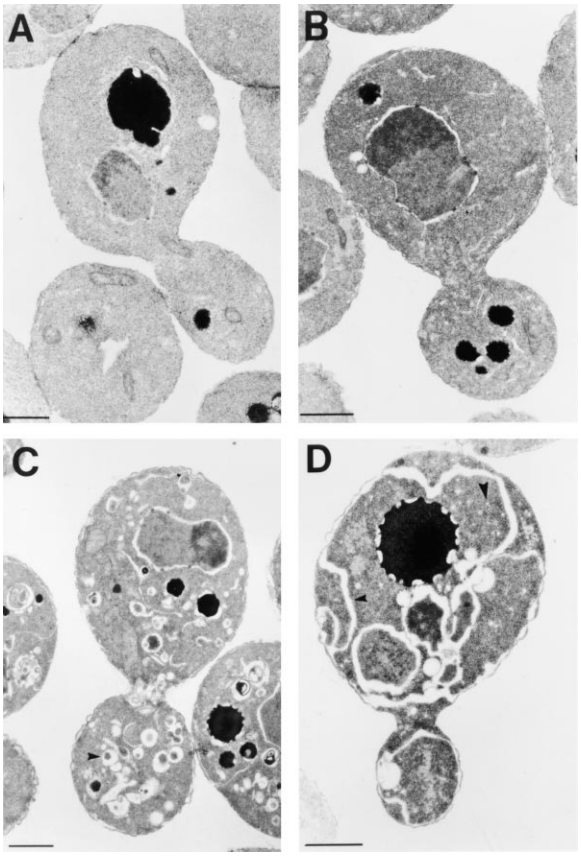

Figure 3. The *trs130* Mutant Accumulates Toroid-Shaped Structures at the Restrictive Temperature

Wild-type cells (A), *trs130*^{ts2} (B and C), and *bet5–1* (D) were shifted to 37°C for 2 hr (A, C, and D) or left at 25°C (B) and prepared for electron microscopy as described before (Rossi et al., 1995). The arrowhead in (C) identifies a toroid-shaped structure that is similar to aberrant Golgi found in *sec7*, *sec14*, *sft1,* and *pik1*. The large arrowhead in (D) shows a cluster of small, ~50 nm diameter transport vesicles, and the small arrowhead in (D) shows dilated ER. The bars in each panel represent 1 μm.

sive temperature (25°C) were phenotypically indistinguishable from wild type (compare Figure 3B with Figure 3A). These morphological results are consistent with a role for TRAPP II in Golgi traffic.

The *trs130* Mutant Accumulates Golgi Forms of CPY and Invertase

If TRAPP II functions in Golgi transport, proteins that utilize the secretory pathway should be arrested in the Golgi. The vacuolar hydrolase carboxypeptidase Y (CPY) is synthesized and translocated into the lumen of the ER (p1 form) before it is transported to and modified in early (p1′ form) and late (p2 form) Golgi compartments (Franzusoff and Schekman, 1989; Banfield et al., 1995). Proteolytic activation in the vacuole yields the mature form (Stevens et al., 1982). Pulse-chase experiments were performed to examine the processing of CPY in *trs130*, as well as wild type and mutants that block ER-to-Golgi (*bet3 and sec18*) and intra-Golgi traffic (*sft1*). After a 4 min pulse, CPY was found in the p1 form in wild type and the mutants (Figure 4A, lanes 1, 3, 5, 7, and 9). Radiolabeled protein was converted to the mature form within 30 min in wild type (lane 2). In contrast, the *sec18* mutant failed to process CPY beyond the p1 form during the chase (lane 4). The p1 form was processed in the *trs130* mutant, indicating that CPY was transported from the ER to the Golgi. In addition, Golgi forms (p1′ and p2) accumulated after 30 min (lane 6). Some mature CPY was also seen following the chase, suggesting that Trs130p was not completely inactivated at 37°C. As reported before (Banfield et al., 1995), *sft1* accumulated the p1′ form of CPY (lane 8), while *bet3* primarily accumulated p1 CPY (lane 10). A small amount of the CPY accumulated in *bet3* was precipitated with anti-α1→6 antibody (data not shown), indicating that it proceeded past an initial block between the ER and Golgi and was then transported to the Golgi.

To examine the processing of invertase, wild-type and mutant cells (*sec18*, *bet3*, *trs130,* and *trs120*) were radiolabeled at 37°C, and the internal and periplasmic forms

