The Sec13p complex and reconstitution of vesicle budding from the ER with purified cytosolic proteins

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SEC13 encodes a 33 kDa protein that participates in vesicle budding from the endoplasmic reticulum (ER). In order to purify a functional form of Sec13p, a **SEC13**–dihydrofolate reductase (mouse) fusion gene (**SEC13:DHFR**) was constructed that complements both *sec13* temperature sensitive and null mutations. Methotrexate–agarose affinity chromatography facilitated the purification of two forms of the Sec13-dhfrp fusion protein: a monomeric form and a high molecular weight complex. The complex form consists of two subunits: Sec13-dhfrp and a 150 kDa protein (p150). Native immunoprecipitation experiments confirm that Sec13p exists in a complex with p150 in wild type cells. Functional analysis supports a role for both subunits in protein transport. Vesicle budding from the ER in a cell-free reaction is inhibited by Fab antibody fragments directed against either Sec13p or p150. The purified Sec13-dhfrp/p150 complex, but not the Sec13-dhfrp monomer, in combination with two other pure protein fractions (Sar1p and a Sec23/Sec24 protein complex) satisfies the requirement for cytosol in a cell-free vesicle budding reaction. The vesicles formed with the purified protein fractions are competent to fuse with the Golgi and are biochemically distinct from the ER membrane fraction from which they derive.

Key words: Sec protein/transport/vesicle budding

Introduction

Vesicle carriers mediate the transport of proteins between organelles (Palade, 1975). The first of these transport events occurs between the endoplasmic reticulum (ER) and the *cis* face of the Golgi apparatus. Genetic studies have identified a large number of genes involved in ER to Golgi transport in yeast including 11 **SEC** genes (Novick *et al.*, 1980), **SAR1** (Nakano and Muramatsu, 1989), two **BET** genes (Newman and Ferro-Novick, 1987), **BOS1** (Newman *et al.*, 1990), **YPT1** (Segev *et al.*, 1988), two **SLY** genes (Dascher *et al.*, 1991; Ossig *et al.*, 1991), **USO1** (Nakajima *et al.*, 1991) and **SED5** (Hardwick and Pelham, 1992). Molecular cloning and biochemical analyses have begun to address the mechanism by which these proteins act together to carry out this complex process. Thus far it appears that the process is fundamentally conserved in diverse eukaryotic species (Pryer *et al.*, 1992; Schekman, 1992).

Vesicle-mediated transport can be divided into two steps: (i) vesicle budding, the formation and release of vesicles from

the donor organelle, and (ii) vesicle consumption, the targeting and subsequent fusion of vesicles with the appropriate acceptor organelle. Morphological and genetic analyses have allowed the classification of some ER to Golgi mutations into two groups corresponding to these steps (Kaiser and Schekman, 1990). Class I genes, defective in vesicle budding, accumulate ER structures at nonpermissive temperatures whereas class II genes, defective in vesicle consumption, accumulate transport vesicles at nonpermissive temperature. Proteins encoded by class I genes are candidates for the biochemical requirements for vesicle formation and include **SEC12**, **13**, **16** and **23** and **SAR1**, a multicopy suppressor of a *sec12* temperature sensitive mutation (Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990). *In vitro* assays that reconstitute transport from the ER to the Golgi have revealed additional requirements (Beckers *et al.*, 1987; Baker *et al.*, 1988; Ruohola *et al.*, 1988; Rexach and Schekman, 1991). These include a specific membrane fraction, cytosol, ATP, GTP and incubation at physiologic temperature. The use of such biochemical assays has elucidated the function of several of the genetically identified requirements (Hicke and Schekman, 1989; d'Enfert *et al.*, 1991a,b; Oka *et al.*, 1991; Hicke *et al.*, 1992). For instance, a cycle of GTP hydrolysis is mediated by the action of two cytosolic proteins, Sar1p (Nakano and Muramatsu, 1989) and Sec23p (Hicke and Schekman, 1989; Yoshihisa *et al.*, 1993), and an ER membrane resident protein, Sec12p (Nakano *et al.*, 1988). Sar1p, a ras-like GTP binding protein, binds GTP (Oka *et al.*, 1991) and is recruited to the ER membrane in a Sec12p-dependent fashion (d'Enfert *et al.*, 1991b). Sec23p stimulates the hydrolysis of Sar1p bound GTP to GDP (Yoshihisa *et al.*, 1993) while Sec12p stimulates the exchange of GDP for GTP on Sar1p to reinitiate the cycle (Barlowe *et al.*, 1993b). Purified Sec23p exists as a heterodimer with a 105 kDa subunit (Sec24p) (Hicke *et al.*, 1992). Though association with Sec24p is not required for Sec23p GAP (GTPase activating) activity, Sec24p is essential for vesicle budding (Hicke *et al.*, 1992). Thus, Sec24p may be an effector molecule of this GTP hydrolysis cycle. The mechanism by which this cycle of GTP hydrolysis stimulates vesicle formation remains unknown.

SEC13, another gene involved in vesicle formation, encodes a 33 kDa protein found both in particulate form and free in the cytosol (Pryer *et al.*, 1993). Sequence analysis reveals an internal repeat structure found in a family of proteins that includes β subunits of trimeric G proteins. The structural or functional role of this motif is not clear. Preliminary biochemical analysis shows that cytosolic Sec13p fractionates predominantly as a large complex of ~700–800 kDa. We have shown that a Sec13p-dependent *in vitro* budding assay requires the high molecular weight form that cannot be replaced by fractions enriched for monomeric Sec13p (Pryer *et al.*, 1993). To define further the cytosolic requirements for vesicle budding from the ER, we have

purified the high molecular weight form of Sec13p. We find that Sec13p, like Sec23p, is part of a heterooligomeric complex, with a protein of 150 kDa (p150). We present evidence that p150 constitutes a new *SEC* gene product. Additionally, we find that the Sec13p/p150 complex in combination with Sar1p and the Sec23p/Sec24p complex fulfil the cytosolic requirements for the budding of vesicles from the ER in an *in vitro* protein transport reaction.

Results

Sec13-dhfr hybrid protein behaves like wild type Sec13p

To facilitate purification of Sec13p, we employed a strain in which the *SEC13* gene is disrupted and *SEC13* function is provided by a hybrid protein of the mouse *DHFR* gene fused to the 3' coding terminus of *SEC13*. Expression of the fusion from a multicopy vector was required to complement fully the null allele of *SEC13*, resulting in a strain (RSY742) which is wild type for growth and secretion at 30°C (Pryer *et al.*, 1993). The *dhfr* domain of the *SEC13:DHFR* allele binds methotrexate, a competitive inhibitor, with a K_i of 4×10^{-12} M (Thillet *et al.*, 1988), allowing affinity purification of the hybrid protein using methotrexate-agarose (Kaufman, 1974).

