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# Sec23p and a Novel 105-kDa Protein Function as a Multimeric Complex to Promote Vesicle Budding and Protein Transport from the Endoplasmic Reticulum

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A cell-free protein transport reaction has been used to monitor the purification of a functional form of the Sec23 protein, a *SEC* gene product required for the formation or stability of protein transport vesicles that bud from the endoplasmic reticulum (ER). Previously, we reported that Sec23p is an 84-kDa peripheral membrane protein that is released from a sedimentable fraction by vigorous mechanical agitation of yeast cells and is required for ER to Golgi transport assayed in vitro. We have purified soluble Sec23p by complementation of an in vitro ER to Golgi transport reaction reconstituted with components from *sec23* mutant cells. Sec23p overproduced in yeast exists in two forms: a monomeric species and a species that behaves as a 250- to 300-kDa complex that contains Sec23p and a distinct 105-kDa polypeptide (p105). Sec23p purified from cells containing one *SEC23* gene exists solely in the large multimeric form. A stable association between Sec23p and p105 is confirmed by cofractionation of the two proteins throughout the purification. p105 is a novel yeast protein involved in ER to Golgi transport. Like Sec23p, it is required for vesicle budding from the ER because p105 antiserum completely inhibits transport vesicle formation in vitro.

## INTRODUCTION

Transport of proteins and membrane between the endoplasmic reticulum (ER) and the Golgi is mediated by the budding and fusion of transport vesicles, processes that require a number of *SEC* and *BET* gene products in yeast (Schekman, 1985; Newman and Ferro-Novick, 1987; Bacon *et al.*, 1989; Baker *et al.*, 1989; Nakano and Muramatsu, 1989). These gene products were identified by mutations that block ER to Golgi transport. A morphological study of transport vesicle intermediates accumulated in ER to Golgi *sec* mutants incubated at the nonpermissive temperature indicates that three proteins are required for vesicle fusion (Sec17p, Sec18p, and Sec22p) and at least four proteins mediate vesicle formation (Sec12p, Sec13p, Sec16p, and Sec23p) (Kaiser and Schekman, 1990). An additional protein required for ER vesicle formation is the GTP-binding protein Sar1p that interacts with Sec12p and has been shown to stimulate vesicle budding in vitro (d'Enfert *et al.*, 1991). Preliminary characterization of these proteins has been obtained by sequencing genes cloned by complementation of *sec* mutants and by raising antibodies to hybrid Sec proteins expressed in *Escherichia coli* (Eakle *et al.*, 1988; Nakano *et al.*, 1988; Hicke and Schekman, 1989; Kaiser and Schekman, unpublished data). Furthermore, biochemical assays for ER to Golgi transport in yeast lysates (Baker *et al.*, 1988; Ruohola *et al.*, 1988; Rexach and Schekman, 1991) depend on Sec proteins and may be used to isolate functional forms of these proteins.

Mammalian counterparts of the Sec18 and Sec17 proteins required for vesicle fusion have been identified biochemically using an in vitro assay for transport between Golgi cisternae. These proteins, *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment protein (SNAP), respectively, have been purified to homogeneity (Block *et al.*, 1988; Clary and Rothman, 1990). In addition to its role in intra-Golgi transport, NSF is required in ER to Golgi transport reconstituted from mammalian tissue culture cells (Beckers

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*et al.*, 1989). Amino acid sequence similarity and functional conservation between SNAP and the yeast Sec17 protein (Clary *et al.*, 1990; Griff *et al.*, 1992) imply a role for SNAP in ER to Golgi transport as well. The identification and purification of NSF and SNAP provide a starting point for a molecular description of transport vesicle targeting and fusion. An understanding of vesicle formation and budding also requires purification and characterization of proteins that drive the process.

The yeast Sec23 protein appears to be one of the factors required for vesicle formation or stability as the *sec23* mutant does not accumulate transport vesicles at the nonpermissive temperature in vivo or in vitro (Kaiser and Schekman, 1990; Rexach and Schekman, 1991). Sec23p is an 84-kDa protein that is peripherally associated with intracellular membrane but can be converted into a soluble form by a number of treatments, including vigorous mechanical agitation during yeast cell lysis (Hicke and Schekman, 1989). Temperature-sensitive transport in *sec23* lysates is reversed by the cytosolic form of wild-type Sec23p (Baker *et al.*, 1988; Hicke and Schekman, 1989). This assay now has been used to purify active Sec23p and an associated 105-kDa protein that is also essential for ER to Golgi transport.