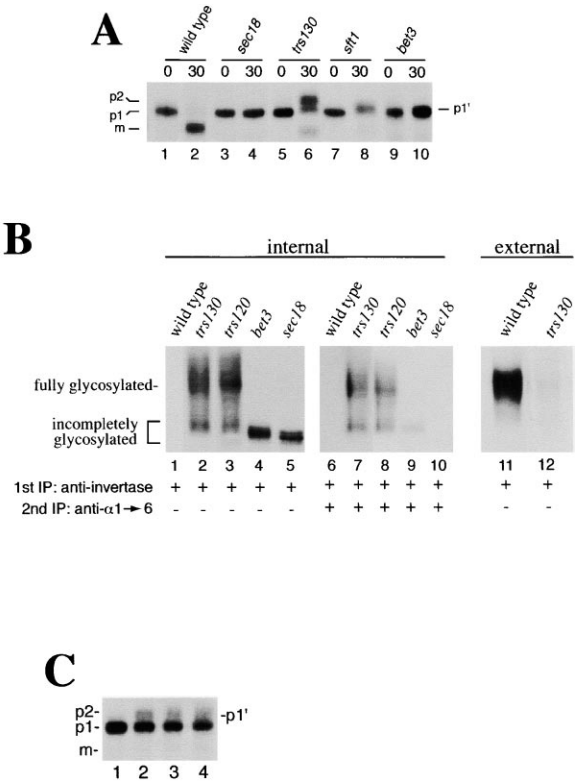

Figure 4. The *trs130* Mutant Accumulates Golgi Forms of CPY and Invertase

(A) The *trs130* mutant accumulates Golgi forms of CPY. Eight units (at OD_{600} = 1) of wild type (lanes 1 and 2), *sec18–1* (lanes 3 and 4), *trs130*^{ts1} (lanes 5 and 6), *sft1–15* (lanes 7 and 8), and *bet3–1* (lanes 9 and 10) cells were preshifted to 37°C for 20 min in 2.8 ml of synthetic minimal medium (with the appropriate supplements) and pulse labeled for 4 min with 250 μCi of [^{35}S]ProMix (Amersham). Samples were removed at the end of the labeling (lanes 1, 3, 5, 7, and 9) and after 30 min of chase (lanes 2, 4, 6, 8, and 10) and then processed for CPY immunoprecipitation as described in Rossi et al. (1995). The ER (p1), late Golgi (p2), and mature (m) forms of CPY are indicated to the left of the panel, and the early Golgi (p1′) form is indicated on the right.

(B) The *trs130* and *trs120* mutants accumulate Golgi-modified forms of invertase. Two units (at OD_{600} = 1) of wild type (lanes 1, 6, and 11), *trs130*^{ts2} (lanes 2, 7, and 12), *trs120* (lanes 3 and 8), *bet3–1* (lanes 4 and 9), and *sec18–1* (lanes 5 and 10) cells were preshifted in synthetic minimal medium containing 2% glucose (with the appropriate supplements) for 20 min at 37°C. The cells were then transferred into medium containing 0.1% glucose to derepress the synthesis of invertase and labeled with 250 μCi of [^{35}S]ProMix for 60 min. Samples were processed for immunoprecipitation of internal invertase (lanes 1–8) and periplasmic invertase (lanes 9 and 10) as described in Rossi et al. (1995). For the analysis of internal invertase, half of the precipitate was analyzed on an SDS–polyacrylamide gel (lanes 1–4), and the other half was re-immunoprecipitated with anti-α1→6 antibody (lanes 5–8) as described before (Lian and Ferro-Novick, 1993).

(C) Bet3p acts at two stages in the secretory pathway. A pulse-chase experiment was performed with the *bet3–2* mutant (Walch-Solimena and Novick, 1999) as described in (A), except the pulse was performed for 4 min at 25°C and then chased for 5 (lane 2), 10 (lane 3), and 30 min (lane 4) at 37°C.

Table 1. Effect of Inactivation of TRAPP Subunits

Subunit	TRAPP I/ TRAPP II	Transport Block
Bet5p	I/II	ER–Golgi
Trs20p	I/II	ER–Golgi
Bet3p	I/II	ER–Golgi
Trs23p	I/II	ER–Golgi
Trs31p	I/II	ER–Golgi
Trs33p	I/II	–
Trs65p	II	–
Trs85p	I/II	ER–Golgi
Trs120p	II	Golgi
Trs130p	II	Golgi

Transport blocks were detected at 37°C in ts mutants either morphologically by EM or by pulse–chase analysis using CPY as a marker protein. Mutants blocked in ER–Golgi transport showed an accumulation of the p1 form of CPY and/or dilated ER and vesicles by EM. Mutants blocked in Golgi transport showed an accumulation of p1′ and p2 CPY and/or Golgi by EM. Minus (–) indicates no block in traffic was detected. *TRS20* and *TRS23* were placed under the control of the *GAL1* promotor and grown in YPD prior to analysis.

of invertase were immunoprecipitated. Wild-type cells showed no accumulation of radiolabeled invertase (Figure 4B, lane 1) but secreted a fully glycosylated form into the periplasm (lane 11). In contrast, the secretion of invertase was defective in the *trs130* mutant (lane 12), and incompletely glycosylated forms of the enzyme accumulated (lane 2) that were precipitated with anti-α1→6 antibody (lane 7). The more highly glycosylated form was precipitated with anti-α1→3 antibody (data not shown). The same result was obtained with a ts mutant, *trs120*, in a second TRAPP II–specific subunit (lanes 3 and 8), and similar results were previously reported for *sft1* (Banfield et al., 1995). While the *bet3* mutant accumulated internal forms of invertase (lane 4), only small amounts of the uppermost form cross-reacted with anti-α1→6 antibody (lane 9), indicating that some invertase accumulated in the Golgi. In contrast to *trs130*, *trs120,* and *bet3*, *sec18* accumulated a form of invertase (lane 5) that was not Golgi modified (lane 10). Thus, phenotypic analysis of the *trs130* mutant indicates that TRAPP II functions after transport to the Golgi.

Inactivation of Subunits Common to TRAPP I and TRAPP II Block ER-to-Golgi Traffic

Using a combination of CPY pulse-chase analysis and EM, we examined mutants in the *trs* genes for a block in membrane traffic. As shown in Table 1, all conditional mutants with defects in subunits that are shared between TRAPP I and TRAPP II blocked ER-to-Golgi membrane traffic. Although both complexes are presumably nonfunctional in these mutants, the TRAPP I phenotype was observed because TRAPP I acts before TRAPP II. In contrast, a block in Golgi traffic was only seen in mutants (*trs130* and *trs120*) defective in TRAPP II–specific subunits. The loss of Trs65p and Trs33p did not alter the assembly of the complex (M. S. and S. F.-N., unpublished data), nor lead to a growth defect (Sacher et al., 2000), and no effect on membrane traffic was observed. While *TRS85* is not essential (Sacher et al., 2000), the absence of this gene product, in a strain containing Bet3p-*myc*, resulted in a reduction in growth at 37°C and a block in ER-to-Golgi transport (Table 1).