Cytosolic Sec13p exists as part of a large complex (Pryer *et al.*, 1993). We tested the fractionation properties of Sec13-dhfrp to determine if the strain bearing the fusion gene could be used to purify the Sec13p complex. The hybrid protein (Figure 1B), like wild type Sec13p (Figure 1A), fractionated as a high molecular weight complex as well as in an apparently monomeric form when subjected to gel filtration chromatography on Superose 6. Complementation of the growth defect of the *sec13* null allele required overexpression of the fusion gene. We have observed previously that overproduction of Sec13p increases the monomeric form, while the amount of complex remains constant (Pryer *et al.*, 1993). This may result from a limiting amount of another member of the Sec13p complex. The *SEC13:DHFR* allele behaved similarly.

Assay of functional Sec13-dhfrp

Fractionation of *SEC13:DHFR* cytosol on methotrexate-agarose resulted in a fraction highly enriched for the hybrid protein but which also contained a number of other proteins (Figure 2A, lane 1). The Sec13-dhfrp in this fraction was predominantly in the monomeric form which is not active in vesicle formation (Pryer *et al.*, 1993) (also, see below). We therefore required an assay to measure the functional form of the hybrid protein for subsequent purification steps.

A two stage *in vitro* budding reaction using gently lysed yeast spheroplasts as a source of membranes has been described previously (Rexach and Schekman, 1991). In the first stage, an *in vitro* translated radiolabeled secretory protein precursor, pre-pro- α -factor, is translocated into the ER. The membrane fraction containing core-glycosylated pro- α -factor is collected and used for the second stage where vesicle formation is measured by the release of core-glycosylated precursor enclosed within a slowly sedimenting compartment. The budding efficiency is calculated as the amount of radiolabeled core-glycosylated pro- α -factor in the slowly sedimenting compartment divided by the total amount of core-glycosylated pro- α -factor. The maximum budding efficiency ranges from 30 to 50%. This assay

Fig. 1. Sec13-dhfrp and Sec13p fractionate in both a high molecular weight form (complex) and a low molecular weight form (monomer). Liquid nitrogen lysed cytosol (200 μ l, 20 mg protein/ml) from a wild type strain (RSY255) (A) or an isogenic chromosomally disrupted *sec13* strain (RSY742) carrying *SEC13:DHFR* on a multicopy vector (B) were chromatographed on a 24 ml Superose 6 gel filtration column. Column fractions (48 fractions of 0.5 ml each) were analyzed by SDS-PAGE followed by transfer to nitrocellulose and probed with affinity purified anti-Sec13p antibodies (1:1000). Fraction numbers and the position of protein standards in kDa used to calibrate the column are indicated, as are the gel molecular weight markers. The positions of Sec13p and Sec13-dhfrp are indicated. L, load fraction.

requires cytosol, temperature, GTP and ATP. Sec13p (and Sec13-dhfrp) is peripherally associated with the membrane fraction (Pryer *et al.*, 1993). Therefore, we modified this assay to measure a cytosolic requirement for Sec13-dhfrp by extracting the membrane fraction with 2.5 M urea to remove all membrane associated Sec13-dhfrp (Pryer *et al.*, 1993). Two other cytosolic factors have been purified that function at the budding step of ER to Golgi transport: Sar1p (d'Enfert *et al.*, 1991b; Barlowe *et al.*, 1993a) and the Sec23p/Sec24p complex (Hicke *et al.*, 1992). Using the urea-extracted membrane fraction, we found that addition of Sar1p and Sec23p/Sec24p complex was not sufficient to allow vesicle budding. However, we tested the Sec13-dhfrp enriched methotrexate eluate and found that mixture of this fraction with Sar1p and the Sec23p/Sec24p complex restored budding to maximum levels. The amount of core-glycosylated pro- α -factor released in the vesicle fraction was proportional to the amount of methotrexate eluate added (not shown) suggesting that the Sec13-dhfrp in this fraction was active and providing a linearly responsive assay for Sec13p activity.

Purification of the Sec13-dhfrp/p150 complex

Desalted methotrexate eluate was applied to a DEAE-Sephacolumn equilibrated with 0.25 M KOAc. Under these salt conditions, one pool of Sec13-dhfrp flowed through the column. Gel filtration chromatography

Fig. 2. The Sec13-dhfrp/p150 complex cofractionates with vesicle budding activity. (A and B): fractionation of the methotrexate eluate by anion exchange on DEAE-Sephacel. The methotrexate eluate (60 ml, 1.5 mg protein/ml) was loaded on to a 20 ml DEAE-Sephacel column equilibrated in 0.25 M KOAc B88GPI, pH 7.4. The column was developed with a 100 ml linear gradient from 0.25 to 0.5 M KOAc and fractions (1.25 ml) were collected. Every third fraction was analyzed by SDS-PAGE followed by silver staining (A). Each fraction (2 μ g protein) was analyzed in the Sec13p-dependent assay for 60 min at 20°C (B). Fractions 18–36 were pooled for further purification. ♦, Position of Sec13-dhfrp; ◆, position of p150. (C and D): the 200 kDa protein was resolved from the Sec13-dhfrp/p150 complex by chromatography on Mono Q. Fractions (0.25 ml) were collected and analyzed by SDS-PAGE followed by silver staining (C). Fraction numbers and the position of molecular weight standards in kDa are indicated. Fractions containing the 200 kDa protein and the Sec13-dhfrp/p150 complex peak were pooled separately as indicated. An aliquot (1 μ g protein) of each fraction was assayed for activity in the Sec13p-dependent budding assay for 60 min at 20°C (D).

demonstrated that the flow-through fraction consisted of the apparently monomeric form of Sec13-dhfrp. This fraction was unable to stimulate vesicle budding in the Sec13p-dependent assay consistent with previous observations that fractions enriched in monomeric Sec13p do not stimulate vesicle formation (Pryer *et al.*, 1993). The column was developed with a 5 column linear gradient from 0.25 to 0.5 M KOAc. Vesicle budding activity eluted as a single peak containing three major proteins: the Sec13-dhfr hybrid protein at 55 kDa, a 150 kDa protein (p150) and a 200 kDa protein (Figure 2A and B). Further fractionation of this material by Mono Q column chromatography resolved the 200 kDa protein, which eluted at 0.48 M KOAc, from the Sec13-dhfrp/p150 complex which eluted at 0.55 M KOAc. Again, the vesicle budding activity eluted as a single peak coinciding exactly with the peak of Sec13-dhfrp and p150 protein (Figure 2C and D). Supplementation of the Sec13-dhfrp/p150 fractions with fractions containing the 200 kDa protein had no effect on budding activity (not shown). Coomassie stained SDS-PAGE analysis of the purification is shown in Figure 3. Further fractionation by Superose 6 gel filtration column chromatography demonstrated that both the Sec13-dhfrp and p150 protein peaks eluted as a high molecular weight species with coincident activity and protein profiles (Figure 4A and C). The recovery and purification of Sec13p activity is quantified in Table I. A large loss in yield after the first column may be explained by irreversible binding of some Sec13-dhfrp to the methotrexate-agarose; additional Sec13-dhfrp was released when column material was heated in SDS-PAGE