## MATERIALS AND METHODS

### *Materials*

Yeast extract, peptone, dextrose (YPD) growth medium contained 2% Bacto-Peptone (Difco, Detroit, MI), 1% yeast extract (Difco), and 2% glucose. SD minimal medium was composed of 6.7 g/l yeast nitrogen base without amino acids (Difco), 2% glucose, and supplemented with amino acids as appropriate. Casamino acids medium contained 6.7 g/l yeast nitrogen base without amino acids (Difco), 1% vitamin assay casamino acids (Difco), and 2% glucose supplemented with 0.01% adenine, 0.01% methionine, 0.01% histidine, and 0.01% tryptophan. Uracil was added to 0.002% to propagate cells that did not carry a plasmid. Liquid cultures were grown in 2.8-l Fernbach flasks with vigorous agitation at 23–25°C or in a 16-l SF116 fermentor (New Brunswick Scientific, New Brunswick, NJ) at 30°C.

Immobilized papain was purchased from Pierce Chemical (Rockford, IL). Concanavalin A-Sepharose, Protein A-Sepharose CL-4B, DEAE-Sepharose Fast Flow, S-Sepharose Fast Flow, Sephacryl S-300 HR, and standards for the calibration of gel filtration columns all were purchased from Pharmacia (Piscataway, NJ). Glass beads (0.5 mm) were obtained from Biospec Products (Bartlesville, OK).

All protein assays were performed with 2- to 25-μl samples in a microtiter dish using protein assay reagent (Bio-Rad, Richmond, CA). Gamma-globulin (1–5 μg; Sigma, St. Louis, MO) was used to establish standard curves.

### *Strains and Plasmids*

RSY255 aka DBY2061 (*ura3-52, leu2-3,112, MATα*) was from D. Botstein (Department of Genetics, Stanford Medical School, Stanford, CA). RSY281 (*sec23-1, ura3-52, his4-619, MATα*) is a *sec23-1* strain derived by crossing LHY3-8C (Hicke and Schekman, 1989) into the genetic background of RSY255 (Kaiser and Schekman, 1990). RSY607 is *ura3-52, leu2-3,112, pep4::URA3, MATα* and was constructed by Nancy Pryer (this laboratory).

Plasmid pCF23 contains a 3.5-kb *Hin*dIII fragment carrying the entire wild-type *SEC23* gene subcloned into pCF35, a multicopy yeast plasmid bearing the *URA3* gene (this laboratory).

### *Sec23p-Dependent In Vitro ER to Golgi Protein Transport Reaction*

*sec23-1* membranes were prepared from strain RSY281. Cytosol fractions were prepared from strains RSY281 (*sec23-1*), RSY255 ($Sec^+$), and RSY255 carrying pCF23 (Baker *et al.*, 1988). Transport reactions were performed at 20 or 30°C for 45 min as described (Baker *et al.*, 1988; Hicke and Schekman, 1989). Sec23p activity was measured in 50-μl incubations that contained 1–25 μl of partially purified Sec23p and 100–200 μg *sec23-1* cytosolic protein mixed on ice. The high concentration of salt in samples collected from DEAE-Sepharose columns was compensated by the addition of Buffer 88 (see below) without KOAc so that the final concentration of KOAc was 0.15 *M* in each reaction. *sec23-1* membranes (10 μl) carrying translocated $^{35}S$-labeled pro-α-factor were added to the cytosol-Sec23p mixture, and the samples were transferred to 30°C. Reactions containing *sec23-1* cytosol with no added Sec23p were conducted at 20 and 30°C for every assay set. A measurement of the background in each assay was obtained by treating a sample of the complete reaction with sodium dodecyl sulfate (SDS) to 1%.

### *Definition of Sec23p Activity Units*

One unit of Sec23p activity is defined as 10% of the difference in $^{35}S$-pro-α-factor cpm, precipitable with anti-α1,6 mannose, between reactions conducted at 30°C containing only mutant Sec23p and those containing a saturating amount of functional wild-type Sec23p.