Our analysis of the trafficking of CPY and invertase in the *bet3* mutant implied that Bet3p operates in more

than one transport event. When we pulse-labeled *bet3* at 25°C and then shifted the mutant to 37°C, a second block was revealed. An early Golgi form of CPY (presumably p1′), as well as some p2 CPY, appeared at 37°C and persisted for the duration of the chase (Figure 4C). Thus, Bet3p plays a role in both ER-to-Golgi traffic and traffic through or from the Golgi.

TRAPP I and TRAPP II Cofractionate on Sucrose Density Gradients

Previous work has shown that Trs130p (TRAPP II) and Trs33p (TRAPP I and TRAPP II) cofractionate with each other and Golgi, but not ER, marker proteins on sucrose velocity gradients (Barrowman et al., 2000). Since we propose that TRAPP II acts in Golgi transport and TRAPP I in ER-to-Golgi transport, we next addressed whether the two complexes localize to the same or different subcompartments of the Golgi. To determine the localization of TRAPP subunits on the Golgi, we fractionated yeast lysates on sucrose density gradients capable of resolving subcompartments of the Golgi. We followed the ER–Golgi SNARE Bet1p as a marker for early Golgi, as well as GDPase and Kex2p as markers for more distal Golgi compartments. Western blots of gradient fractions revealed that Trs130p fractionated in one peak with Bet1p (Figure 5A), and did not cofractionate with either GDPase or Kex2p (Figure 5B). TRAPP subunits Trs33p (Figure 5A) and Bet3p (data not shown), which are members of both TRAPP subcomplexes, also cofractionated with Trs130p and Bet1p. These and previous localization results (Barrowman et al., 2000) indicate that TRAPP I and TRAPP II cofractionate to an early Golgi compartment of similar size and density.

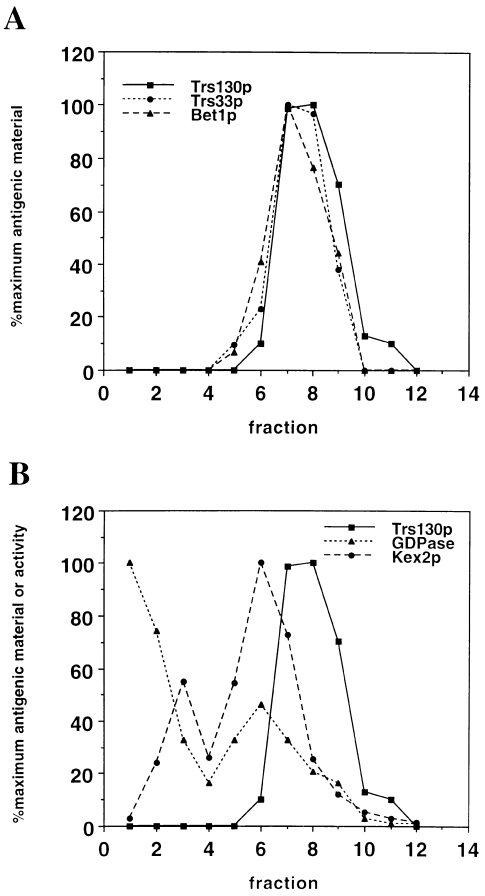

Figure 5. The TRAPP I and TRAPP II Complexes Cofractionate with Each Other and an Early Golgi Marker on Sucrose Density Gradients

A lysate was prepared from wild-type yeast cells and fractionated on a sucrose density gradient as described in Barrowman et al. (2000), except that the gradients were centrifuged for 18 hr to allow the membranes to reach equilibrium.

(A) TRAPP subunits cofractionate with the SNARE Bet1p. Proteins were visualized from aliquots (50 μl) of gradient fractions by ECL using antibodies against Bet1p (1:1000; closed triangles), Trs33p (1:2500; closed circles), and Trs130p (1:1000; closed squares).

(B) Trs130p does not cofractionate with GDPase or Kex2p. Aliquots (50 μl) were assayed for GDPase (closed triangles) and Kex2p (closed circles) activity as described before (Sacher et al., 1998), or analyzed by Western blot analysis using anti-Trs130p antibody (1:1000; closed squares).

TRAPP I, but Not TRAPP II, Binds ER-Derived COPII Vesicles In Vitro

Many factors have been proposed to mediate ER–Golgi vesicle tethering in yeast, including TRAPP I, Ypt1p, the Sec34p/Sec35p complex (which has six putative members) and Uso1p (Barlowe, 1997; VanRheenen et al., 1999; Barrowman et al., 2000). How these components mediate this event is unclear. A key question that remains is the identity of the factor that confers specificity. With a means to produce radiolabeled COPII vesicles in vitro, and the ability to separate the two forms of TRAPP, the role of TRAPP I in vesicle tethering can now be addressed.