Fig. 3. Coomassie stained SDS-PAGE of fractions from the Sec13-dhfrp/p150 complex purification: lane 1, 10 μ g protein from liquid nitrogen lysed cytosol, lane 2, 1 μ g protein from methotrexate eluate, lane 3, 1 μ g protein from DEAE pool, lane 4, 1 μ g protein from Mono Q pool. The positions of molecular weight standards in kDa are indicated.

sample buffer (not shown). The final fraction has a 200-fold higher specific activity than the crude cytosol.

Coprecipitation of the Sec13p/p150 complex in wild type cells

In order to characterize the role of Sec13p and p150 in vesicle budding, we raised antibodies to native Sec13p and

Table I. Purification of Sec13-dhfrp/p150 complex

Fraction	Vol. (ml)	Conc. (mg/ml)	mg	Units ^a	Sp. act (U/mg)	Recovery (%)
Cytosol	330	26	8580	1×10^6	125	100
MTX eluate	60	1.5	90	69 210	769	7
DEAE eluate	22.5	0.18	4.05	45 000	11 111	4.5
Mono Q pool	2	0.7	1.4	35 000	25 000	3.5

^aUnit defined in Materials and methods.

p150. Sec13-dhfrp monomer was injected into rabbits to generate antisera that recognized native Sec13p. Purified Sec13-dhfrp/p150 complex was dissociated with 2 M urea and pure p150 was resolved by gel filtration. Fractions containing p150 were used to immunize rabbits. Sec13-dhfrp antiserum recognized the hybrid protein (Figure 4B) and wild type Sec13p (Figure 1A) on an immunoblot. p150 antiserum reacted with the intact protein and with a series of breakdown products present in the purified fraction of Sec13-dhfrp/p150 complex (Figure 4B).

To test whether the wild type form of Sec13p also is complexed with p150, we performed native immunoprecipitations from radiolabeled wild type cells. Anti-Sec13p immune serum immunoprecipitated two proteins not present in precipitations with preimmune serum (Figure 5, lanes 1 and 2). These proteins correspond in size to Sec13p (33 kDa) and p150 (150 kDa). Excess unlabeled Sec13-dhfrp antigen competed for the immunoprecipitation of these two proteins but not for proteins precipitated with the preimmune serum (Figure 5, lanes 2 and 3). Similarly, native immunoprecipitation reactions using anti-p150 antibodies yielded two proteins of 33 kDa and 150 kDa that were not precipitated in the presence of excess p150 (Figure 5, lanes 4–6). These data indicate that wild type Sec13p associates specifically with p150.

Anti-Sec13p and anti-p150 Fab fragments inhibit vesicle formation

Functional Sec13p required the participation of p150 in a heterooligomeric complex. We tested the direct involvement of Sec13p and p150 in vesicle budding using antibodies directed against the native proteins. Both anti-Sec13p and anti-p150 immune sera completely inhibited the ability of whole cytosol to facilitate vesicle budding, whereas preimmune sera had no effect at the same concentration (not shown). Because both antisera precipitate the Sec13p/p150 complex, we repeated these experiments using Fab fragments derived from immune IgG fractions. Anti-Sec13p Fab fragments inhibited vesicle budding and Fab inhibition was prevented by the addition of excess Sec13-dhfrp monomer or purified Sec13-dhfrp/p150 complex (Figure 6A). Anti-p150 Fab fragments also inhibited budding; however, inhibition was prevented only in the presence of excess Sec13-dhfrp/p150 complex and not by excess Sec13-dhfrp monomer (Figure 6B). These results confirm the monospecificity of the two antisera and demonstrate that both Sec13p and p150 are required in the vesicle budding reaction.

Three cytosolic fractions are required for vesicle budding

In the course of purifying the active form of Sec13-dhfrp it became clear that the cytosolic protein requirements for vesicle budding were satisfied by three purified protein

fractions: Sar1p, the Sec23p/Sec24p complex and the Sec13-dhfrp/p150 complex. Figure 7 shows the results of titration experiments in which two components were provided in fixed amounts and a third varied. Maximum levels of budding were achieved with 0.24 μ g Sar1p (11 pmol), 1.2 μ g Sec23p/Sec24p complex (6.3 pmol) and 0.4 μ g Sec13-dhfrp/p150 complex (2 pmol) using 500 μ g of membranes. Though these three components appeared sufficient to replace the cytosolic requirement for budding *in vitro*, it remained possible that the participation of one or more additional factors was masked by a large excess of the described fractions. To address this issue, quantitative immunoblotting was performed to determine the amount of each of the required components present in crude cytosol. We then calculated the level of each that was provided in incubations conducted with cytosol as compared with the reconstituted reaction. In comparison with crude cytosol, vesicle budding reactions conducted with purified components required ~2-fold less Sec23p/Sec24p complex and 2-fold more Sar1p and the Sec13p/p150 complex (Table II). Thus, the amount of the three purified protein fractions required for maximum budding was similar to their representation in a functionally equivalent amount of unfractionated cytosol. We also analyzed the kinetics of budding with the purified components as compared with unfractionated cytosol. Vesicle release using the reaction reconstituted with purified proteins proceeded with kinetics indistinguishable from those using unfractionated cytosol (Figure 8A, crude cytosol; Figure 8B, purified proteins). In addition, although crude cytosol sustains a full round of vesicle budding, targeting and fusion to the Golgi complex (Rexach and Schekman, 1991), the purified proteins did not stimulate transfer of glycosylated pro- α -factor to the Golgi complex (not shown). Thus Sar1p, Sec23p/Sec24p complex and Sec13-dhfrp/p150 complex are sufficient to fulfil the cytosolic requirements for vesicle budding from the ER, but not for vesicle targeting and fusion with the Golgi.