### *Sec23 Protein Purification*

Twelve liters of RSY255 transformed with pCF23 were grown to mid-logarithmic phase ($OD_{600}$ 4–6) in a 16-l SF116 fermentor and harvested in a Sharples centrifuge. Cells (35–45 g wet wt) were washed once in cold $dH_2O$ and once in cold Buffer 88 (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-KOH, pH 6.8, 0.15 M KOAc, 250 mM sorbitol, 5 mM MgOAc), resuspended in 250 ml Buffer 88, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and transferred to a 360-ml bead-beater (Biospec Products) half-filled with acid-washed glass beads (0.5 mm diam). Cells were lysed in the bead-beater at 4°C with six to eight 1-min bursts. Lysis efficiency was examined with a light microscope and was always >50%. The lysate was removed from the glass beads with a pipette, the beads were rinsed with an equal volume of Buffer 88, and the rinse was combined with the lysate. Unbroken cells and rapidly sedimenting membranes were removed by centrifugation at 4000 × *g* in a GSA rotor (Sorvall, Dupont, Wilmington, DE) for 10 min at 4°C, and the supernatant fraction was further centrifuged at 100 000 × *g* in a Ti45 rotor for 1 h at 4°C to remove all remaining membranes. Cytosol was split into three equal volumes and either used immediately or quick-frozen in liquid $N_2$ and stored at −85°C until needed.

Purification was initiated with one-third the volume of cytosol obtained from the preparation described above (typically 0.5–1 g cytosolic protein). Cytosol was adjusted to 0.5 M KOAc with 5 M KOAc, and the conductivity was measured. Cytosol in 0.5 M KOAc was applied at 55 ml/h to a 45-ml DEAE-Sepharose column (2.5 cm × 9.5 cm) equilibrated in Buffer 88, 0.5 M KOAc. The column was then washed with 1.5 column volumes Buffer 88, 0.5 M KOAc and eluted with an 180-ml linear gradient of 0.5–0.75 M KOAc in Buffer 88. Fractions (9 ml each) were assayed for Sec23p activity, total protein, and conductivity. Aliquots of each fraction were resolved by SDS- polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting to detect Sec23p antigen cross-reactivity. The peak of Sec23p activity was pooled and dialyzed overnight at 4°C against Buffer 88, 50 mM KOAc, until the conductivity matched that of the buffer. The dialyzed pool was then loaded onto a 5-ml column of S-Sepharose Fast Flow (1.0 cm × 6.0 cm) equilibrated in Buffer 88, 50 mM KOAc. Fractions of 5 ml were collected during the loading of the column. The column was washed with 6 ml Buffer 88, 50 mM KOAc, and eluted with a 25-ml linear gradient of 50 mM to 0.5 M KOAc in Buffer 88. One-milliliter

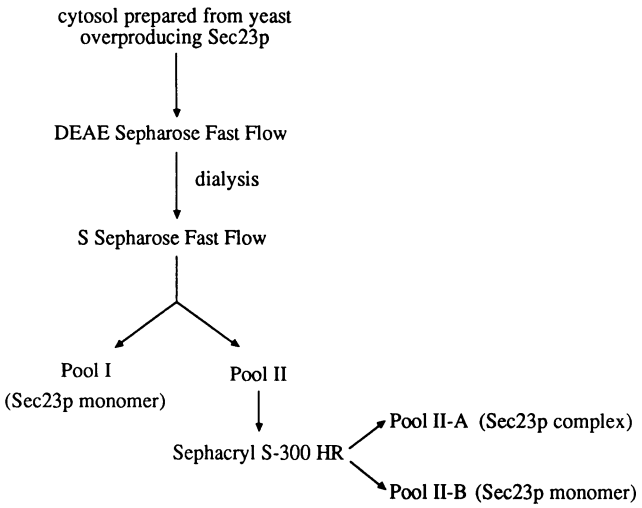


**Figure 1.** Sec23 protein purification scheme.

fractions were collected during the wash and elution steps. All flow rates were ~100 ml/h to minimize the time Sec23p spent associated with the column medium. Activity, total protein, and Sec23p antigen cross-reactivity were determined in all fractions. Two peaks of activity were resolved and were pooled separately. Each pool was then fractionated on a 350-ml Sephacryl S-300 HR column (2.5 cm × 70 cm) equilibrated in Buffer 88. Fractions of 5.5 ml were collected at a flow rate of 50 ml/h. Fractions collected from Sephacryl S-300 were assayed for Sec23p activity, and aliquots were evaluated by SDS-PAGE developed with silver-stain.