To begin these studies, we first tested if immobilized TRAPP can bind COPII transport vesicles formed in vitro. Initially, binding studies were performed with a mixture of both forms of the TRAPP complex (TRAPP I/II). TRAPP I/II was immobilized by treating IgG-Sepharose beads with a lysate prepared from a strain in which Bet3p is fused to protein A (Sacher et al., 2000). As a control, beads were treated with a lysate prepared from an isogenic untagged strain. Vesicles were formed in vitro by incubating donor permeabilized yeast cells, containing radiolabeled pro-α-factor, with cytosol and ATP. Under these conditions vesicles, but not ER, are released from the cells (Groesch et al., 1990). As shown in Figure 6A, beads containing TRAPP I/II bound approximately six times more radiolabel than control beads (compare solid bars in TRAPP I/II and control data sets), and the binding of the vesicles to the beads showed a linear response over the range tested (not shown). We estimated that ~10%–30% of the vesicles in the reaction bound to the beads. The radiolabel precipitated on the TRAPP I/II beads represents pro-α-factor contained within a membrane since pretreatment of the vesicle fraction with 1% Triton X-100 abolished binding of radiolabel to the beads (Figure 6A, compare hatched bars in TRAPP I/II and control data sets). Furthermore, binding of radiolabeled pro-α-factor was dependent upon vesicle production as inhibition of vesicle formation with apyrase significantly reduced binding to background levels (Figure 6A, compare open bars in TRAPP I/II and control data sets).

To establish which form of the TRAPP complex bound to COPII vesicles, we prepared a lysate from the Bet3p–

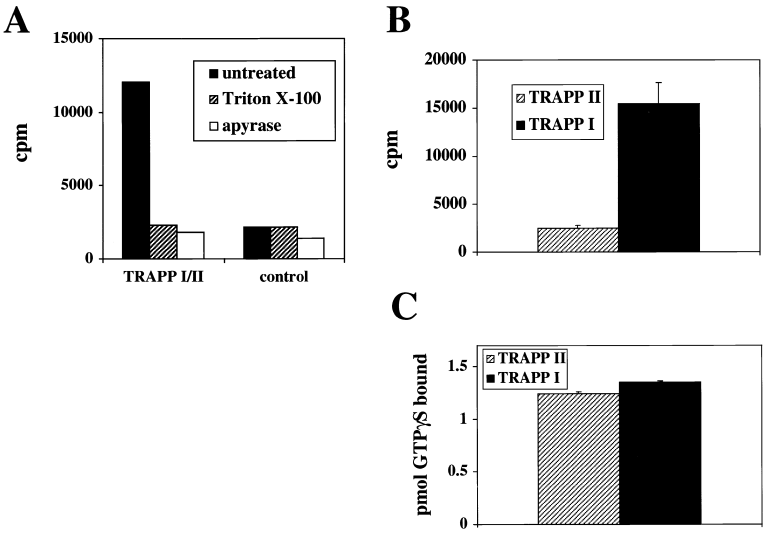

Figure 6. TRAPP I Binds ER-Derived Transport Vesicles In Vitro

(A) Beads incubated with a Bet3p-Protein A–tagged lysate bind transport vesicles. Aliquots (20 μl) of IgG-Sepharose beads with or without TRAPP were incubated with ~100 μl of a supernatant fraction containing ER-derived vesicles (the equivalent of one reaction) for 2 hr at 4°C. The radiolabel bound to the washed beads was determined by scintillation counting. In the reactions shown, vesicles were either untreated (closed bars) or treated with 1% Triton X-100 for 5 min prior to the binding reaction (hatched bars). Vesicles fail to form when the reaction is performed in the presence of apyrase, and no binding was observed (open bars). The experiment was performed four times, and a representative result is shown.

(B) TRAPP I beads, but not TRAPP II beads, bind vesicles. IgG-Sepharose beads containing either TRAPP II (hatched bar), TRAPP I (closed bar), or control beads (not shown) were prepared as described in the Experimental Procedures. Aliquots (35 μl) of the TRAPP I– and TRAPP II–containing beads were analyzed by Western blot analysis (ECL) using anti-Trs33p (1:2500). Binding reactions were as described above, and the cpm bound to the TRAPP I– or TRAPP II–containing beads are expressed as a function of the Trs33p present in the binding reaction. The control beads bound 2547 ± 125 cpm.

(C) TRAPP II bound to beads is active for nucleotide exchange. TRAPP I and TRAPP II beads were prepared as above. Aliquots of the TRAPP II beads (hatched bar), TRAPP I beads (closed bar), as well beads prepared from a fraction (fraction 23) containing neither complex and beads with column buffer alone (which measures the intrinsic exchange rate of Ypt1p) were assayed for 15 min at room temperature to test their ability to stimulate uptake of [^{35}S]GTPγS onto Ypt1p. The intrisic exchange rate for Ypt1p was 0.51 ± 0.01 pmol of GTPγS, and the value for fraction 23 was 0.52 ± 0.01 pmol of GTPγS. When an untagged lysate was fractionated by gel filtration, the exchange rate in fractions 9 and 13 (0.52 ± 0.02 and 0.47 ± 0.01 pmol of GTPγS, respectively) was the same as the intrinsic rate for Ypt1p.

protein A tagged strain and separated TRAPP I and TRAPP II by gel filtration. Fractions enriched for either complex were incubated with IgG-Sepharose beads, and the washed beads were assayed for their ability to bind COPII vesicles in vitro. The results shown were normalized to the amount of Trs33p that bound to the beads. As a control, we also treated beads to a column fraction (fraction 23) that did not contain either TRAPP complex (see Figure 1A). As shown in Figure 6B, vesicles bound to TRAPP I, but binding to TRAPP II was the same as the control (see legend to Figure 6).