Vesicles made with pure protein fractions are functional intermediates in ER to Golgi vesicle transport

The inability of the three cytosolic factors to support overall transport could result from two possible situations. First, the vesicles released may not be bona fide transport vesicles, but simply ER fragments arising during the incubation. Alternatively, the three protein fractions produce functional vesicle intermediates, but fusion of the vesicles with the Golgi acceptor compartment requires additional factors distinct from those required for vesicle budding. To address these possibilities, we monitored the vesicle release reaction for the appearance of ER resident proteins in the vesicle-containing fraction. We found that neither Sec61p (Stirling

Fig. 4. Purified Sec13p-dhfr/p150 complex elutes at the position of the high molecular weight form of Sec13p. Purified Sec13p-dhfr/p150 complex (70 μ g, 100 μ l) was chromatographed on a 21 ml Superose 6 column and 0.5 ml fractions were collected. (A) Coomassie stained SDS-PAGE of column fractions. The fraction numbers and the position of molecular weight standards in kDa are indicated. The elution profile of molecular weight standards to calibrate the column was as follows: thyroglobulin (mol. wt 669 000), fraction 27; ferritin (mol. wt 440 000), fraction 30; catalase (mol. wt 232 000), fraction 32; and bovine serum albumin (mol. wt 68 000), fraction 34. (B) Immunoblot where the top half was probed with anti-p150 serum (1:10 000) and the bottom half with affinity purified anti-Sec13p antibodies (1:1000). The blots were then developed with the ECL detection system. The positions of molecular weight standards in kDa are indicated. (C) Aliquots (16.8 μ l) of each fraction were assayed for vesicle budding activity in the Sec13p-dependent assay.

et al., 1992), an ER integral membrane protein, nor Kar2p (BiP, Rose *et al.*, 1989), an ER luminal protein, were released during the time course of the reaction (Figure 8)

Fig. 5. Coprecipitation of native Sec13p and p150 from wild type radiolabeled cells. Wild type cells (RSY255) were radiolabeled with [35 S]methionine as described in Materials and methods. Whole cell lysates were centrifuged at 10 000 g for 10 min and proteins in the supernatant fluid precipitated with 30% ammonium sulfate to enrich for the Sec13p/p150 complex. Aliquots of the 30% ammonium sulfate pellet were immunoprecipitated with 1 μ l preimmune serum (lanes 1 and 4), 2 μ l affinity purified anti-Sec13p antibodies (lanes 2 and 3) or 1 μ l anti-p150 serum (lanes 5 and 6). Lane 3 contained 1.2 μ g unlabeled Sec13-dhfr/p150 complex to compete with specific antibody-antigen interactions. Immune complexes were collected using protein A-Sepharose and analyzed by SDS-PAGE followed by autoradiography. ♦, position of Sec13p; ♦♦, position of p150.

consistent with the idea that the vesicles are produced by an authentic protein sorting reaction and not by fragmentation of the ER.

To determine whether the vesicles formed using the purified cytosolic proteins were functional, we incubated the vesicle fraction formed in the reconstituted reaction with fresh membranes to provide an acceptor compartment and crude cytosol to supply cytosolic factors needed for fusion of vesicles with the Golgi. Under these conditions, we observed that the glycosylated pro- α -factor received α 1,6-mannose addition indicative of its transfer into the Golgi (Figure 9A). Additionally, the chase of the vesicles was more efficient than the overall transport reaction (25–30% versus 15–20%). We next asked whether the chase of these vesicles was sensitive to the same reagents that inhibit the overall ER to Golgi transport reaction. Fab fragments directed against Ypt1p, a small GTP binding protein involved in ER to Golgi transport *in vivo* (Segev *et al.*, 1988), inhibit ER to Golgi transport *in vitro* at a step after the budding of ER derived vesicles, but prior to their fusion with the Golgi (Rexach and Schekman, 1991). Anti-Ypt1p Fab fragments inhibited the chase of the vesicles by 72% (Figure 9B). This inhibition was prevented by the addition of recombinant Ypt1 protein. In contrast, the addition of 20 μ M GTP γ S had no effect on the vesicle chase reaction (Figure 9B), while transport of pro- α -factor from the ER is completely inhibited by this reagent (Baker *et al.*, 1988). Thus, the chase of the vesicles produced in the reconstituted reaction requires a protein (Ypt1p) thought to be involved in the targeting of vesicles to the Golgi, but these vesicles are biochemically distinct from the donor membranes from which they derive. Transport of glycosylated pro- α -factor from the ER is

Fig. 6. Fab fragments directed against Sec13p or p150 inhibit vesicle budding. Wild type (RSY607) liquid nitrogen cytosol (200 μ g) was incubated in the presence of Fab fragments supplemented as indicated with 6 μ g Sec13-dhfrp monomer or 5 μ g purified Sec13-dhfrp/p150 complex for 30 min on ice prior to the addition of membranes and then incubated for 60 min at 20°C in a standard budding reaction. Each column represents the budding activity relative to a control incubation with cytosol and buffer. (A) 1 μ g anti-Sec13p Fab fragments; (B) 5 μ g anti-p150 Fab fragments.

sensitive to GTP γ S while targeting and fusion of the vesicle intermediate is not, indicating that these vesicles have proceeded past the GTP γ S sensitive step and are indeed an intermediate in the ER to Golgi transport reaction.

Discussion

We have employed an *in vitro* budding reaction to purify a functional form of the *SEC13* gene product. The purification was facilitated by the construction of a fusion of *SEC13* with the mouse *DHFR* gene that functions *in vivo* and *in vitro*, allowing us to use affinity chromatography on methotrexate-agarose. We found that both the hybrid protein and native Sec13p exist in a high molecular weight complex with a single other subunit of 150 kDa (p150). Preliminary peptide sequence analysis reveals that the p150 is not encoded by any of the previously identified genes involved in ER to Golgi transport (N.Salama, A.Admon and R.Schekman, unpublished data), indicating that we have isolated a novel factor involved in ER to Golgi transport.

The cytosolic protein requirements for vesicle budding from the ER are satisfied by three protein fractions: Sec13p/p150, Sar1p and Sec23p/Sec24p. These fractions represent some but not all of the genetic requirements for vesicle budding established by phenotypic inspection of *sec*

Fig. 7. Titration of three cytosolic fractions required for vesicle budding. Standard budding reactions were performed for 60 min at 20°C with 500 μ g membranes. (A) Various amounts of Sar1p with 1.5 μ g Sec23p/Sec24p complex and 0.8 μ g Sec13-dhfr/p150 complex. (B) Various amounts of Sec23p/Sec24p complex with 0.24 μ g Sar1p and 0.8 μ g Sec13-dhfr/p150 complex. (C) Various amounts of Sec13-dhfr/p150 complex with 0.24 μ g Sar1p and 1.5 μ g Sec23p/Sec24p complex.

mutant cells. Several of the genes encoding these proteins, *SAR1*, *SEC23* and *SEC13*, show genetic interaction with each other as well as with *SEC12* and *SEC16* (Nakano and Muramatsu, 1989; Kaiser and Schekman, 1990). *SEC12* encodes an integral membrane glycoprotein with an N-terminal cytosolic domain that interacts with Sar1p and is required for vesicle budding from the ER (Nakano *et al.*, 1988; d'Enfert *et al.*, 1991a,b). *SEC16* encodes a large (~240 kDa) hydrophilic protein of unknown location whose