TRAPP II may fail to bind to COPII vesicles in our studies because it lacks function when bound to IgG-Sepharose beads. Previous studies have shown that TRAPP I is an exchange factor for Ypt1p (Wang et al., 2000). Since Ypt1p acts in ER–Golgi and Golgi traffic, both TRAPP I and TRAPP II may exchange nucleotide on Ypt1p. Beads containing the TRAPP I or TRAPP II complex were prepared as described above and assayed for their ability to stimulate the uptake of [^{35}S]GTPγS onto Ypt1p. As shown in Figure 6C, when normalized to the amount of Trs33p that bound to the beads, TRAPP I and TRAPP II had similar activities. These data demonstrate that although TRAPP II is active on beads, it cannot recognize COPII vesicles. Furthermore, our findings indicate that both forms of TRAPP can exchange nucleotide on Ypt1p.

The Binding of TRAPP I to COPII Vesicles Is Specific and Does Not Require Other Tethering Factors

If TRAPP I specifically recognizes and binds to COPII vesicles, the vesicle binding event we reconstituted in vitro may not require other tethering factors. To address this possibility, we formed COPII vesicles from donor cells and cytosol prepared from mutants (*ypt1–3*, *uso1–1*, *sec34–1,* and *sec35–1*) that harbor lesions in the genes that encode the different tethering factors. Although fractions prepared from these mutants were defective for transport in vitro (Figure 7A, top panel), this defect was not at the level of TRAPP I–dependent tethering as vesicles produced from these mutants bound to TRAPP I as efficiently as wild type (Figure 7A, bottom panel). Excess amounts of GDI, which inhibits Ypt1p activity by extracting the GDP-bound form of Ypt1p from membranes (Garrett and Novick, 1995), also did not interfere with binding. Furthermore, when antibody to the vesicle SNARE Bos1p was added in amounts sufficient to block transport in vitro, COPII vesicle binding to TRAPP I was not affected (data not shown).

Previous studies have shown that Bet3p, a component of TRAPP I and TRAPP II, binds tightly to Golgi membranes (Barrowman et al., 2000). Golgi membranes, as well as COPII vesicles, are released from permeabilized yeast cells in the presence of cytosol and an ATP-regenerating system (Ruohola et al., 1988). Therefore, the radioactivity we are monitoring in our binding studies could represent vesicles bound to Golgi, or labeled α factor that was delivered to the Golgi. The finding that TRAPP I, but not TRAPP II, binds membrane-bound α factor makes this seem unlikely. Nonetheless, to rule out this possibility, we formed COPII vesicles with purified COPII coat components as described before (Barlowe et al., 1994; Barlowe, 1997). The protocol we employed yields vesicles that are free of cytosol and Golgi, eliminating the possible artifacts described above. Furthermore, unlike the binding studies shown in Figure 6, these

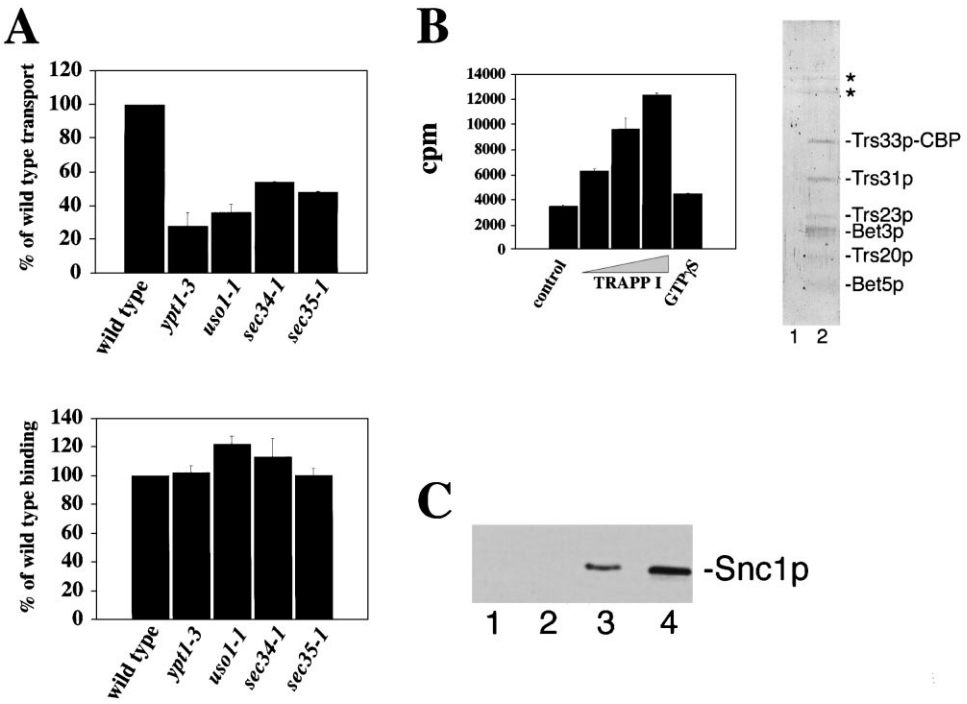

Figure 7. TRAPP I–Dependent Binding of COPII Vesicles Does Not Require Other Tethering Factors

(A) Top panel: Fractions prepared from the *ypt1–3*, *uso1–1*, *sec34–1,* and *sec35–1* mutants display defects in ER-to-Golgi traffic in vitro. Fractions were prepared as described before (Groesch et al., 1990) except cells were converted to spheroplasts during a 1 hr incubation at 25°C, regenerated at 25°C for 90 min, and assayed at 25°C. Results are expressed as percentage of wild-type transport. Bottom panel: TRAPP I binds to COPII vesicles in the absence of other tethering factors. Vesicle budding reactions were performed with donor cells and cytosol prepared from either wild type, *ypt1–3*, *uso1–1*, *sec34–1,* or *sec35–1*.