Table II. Comparison of protein levels in unfractionated cytosol versus reconstituted system

Protein fraction	ng protein per μ g unfractionated cytosol	μ g protein in saturating amount of unfractionated cytosol ^a	μ g protein needed to saturate reconstituted system
Sec23p/Sec24p	6.2	2.0	1.2
Sar1p	0.3	0.1	0.24
Sec13p/p150	0.6	0.2	0.4 ^b

^a330 μ g.^bSec13-dhfrp/p150. **Fig. 8.** Kinetics of vesicle budding in the presence of unfractionated cytosol or the purified proteins. Budding reactions were incubated for the indicated times at 20°C and processed immediately to monitor the release of radiolabeled pro- α -factor (squares), Kar2 protein (circles) or Sec61 protein (triangles). Reactions contained 330 μ g wild type cytosolic protein (panel A) or 0.24 μ g Sar1p, 1.2 μ g Sec23p/Sec24p complex and 1.1 μ g Sec13-dhfrp/p150 complex (panel B).

role in vesicle budding has not been established (C.Kaiser, unpublished data).

Among the purified cytosolic proteins, one novel interaction was detected that suggests an intimate connection between GTP hydrolysis and vesicle budding or release. Sar1p is a small GTP binding protein with a low GTPase activity (Nakano and Muramatsu, 1989; Barlowe *et al.*, 1993a). The Sec23p subunit of the Sec23p/Sec24p complex specifically activates GTP hydrolysis by Sar1p (Yoshihisa *et al.*, 1993). The rate of GTP hydrolysis by Sar1p is further stimulated by the cytosolic domain of Sec12p which specifically stimulates GTP/GDP nucleotide dissociation from Sar1p (Barlowe *et al.*, 1993b). Inasmuch as *sec12* and *sec13* show synthetic lethal genetic interaction and *sec13* is partially suppressed by overproduction of Sar1p, there is a good chance that the Sec13p complex will either influence the cycle of GTP hydrolysis or be regulated by the Sar1p-GTP complex.

At this point it is not possible to speak with any precision about the role of the Sec13p complex in vesicle formation. Fab antibody fragment inhibition demonstrates that both subunits of the Sec13p complex are required for budding. Therefore, cloning and sequence analysis of the p150 subunit may provide further clues. *SEC13* contains a repeated motif that is characteristic of the β subunit of heterotrimeric G

proteins and transducin (Pryer *et al.*, 1993). The function of this motif is unknown, though many of the proteins that contain this sequence are engaged in multisubunit complexes, some of which bear no other resemblance to G proteins (Dalrymple *et al.*, 1989). It is possible that this kind of subunit may be designed to allow cyclic and regulated dissociable interactions with other proteins.

One way in which the Sec13p complex and the other purified cytosolic proteins could act in vesicle budding is to regulate the assembly of a coat protein complex involved in vesicle formation. A non-clathrin coat protein complex (coatamer) has been postulated to be involved in vesicle budding within the Golgi apparatus (Orci *et al.*, 1989). A comparable yeast coat protein has been described that contains a subunit encoded by *SEC21*, a gene required for protein transport between the ER and the Golgi apparatus (Hosobuchi *et al.*, 1992). Surprisingly, the Sec21p complex is completely resolved from the three protein fractions required for ER vesicle budding and the urea-extracted membranes used in the vesicle budding reaction contain less than 10% of the Sec21p found in an equivalent fraction of unextracted membranes (M.Hosobuchi, unpublished results).

The level of residual Sec21p complex present in our reconstituted reaction may not be rate-limiting for vesicle formation or the coat complex may be required in vesicle

Fig. 9. Chase of vesicles. (A) Vesicle chase reactions (squares) or overall ER to Golgi transport reactions (circles) were incubated for the indicated times at 20°C and transport was calculated as the percent of the glycosylated pro- α -factor containing α 1,6-mannose residues. (B) Vesicle chase reactions with no additions, 1 μ g anti-Ypt1p Fab fragments, 1 μ g anti-Ypt1p Fab fragments and 1 μ g recombinant Ypt1p, 20 μ M GTP γ S, or 20 μ M GTP γ S and 1 mM GTP, were incubated for 60 min at 20°C and the % transport determined.

targeting or fusion but not in vesicle formation. Unfortunately, the *sec21* mutant phenotype does not unambiguously differentiate between these two models. *sec21* mutant cells accumulate ER structures and a number of transport vesicles intermediate between wild type cells and mutants that block vesicle consumption (such as *sec17*) (Kaiser and Schekman, 1990). A *sec21 sec17* double mutant accumulates the large number of vesicles that accumulate in *sec17*. This result can be interpreted to favor a role for SEC21 in targeting, budding or both, and for this reason no assignment has been made. Alternatively, the residual Sec21p/coatamer on the membranes may be organized in a stable urea-resistant prebudding structure that requires the purified cytosolic components for release from the ER membrane. A Sec21p-depleted membrane fraction will be required to address this issue.

Additional peripheral and integral membrane proteins of the ER are likely to emerge as key factors in the mechanism and regulation of transport vesicle formation. Alternative fractionation schemes designed to remove or selectively inactivate these proteins will be required to move forward toward our goal of achieving a vesicle budding reaction reconstituted with pure proteins and defined lipids.

Materials and methods

Strains, materials and general methods

Yeast strains used in this study were RSY255 (*leu2-3,112, ura 3-52, MAT α* ; D.Botstein), RSY607 (*leu2-3,112, ura 3-52, pep4::URA3, MAT α*), RSY742 (*pNS1353, leu2-3,112, ura 3-52, sec13::URA3, MAT α*) and RSY799 (*pNS1354, leu2-3,112, ura 3-52, MAT α*). Plasmid pNS1353 (*SEC13::DHFR, LEU2*, 2 μ m) was described previously (Pryer *et al.*, 1993). pNS1354 was constructed by removing the *Xba*I–*Bam*HI fragment from pNS1350 (*SEC13::DHFR, URA3*, 2 μ m) generating a construct in which the first 17 amino acids of SEC13 are fused to the mouse DHFR gene.

Yeast peptone dextrose (YPD) contained 2% bacto-peptone (Difco, Detroit, MI), 1% yeast extract (Difco) and 2% glucose. Minimal medium was composed of 1.67 g/l yeast nitrogen base without amino acids and ammonium sulfate (Difco), 2% glucose and supplemented with 100 μ M (NH₄)₂SO₄ and amino acids where appropriate. Liquid cultures were grown with vigorous agitation at 30°C or in a 200 l New Brunswick fermenter.