(B) Chemically pure TRAPP I binds COPII vesicles. TRAPP I was purified as described in the Experimental Procedures. COPII vesicles were produced from purified COPII components as described before (Barlowe et al., 1994; Barlowe, 1997). Increasing amounts (10, 20, and 40 μl) of beads containing TRAPP I or 40 μl of empty beads (control) was incubated with vesicles produced in the presence of ATP. Empty beads (0–30 μl) were added to samples so that all reactions contained 40 μl of beads. Approximately equal amounts of ConA precipitable counts (a measure of vesicles) were used in all the binding assays, including those containing GTPγS. For the GTPγS budding reaction, GTPγS at 100 μM was used in place of GTP. A silver-stained gel showing purified TRAPP I (lane 2) is shown to the right of the graph. The bands marked with an asterisk were not contaminants of the preparation as they were also seen in lane 1, which only contained sample buffer. The minor TRAPP I component, Trs85p (see Figure 1B, lane 3), was not revealed with silver stain but could be detected by Western blot analysis using the ECL method (not shown).

(C) Snc1p-containing membranes do not bind to TRAPP I–coated beads. A lysate containing post-Golgi vesicles was prepared from a *sec6–4* mutant as described in the Experimental Procedures. The lysate was incubated for 2 hr at 4°C with beads containing (lane 1) or lacking (lane 2) purified TRAPP I. The beads were washed and protein was eluted by boiling in SDS–PAGE sample buffer. Samples were analyzed by Western blot analysis using anti-Snc1p antibody (1:1000 dilution). The amount of Snc1p before (lane 3) and after (lane 4) the temperature shift was compared.

experiments were performed with chemically pure TRAPP I that was immobilized on calmodulin agarose beads (see Figure 7B), and purified as described in the Experimental Procedures from a strain in which Trs33p was TAP tagged. Western blot analysis revealed that purified TRAPP I did not contain any TRAPP II–specific contaminants (not shown).

Using this highly purified system, COPII vesicles bound to TRAPP I in a concentration-dependent manner (Figure 7B). We estimated that at the highest concentration of TRAPP I used in these studies, ~25%–30% of the vesicles bound to the beads. As these binding studies were performed in the absence of cytosol, we can conclude that cytosol is not required for TRAPP I–dependent vesicle binding. Consistent with this conclusion, we have found that the addition of cytosol to cytosol-free vesicles does not increase their efficiency of binding (not shown). If TRAPP I interacts directly with the COPII coat, an increase in binding should be observed in the presence of GTPγS, a nonhydrolyzable analog of GTP that is known to stabilize the COPII coat (Barlowe et al., 1994). Instead, binding was drastically reduced (Figure 7B), suggesting that COPII vesicles must uncoat before TRAPP I can bind.

To determine whether other types of vesicles bind to TRAPP I, chemically pure TRAPP I was incubated with a 10,000 × *g* supernatant prepared from a *sec6–4* mutant grown at 25°C and then shifted for 1 hr at 37°C, to accumulate post-Golgi vesicles (Walworth and Novick, 1987). Snc1p, the post-Golgi vesicle SNARE, accumulated intracellularly under these conditions (Figure 7C, compare lane 3 with lane 4). The membranes that accumulated were sealed, since a 4.5-fold increase in invertase activity was found upon the addition of Triton X-100. Prior to the shift, Snc1p was also found on membranes (lane 3). Previous studies have shown that these

membranes include the plasma membrane, endosomes, and Golgi (Protopopov et al., 1993; Lewis et al., 2000). As shown in Figure 7C, Snc1p-containing membranes do not bind to TRAPP I (compare lane 1 with control in lane 2). Thus, the binding of COPII vesicles to TRAPP I is specific.

Discussion

We present compelling evidence that TRAPP subunits are distributed in two functionally distinct complexes. The smaller complex, TRAPP I, contains seven (Bet5p, Trs20p, Trs23p, Bet3p, Trs31p, Trs33p, and Trs85p) of the ten previously identified subunits. This complex is required for membrane traffic between the ER and Golgi in vitro. Consistent with this role, we find that mutants with defects in TRAPP I subunits block the transport of CPY in a pre-Golgi compartment. The larger complex, TRAPP II, contains all ten of the previously identified TRAPP subunits in equimolar amounts (Sacher et al., 1998, 2000). Fractionation of lysates containing either Trs65p-*myc*, Trs120p-*myc,* or Trs130p-*myc* indicated that these subunits were found exclusively in TRAPP II, and no other pool was detected (M. S. and S. F.-N., unpublished data). Genetic, biochemical, and morphological analysis of the *trs130* mutant indicates that TRAPP II functions in Golgi traffic or in exit from the Golgi.

That the different forms of TRAPP mediate different transport events and interact differentially with COPII vesicles suggests that TRAPP I is a stage-specific tethering factor. TRAPP I binds COPII vesicles, but not post-Golgi vesicles and other membranes (endosomes, plasma membrane, Golgi). It is unlikely that TRAPP I recognizes the COPII coat on these vesicles because COPII coat components do not bind to TRAPP I under conditions where Ypt1p binds (M. S. and S. F.-N., unpublished data). Furthermore, the observation that GTPγS blocks vesicle binding in vitro suggests that COPII vesicles must uncoat before they bind to TRAPP I. Like the TRAPP complexes, the small GTP binding protein Ypt1p acts in both ER-to-Golgi and Golgi traffic. Our finding that Ypt1p is activated by two distinct but related exchange factors (TRAPP I and TRAPP II) explains how this small GTP binding protein can act in two different transport events. This result also implies that the binding of each TRAPP complex to vesicles occurs via a stage-specific receptor and not Ypt1p.