Yeast genetic manipulations and molecular techniques were as described by Sambrook *et al.* (1989) and Guthrie and Fink (1991).

Immobilized papain was purchased from Pierce Chemical (Rockford, IL). Concanavalin A–Sephacrose (Con A–Sephacrose), protein A–Sephacrose CL-4B, DEAE–Sephacrose Fast-flow, G-25 Sephadex, prepacked Mono Q and Superose 6 columns, and standards for the calibration of gel filtration columns were all purchased from Pharmacia (Piscataway, NJ). All other reagents were purchased from Sigma (St Louis, MO) except where a different vendor is indicated.

Protein assays were performed with 1–20 μ l samples in a microtiter plate using protein assay reagent (Bio-Rad, Richmond, CA). Bovine serum albumin (BSA) was used as a protein standard. SDS–PAGE was performed according to Laemmli (1970) using 10% or 7.5% acrylamide. Electrophoresed proteins were visualized by staining with Coomassie blue (Sasse and Gallagher, 1989) or by silver staining (Hicke *et al.*, 1992). For immunoblotting, electrophoresed proteins were transferred to nitrocellulose filters (Schleicher and Schuell, Keene, NH) as previously described (Towbin *et al.*, 1979). Filters were blocked and antibody incubations were performed with 2% nonfat dry milk in 20 mM Tris–HCl, pH 7.4, 150 mM NaCl (TBS) with 0.05% Tween 20 (Bio-Rad). Detection of filter-bound antibodies was performed using either the ECL detection system (Amersham, Arlington Heights, IL) or ¹²⁵I-labeled protein A (ICN, Irvine, CA) (Harlow and Lane, 1988). Antibodies were used at the following dilutions: affinity purified anti-Sec13p, 1:1000; p150 serum, 1:10 000; affinity purified anti-Sar1p, 1:400 (Barlowe *et al.*, 1993a); affinity purified anti-Sec23p, 1:50 (Hicke *et al.*, 1992), Sec61p serum, 1:1000 (Stirling *et al.*, 1992), Kar2p serum, 1:20 000 (J.Brodsky, this laboratory) and anti-dhfr serum, 1:10 000 (kindly given by Walter Neupert, Institute for Physiological Chemistry, University of Munich).

Sec13p-dependent *in vitro* vesicle budding assay

Development of a Sec13p-dependent assay required modifications in the preparation of both the membrane and cytosolic fractions used in the budding reaction (Rexach and Schekman, 1991). Preparation of spheroplasts from wild type cells (RSY255) and their lysis by freeze–thawing to produce membranes were as described by Rexach and Schekman (1991). After stage I, the membranes (60 OD₆₀₀ unit cell equivalents of lysed spheroplasts) were extracted to remove peripherally associated proteins by resuspending in 1 ml B88 (20 mM HEPES, pH 6.8, 0.15 M KOAc, 250 mM sorbitol, 5 mM MgOAc) containing 2.5 M urea, incubating for 20 min on ice and then washing the extracted membranes twice by centrifugation at 13 000 g for 30 s followed by resuspension in 1 ml B88. For stage II, the membranes were resuspended to ~500 μ g protein/5 μ l in B88. The cytosolic fraction was replaced with purified protein fractions. Reaction mixtures contained 5 μ l extracted membranes (~500 μ g protein), an ATP regenerating system (Baker *et al.*, 1988), 0.1 mM GTP, Sar1p (0.3 μ g), Sec23p/Sec24p complex and Sec13p-containing fractions in a total volume of 50 μ l. Sec23p/Sec24p

complex was used in pure form or as a 0.5–0.75 M KOAc step eluate of whole cytosol applied to DEAE–Sephacryl Fast-flow (Pryer *et al.*, 1993). This fraction is enriched in Sec23p/Sec24p activity, but is devoid of Sar1p and Sec13p (Pryer *et al.*, 1993). Pure Sec23p/Sec24p (0.5 μ g) complex or 18.2 μ g protein of the DEAE step was used in each reaction. Reactions were incubated for 30 min at 30°C. Medium speed supernatant (MSS) fractions were prepared by centrifuging 30 s at 13 000 g in a TOMY refrigerated microcentrifuge (Tomy Seiko, Tokyo, Japan) at 4°C. The MSS fractions were treated with 250 μ g/ml trypsin for 10 min on ice followed by the addition of 250 μ g/ml trypsin inhibitor. Reactions were processed as described previously (Rexach and Schekman, 1991) and core-glycosylated, protease-protected Con A–Sephacryl precipitable material was quantified by counting in a Beckman LS7500 liquid scintillation system (Beckman, Palo Alto, CA). The budding efficiency is expressed as the ratio of Con A–Sephacryl binding pro-alpha-factor in the MSS to that present in the total reaction. The release of ER resident markers Kar2p and Sec61p was monitored by taking an aliquot of the MSS or unfractionated reactions and mixing with an equal volume of 2 $\times$ SDS–PAGE sample buffer. Samples were fractionated by SDS–PAGE followed by transfer to nitrocellulose. Immunoblots were probed with anti-Sec61p serum or anti-Kar2p serum at the dilutions indicated above and were developed with 125 I-labeled protein A (ICN Radiochemicals, Irvine, CA) (Harlow and Lane, 1988). The amount of radioactivity present in each band was quantified using a PhosphorImager (Molecular Dynamics, Menlo Park, CA).

Definition of Sec13p/p150 complex activity units

One unit of Sec13p/p150 activity is defined as 50% maximal budding in a 30 min budding reaction at 20°C using urea-extracted membranes supplemented with Sar1p and the Sec23p/Sec24p enriched fraction (see above).

Sec13-dhfrp/p150 complex purification

RSY742 (200 l culture) were grown to 10 OD₆₀₀ (optical density at 600 nm) units of cells per ml in a New Brunswick fermenter and harvested with a Sharples centrifuge. The cells (~1500 g wet weight) were washed once in cold H₂O and once in cold B88. Washed cells were resuspended with ~500 ml B88 and quick frozen in liquid nitrogen yielding ~1600 g frozen cell pellet which was stored at –80°C. A portion of the frozen pellet (500 g) was mixed with liquid nitrogen in a Waring blender to prepare a cell lysate (Dunn and Wobbe, 1989). The blended lysate was thawed to 4°C and diluted with 250 ml B88 containing protease inhibitors (PI; 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml pepstatin A, 0.5 μ g/ml leupeptin). All remaining steps in the purification were carried out at 4°C. Unbroken cells and large membrane aggregates were removed by centrifugation for 10 min at 4000 g in a GSA rotor (Sorvall, Dupont, Wilmington, DE). The supernatant fraction was further clarified by centrifugation at 100 000 g in a Ti45 rotor (Beckman, Palo Alto, CA) for 1 h. Clarified supernatant material (liquid nitrogen cytosol) was applied to a 40 ml methotrexate–agarose column (2.5 cm $\times$ 10 cm) equilibrated in B88PI at 2 ml/min. The column was washed with 10 column volumes of 1 M KCl, 0.1% Triton X-100 in B88PI followed by 10 column volumes of B88PI at 5 ml/min. One column volume of 50 mM methotrexate in B88 with 10% glycerol (B88GPI) was allowed to flow into the column which was then clamped for 3 h. Elution was achieved using 1 column volume of 50 μ M methotrexate in B88GPI and the first 60 ml of the eluate was collected for further processing. The eluate was divided into two 30 ml fractions and each was loaded on to a 100 ml G-25 Sephadex column (2.5 cm $\times$ 20 cm) equilibrated in 0.25 M KOAc B88GPI, pH 7.4. Protein peak fractions from both columns were combined and loaded on to a 20 ml DEAE–Sephacryl Fast-flow column (XK16/20, Pharmacia) equilibrated in 0.25 M KOAc B88GPI, pH 7.4, using a Pharmacia FPLC. The column was washed with 100 ml of the starting buffer, then eluted with a 100 ml linear gradient from 0.25 to 0.5 M KOAc. Column fractions were analyzed by SDS–PAGE and immunoblotting with affinity purified anti-Sec13p antibodies. Fractions containing Sec13-dhfrp/p150 complex (eluting at ~0.35 M KOAc) were pooled and applied to a 1 ml Mono Q column equilibrated in 0.4 M KOAc B88GPI, pH 7.4. The salt concentration was raised to 0.48 M KOAc by a 1.4 ml linear gradient and the column washed with 10 ml of 0.48 M KOAc B88GPI, pH 7.4, to remove the 200 kDa protein contaminant. Sec13-dhfrp was eluted with a 3.6 ml gradient to 0.7 M KOAc and fractions were again analysed by SDS–PAGE. Fractions of Sec13-dhfrp/p150 complex, eluting at ~0.55 M KOAc, were pooled, frozen in liquid nitrogen and stored at –80°C. Roughly 1.5 mg of Sec13-dhfrp/p150 complex was obtained with a yield of 3.5% of the starting activity (Table I).

Production of anti-native Sec13p antibodies

Sec13-dhfrp monomer was prepared from 200 g of frozen cells using a 20 ml methotrexate column. A pooled methotrexate eluate fraction (17 ml) was

applied to a 300 ml Sephacryl-300 column (2.5 cm $\times$ 75 cm) equilibrated in B88GPI. The protein peak fractions at the position of monomeric Sec13-dhfrp were pooled. Two rabbits (9620 and 9621) were each injected with 1 mg purified Sec13-dhfrp emulsified in Freund's complete adjuvant (Harlow and Lane, 1988). After the primary injection, we performed boost injections using 1 mg Sec13-dhfrp emulsified in Freund's incomplete adjuvant approximately every 4 weeks for 8 months (Harlow and Lane, 1988). Immune sera recognized a 33 kDa species in whole cell lysates that was overexpressed in cells containing a high copy SEC13 plasmid.

We used two affinity columns to purify the Sec13p antibodies: a dhfr affinity column to adsorb anti-dhfr antibodies and a Sec13-dhfrp column to affinity purify the anti-sec13p antibodies. Dhfr was prepared from 226 g of RSY799 frozen cell pellet as described for the Sec13-dhfrp monomer except 30 ml of methotrexate eluate was concentrated in a Centriprep-30 (Amicon, Beverly, MA) to 4.5 ml (90 min) and 2 ml of this material was loaded on to an 80 ml Sephacryl-300 column (1 cm $\times$ 100 cm) equilibrated in B88GPI. Column fractions were analyzed by SDS–PAGE and immunoblotting using anti-dhfr antibodies. Fractions containing pure dhfr were pooled (8.5 ml) and exchanged to 0.1 M HEPES, pH 7.4, by gel filtration on a 40 ml G-25 Sephadex column. Peak protein fractions were pooled and concentrated in a Centriprep-10 (Amicon) yielding 1.2 mg of protein in 1.2 ml. This material was coupled to Affi-Gel 15 (Bio-Rad) according to the manufacturer's instructions. The resulting 0.2 ml of coupled resin was poured into a 1 ml column. Similarly, 3.5 mg of Sec13-dhfrp was coupled to 0.3 ml of Affigel-15. Anti-Sec13p serum (0.5 ml) was diluted 1:10 with TBS and serially applied three times to the dhfr column. The resulting flow-through was applied three times to the Sec13-dhfrp column and the bound material was washed with 20 column volumes of TBS. Bound antibodies were eluted with 8 column volumes of 0.2 M glycine, pH 3.0, into a tube containing 2 column volumes of 1 M Tris–HCl, pH 9.0. The eluted antibodies were concentrated to 0.2 mg/ml in a Centriprep-30 and assayed for their ability to recognize Sec13p on immunoblots of yeast cell lysates.

Preparation of anti-p150 sera

p150 protein was prepared by dissociating the Sec13-dhfr/p150 complex in 2 M urea. Briefly, 1 ml of the Mono Q column fraction of Sec13-dhfr/p150 complex was diluted 1:1 with 2 $\times$ urea buffer (4 M urea, 0.4 M NaCl, 0.25 M Tris–HCl, pH 7.5). This material was loaded on to a 104 ml Superose 6 column, equilibrated in 1 $\times$ urea buffer + PI, using a Pharmacia FPLC system. Fractions were analyzed by SDS–PAGE and those fractions containing p150 were pooled and concentrated in a Centricon 30 microconcentrator (Amicon). Purified p150 (100 μ g) was emulsified in Freund's complete adjuvant and injected into each of two rabbits (442 and 443). Boost injections of 100 μ g p150 emulsified in Freund's incomplete adjuvant were performed approximately every 4 weeks for 5 months. Immune sera recognized a protein of 150 kDa on immunoblots that was enriched throughout the Sec13-dhfrp/p150 complex purification.

Preparation of Fab fragments and inhibition of whole cytosol vesicle budding reactions

Fab fragments were generated from anti-Sec13p or anti-p150 IgG fractions purified using protein A–Sephacryl as described by Harlow and Lane (1988). Antibodies eluted from the protein A column were exchanged to digestion buffer (20 mM NaH₂PO₄, 10 mM EDTA, pH 6.2) using a Centriprep-30. Immobilized papain beads (500 μ l), washed three times with digestion buffer containing 40 mM cysteine–HCl, were mixed with 1 ml of the antibodies and incubated overnight at 37°C with rotation. Fc fragments were removed by adsorption on a protein A column (1 ml) equilibrated in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄ (PBS). Protein in the column flow-through was pooled and exchanged to PBS with a Centriprep-10. Digestion was confirmed by SDS–PAGE and yielded ~0.5 ml of 0.2 mg/ml Fab fragments.