Based on the findings reported here and the phenotype of the *trs130* mutant, a possible role for TRAPP II is to bind retrograde vesicles in the Golgi. Such vesicles would arise from a later Golgi compartment and bind to early Golgi. In this respect, it is interesting to note that we have found that *trs130^{ts2}* displays synthetic interactions with *ret2–1* (a mutant with a defect in a COPI subunit) and *arf1*Δ (a mutant that lacks Arf1p that is required for COPI vesicle formation), but not with *sec13–1*, *sec23–1*, *sec24–1,* and *sec31–1* (mutants defective in COPII subunits). Alternatively, TRAPP II may play a role in exit from the Golgi. Golgi-to-endosome/vacuole trafficking is unlikely, since the *trs120* and *trs130* mutants do not accumulate endosomal-like structures, and defects in traffic on the endocytic/vacuolar pathway have not been observed thus far (Jiang et al., 1995; our unpublished data).

Recent work has implicated large protein complexes such as TRAPP, the Exocyst, the Vps52p/53p/54p complex, and the Vam2p/Vam6p complex (Sacher et al., 1998; Guo et al., 1999; Barrowman et al., 2000; Conibear and Stevens, 2000; Price et al., 2000) as possible determinants of specificity in membrane traffic (Guo et al., 2000). These complexes act at different stages of the secretory pathway and appear to be specific to each transport step. The subunits of these complexes do not share homology with each other, or with subunits in any of the other complexes. Although a subcomplex of the Exocyst exists (Guo et al., 1999), it is functionally linked to the other Exocyst components and all members of this complex act at the same stage of the secretory pathway. Our results showing that TRAPP I binds to COPII, but not post-Golgi, vesicles is important since it clearly demonstrates that one of these putative tethering complexes binds to a specific class of vesicles.

The interaction of a transport vesicle with its target membrane is a multistage process that involves recognition followed by one or more tethering events. The binding of a COPII vesicle to TRAPP I may be the first of these events, implicating TRAPP I as the tether that is most likely to confer specificity. Consistent with this proposal, we see specificity in our binding assay. We propose that TRAPP I binds to an uncoated ER-derived vesicle through an unidentified ligand. Since the known tethering factors are not needed for the binding of TRAPP I to vesicles, this ligand does not appear to be Ypt1p, Uso1p, or the Sec34p/Sec35p complex. Once TRAPP I binds to the vesicle, it activates Ypt1p, converting it from its GDP-bound form to its GTP-bound form. The activation of Ypt1p by TRAPP I may be the signal that the vesicle has reached its correct acceptor compartment. This then leads to the recruitment of other tethers, as the binding of COPII vesicles to TRAPP I may be reversible in vivo in their absence. Consistent with this hypothesis is the recent finding that p115, the mammalian ortholog of Uso1p, is recruited to membranes by activated Rab1 (Allan et al., 2000). The binding of Uso1p to membranes has also been shown to be dependent on Ypt1p (Cao et al., 1998), and Uso1p-dependent vesicle tethering requires the Sec34/Sec35p complex in vitro (VanRheenen et al., 1998, 1999). These late tethering events may be a prerequisite to SNARE pairing (Allan et al., 2000), the final step in docking a COPII vesicle to the Golgi.

Since TRAPP I resides in the cytosol and on membranes (Sacher et al., 1998), an alternate proposal is that cytosolic TRAPP I may bind to COPII vesicles. The activation of Ypt1p may then enable TRAPP I to bind to its receptor on the Golgi, where other tethering factors are recruited. A third possiblity is that TRAPP I acts solely to activate Ypt1p, which, in combination with other tethering factors, allows the vesicle to bind to the Golgi. This can be ruled out as genetic studies have shown that the complete loss of Ypt1p and the other tethers can be bypassed, while the complete loss of TRAPP cannot (Sapperstein et al., 1996; VanRheenen et al., 1998, 1999; Barrowman et al., 2000).

Using an in vitro vesicle binding assay that employs COPII vesicles and chemically pure TRAPP I, we have

described a role for TRAPP I in vesicle tethering. Since the ligand on the ER-derived vesicle that recognizes TRAPP I is not one of the known tethering factors, a challenge for the future will be to identify this factor through the use of biochemical and genetic approaches.

Experimental Procedures

In Vitro Reconstitution Experiments

The in vitro transport assay and the preparation of the fractions were performed as described previously (Ruohola et al., 1988; Groesch et al., 1990; Lian and Ferro-Novick, 1993; Sacher et al., 1998). For the reconstitution experiments, lysates were prepared from SFNY 26–3a (*MAT*a *ura3–52*) or SFNY 904 (Sacher et al., 2000). A total of 1050 units of cells at $OD_{600} = 1$ were converted to spheroplasts as described before (Sacher and Ferro-Novick, 2001) and lysed in 2.5 ml of 20 mM HEPES (pH 7.2) by pipetting and gently vortexing the solution. After cell lysis, 10× concentrated Transport Assay buffer was added to the lysate to give a final composition of 25 mM HEPES (pH 7.2), 115 mM potassium acetate, 2.5 mM magnesium acetate. The lysates were centrifuged at 100,000 × *g* for 1 hr, and aliquots (300–500 μl) containing 5 mg of protein were then passed over a 25 ml Superdex-200 column (Pharmacia) at a flow rate of 0.4 ml/min, using 1× Transport Assay buffer as the column buffer. Samples of 1 ml were collected into 25 tubes. This chromatographic procedure was repeated two more times, and the TRAPP I– (fraction 13) and TRAPP II– (fraction 9) containing fractions from each column run were pooled separately and concentrated 10-fold using a Microcon 10 microconcentrator (Amicon) according to the manufacturer's instructions. Sorbitol (4 M) was added to give a final concentration of 250 mM. The amount of Trs33p in fraction 9 was found on average to be 1.3-fold higher than fraction 13. Reconstitution assays contained 25 μl of wild-type permeabilized yeast cells, 50–70 μl (1 mg of protein) of Bet3p-depleted cytosol, 7–10 μl (250 μg of protein) of Bet3p-depleted membranes, and 40–80 μl of the TRAPP I– or TRAPP II–containing fraction. The samples were processed for concanavalin A precipitation and outer chain immunoprecipitation as described before (Lian and Ferro-Novick, 1993).

Vesicle Binding Assay

Vesicles containing radiolabeled pro-α-factor were formed in vitro as described before (Lian and Ferro-Novick, 1993), and the donor membranes were removed during a 1 min centrifugation at 13,000 × *g* in a microcentrifuge. To prepare beads containing TRAPP I and/or TRAPP II, yeast spheroplasts were generated and lysed from SFNY904 as described before (Sacher and Ferro-Novick, 2001). The proportion of cells to buffer was 75 U of cells at OD_{600} per ml of buffer. Prior to centrifugation, NaCl was added to a final concentration of 0.3 M, and the lysate was incubated on ice for 15 min. The lysate was centrifuged at 60,000 × *g* for 1 hr in a Beckmann 70Ti rotor. The protein concentration of the resulting cytosol was ~10–15 mg/ml. This cytosol was incubated with IgG-Sepharose for 2–4 hr with rotation (Sacher and Ferro-Novick, 2001). The beads were then pelletted at 1500 × *g* for 3 min and washed two times with 5 ml of Transport Assay buffer (see above) containing 250 mM sorbitol (TB+sorbitol). To prepare beads containing either TRAPP I or TRAPP II, cytosol (10 mg) prepared from 2000 OD units of SFNY 904 was fractionated on a Superdex-200 gel filtration column as described above. This was repeated twice and fractions 9 (TRAPP II), 13 (TRAPP I), and 23 (control) were pooled separately and bound to 150 μl of a 50% slurry of IgG-Sepharose. The beads were stored as a 50% slurry in assay buffer at 4°C for 1–2 weeks.

Binding assays contained 10–40 μl of an IgG-Sepharose bead slurry and ~100 μl of a supernatant fraction from one vesicle budding reaction (700,000 cpm). The reaction was incubated on a nutator at 4°C for 2 hr and washed three times with 500 μl of TB+sorbitol, and the beads were then transferred using 50 μl of assay buffer to a scintillation vial for liquid scintillation counting. To assess the activity of TRAPP I and TRAPP II when bound to IgG-Sepharose beads, samples were prepared as described above and the beads were assayed for nucleotide exchange activity as before (Wang et al., 2000).

To accumulate post-Golgi vesicles in the *sec6–4* mutant, NY 17 (*MAT*a *ura3–52 sec6–4*) was incubated for 1 hr at 37°C and a 10,000 × *g* supernatant was prepared as described before (Walworth and Novick, 1987), except 75 units of cells at $OD_{599} = 1$ were lysed in 1.1 ml of lysis buffer. Binding reactions were performed with 75 μl of lysate and 10 μl of an IgG-Sepharose bead slurry as described above. The vesicles and other Snc1p-containing membranes were followed by Western blot analysis using antibody to the SNARE Snc1p (1:1000 dilution).

To obtain chemically pure TRAPP I, Trs33p was tagged with the TAP tag and purified as described before (Rigaut et al., 1999) with the following minor modifications. Cells were lysed in buffer containing 20 mM HEPES (pH 7.2), 150 mM NaCl, and the salt concentration was adjusted to 300 mM prior to centrifugation. The lysate (1.3 g) was centrifuged at 170,000 × *g* for 4 hr to pellet TRAPP II before it was incubated with IgG-Sepharose beads. To release TRAPP from the beads, 350 U of TEV Protease were used and the incubation was performed at room temperature for 2 hr. Binding reactions were performed in buffer B88 (Barlowe, 1997) containing 2 mM $CaCl_2$. Western blot analysis of the purified TRAPP I complex, using the ECL method, confirmed that it was devoid of Trs130p, a TRAPP II–specific component.

Acknowledgments

We are grateful to Randy Schekman for providing the purified COPII components used to make COPII vesicles for the experiments in Figures 7B and 7C. We would also like to thank the following investigators who supplied us with strains and reagents for this study: Pierre Cosson, Rainer Duden, Alex Franzusoff, Chris Kaiser, Francois Letourneur, and Peter Novick. We gratefully acknowledge the technical assistance of Lora Cavallo and Elaine Downie. We also thank Peter Novick, Graham Warren, Chavela Carr, and Eric Grote as well as members of the Ferro-Novick laboratory for helpful discussions and critical comments on this manuscript.

Received June 30, 2000; revised January 3, 2001.

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