Budding reactions contained 500 μ g urea-extracted membranes mixed with 200 μ g of wild type cytosolic protein (RSY607 liquid nitrogen cytosol), an ATP regenerating system and 0.1 mM GTP. For Fab inhibition studies, we incubated cytosol in the presence of Fab fragments or Fab fragments plus excess competing antigen for 30 min on ice prior to the addition of membranes. Reactions were incubated for 60 min at 20°C and processed as described above.

Native immunoprecipitations from wild type radiolabeled cells

Wild type cells (RSY255) in minimal medium supplemented with uracil (0.02%), leucine (0.03%) and 100 μ M (NH₄)₂SO₄ were grown to 0.1–0.2 OD₆₀₀ units of cells/ml. Cells were harvested by sedimentation in a clinical centrifuge at top speed for 2 min, resuspended to 5 OD₆₀₀ units of cells/ml in minimal medium with uracil, leucine and (35 S) Express protein labeling mix (Dupont NEN) at 30 μ Ci/OD₆₀₀ unit of cells and incubated for 30 min

at 30°C. Labeling was terminated by adding an equal volume of 20 mM NaN_3 and incubating for 5 min on ice. The cells were washed once with 20 mM NaN_3 , then resuspended at $10 \mu\text{l}/\text{OD}_{600}$ unit of cells (TBS, 0.1% Triton X-100, PI) and transferred to an Eppendorf tube. Acid washed glass beads (0.5 mm diameter) were added to the meniscus and the suspension was vortexed 10 times for 10 s with 20 s intervals at 4°C. An equal volume of lysis buffer was added and the supernatant fraction clarified at 10 000 g in a TOMY refrigerated microfuge to generate a medium speed supernatant (MSS) containing 0.05 OD_{600} units of cell equivalent/ μl with $\sim 2 \times 10^7$ c.p.m./ OD_{600} unit of cell equivalent. From 6 to 10×10^6 c.p.m. ($\sim 5 \mu\text{l}$) were used per immunoprecipitation reaction. To reduce the background of proteins precipitated by both preimmune and immune sera, we prepared an enriched fraction of the Sec13p/p150 complex by selective precipitation of the MSS fraction using 30% ammonium sulfate (Pryer *et al.*, 1993). Saturated $(\text{NH}_4)_2\text{SO}_4$, pH 7.0, (0.428 $\mu\text{l}/\mu\text{l}$ MSS) was added and incubated for 30 min on ice. The precipitate was collected by centrifugation at 10 000 g for 10 min and resuspended to 90 μl /reaction with lysis buffer containing 10% glycerol. Preimmune serum (1 μl) was added to each reaction and incubated for 30 min on ice. Nonspecific protein aggregates were removed by adding 25 μl 10% protein A–Sephacrose CL-4B in B88 with 2% BSA, incubating for 30 min at 4°C with rotation and centrifuging 10 min at 10 000 g. The clarified supernatant fraction was transferred to a fresh tube containing immune antiserum (2 μl affinity purified anti-Sec13p antibodies or 1 μl anti-p150 serum) in the presence or absence of nonradioactive Sec13-dhfrp-containing fractions (1.2 μg monomer or 1.4 μg complex) and incubated for 30 min on ice. After centrifugation at 10 000 g for 10 min, immune complexes were precipitated from the supernatant by adding 25 μl 10% protein A–Sephacrose CL-4B in B88 with 2% BSA and incubating 30 min with rotation at 4°C. The immune complexes were collected by centrifugation for 30 s at 5000 g, washed twice with 1 ml lysis buffer, twice with 0.5 M NaCl, 20 mM Tris–HCl, pH 7.5, 1% Triton X-100 and twice with 50 mM NaCl, 10 mM Tris–HCl, pH 7.5. The washed precipitates were resuspended in 15 μl $2 \times \text{SDS}$ –PAGE sample buffer, heated for 5 min at 95°C and analyzed by SDS–PAGE followed by autoradiography.

Quantification of Sec proteins in unfractionated cytosol

Immunoblotting was used to quantify the abundance of Sec proteins present in unfractionated cytosol. Increasing amounts of the purified Sec protein fractions and wild type cytosolic protein were fractionated by SDS–PAGE followed by transfer to nitrocellulose. The immunoblots were probed with affinity purified anti-Sar1p antibodies, affinity purified anti-Sec23p antibodies or anti-p150 sera at the dilutions indicated above. The blots were developed with ^{125}I -labeled protein A (ICN Radiochemicals, Irvine, CA) (Harlow and Lane, 1988) and the amount of radioactivity present in each band quantified using a PhosphorImager (Molecular Dynamics).

ER to Golgi transport and vesicle chase reactions

ER to Golgi transport reactions were performed in two stages as described by Rexach and Schekman (1991), except that the membrane fraction used for the second stage was extracted with 2.5 M urea as described above. The vesicle chase reactions were performed with the MSS fraction from a budding reaction that contained Sar1p (0.3 μg), purified Sec23p/Sec24p (1.5 μg) complex and Sec13-dhfrp/p150 (0.8 μg) complex incubated for 60 min at 20°C. One-fifth of this fraction (10 μl) was used for each chase reaction in combination with 250 μg unextracted membranes, 120 μg cytosolic protein, an ATP regenerating system (Baker *et al.*, 1988) and 0.2 mM GTP in a total volume of 25 μl for 60 min at 20°C. Upon completion of the incubation, 15 μl was processed for anti- α 1,6-mannose immunoprecipitation and 5 μl was processed for Con A–Sephacrose precipitation as described by Rexach and Schekman (1991). The % transport was calculated as the anti- α 1,6-mannose immunoprecipitable c.p.m. divided by the total Con A–Sephacrose precipitable c.p.m. multiplied by 100. For inhibition with Ypt1p Fab fragments, 1 μg anti-Ypt1p Fab fragments in the presence or absence of 1 μg recombinant Ypt1p was added to the reaction as described by Rexach and Schekman (1991). For reactions to be treated with GTP γ S, the MSS fraction was centrifuged for 30 min at 100 000 g in a TLA 100.3 rotor (Beckman) to separate the vesicles from the GTP contained in the vesicle formation reaction. The vesicle pellet was resuspended in half the original volume of B88 and 10 μl added to each chase reaction as described above, except 20 μM GTP γ S or 20 μM GTP and 1 mM GTP was added in place of 0.2 mM GTP.

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