Purification of Sec23p from cells containing one copy of the *SEC23* gene was performed with the following modifications. A *pep4* strain (RSY607) carrying a mutation in the vacuolar protease A structural gene was used to minimize proteolysis during the purification. Cells (40–70 g wet wt) were lysed and centrifuged as above. The cytosolic and membrane fractions were separated. To recover Sec23p still associated with membranes, the sedimentable fraction was mixed with 100 ml Buffer 88, 0.75 M KOAc, 1 mM DTT, 0.5 mM PMSF, 0.5 mg/ml leupeptin, 0.7 μg/ml pepstatin and resuspended with three strokes in a Potter-Elvehjem homogenizer. The homogenate was mixed with another 100 ml of the same buffer, stirred for 30 min at 4°C, and centrifuged at 100 000 × *g* for 60 min. Soluble protein from this salt extraction was combined with the cytosol fraction, and the entire mixture was adjusted to 0.5 M KOAc and used for purification.

Isolation of Sec23p from untransformed cells was performed on an approximately threefold larger scale than from the overproducer strain. Only one peak of activity eluted from S-Sepharose and this was concentrated by adsorption to a 1.5-ml bed volume DEAE-Sepharose column equilibrated in Buffer 88. The column was washed with six volumes of Buffer 88 and eluted with Buffer 88 containing 0.75 M KOAc. Fractions containing proteins eluted in 0.75 M KOAc were pooled and subjected to gel filtration on Sephacryl S-300. Approximately 2–3 mg Sec23p complex was obtained from 40 to 70 g wet cells.

### *Silver Staining of Polyacrylamide Gels*

Silver staining was performed according to a modified protocol of Morrissey (1981). Gels were fixed in 50% methanol, 50% $dH_2O$, 0.4% formaldehyde for >1 h at room temperature, incubated in 5 μg/ml DTT for 30 min, and stained in 0.1% silver nitrate for 30 min. After rinsing quickly in $dH_2O$, the gels were developed in 3% sodium carbonate, 0.2% formaldehyde and the development was stopped by the addition of one-tenth volume 2.3 M citric acid.

### *Antiserum Prepared Against Native Sec23p*

Two rabbits (9049 and 9188) were each injected with 100 μg purified Sec23p monomer emulsified in Freund's complete adjuvant. Approximately 4 wk later the rabbits were each injected with 50 μg pure Sec23p monomer emulsified in Freund's incomplete adjuvant. These boost injections were repeated every 3 wk with 50 μg protein each for ~4 months. Immune antiserum from both rabbits recognized an 84-kDa protein in yeast lysates not recognized by preimmune sera. Affinity-purified Sec23p antibody was prepared by adsorption to TrpE-Sec23p hybrid protein conjugated to Sepharose as described (Hicke and Schekman, 1989), except that the antibodies bound to the column were recovered by serial elution with 0.2 M glycine-HCl, pH 2.5 and 0.1 M $Na_2CO_3$. The eluate was dialyzed against 50 mM tris(hydroxymethyl)aminometnane-HCl, pH 7.5, 150 mM NaCl and stored at 4°C in the presence of 0.5% bovine serum albumin and 10 mM $NaN_3$. The affinity-purified antibody was used for the experiment in Figure 5 at a dilution of 1:50. IgGs added to the in vitro transport reactions were purified from antisera by adsorption to and elution from protein A-Sepharose and concentrated to 12–16 mg/ml with Centricon 30 Microconcentrators (Amicon, Danvers, MA). Fab fragments were produced from an IgG fraction using immobilized papain as described by the manufacturer (Pierce).

### *Antiserum Prepared Against p105*

p105 antisera were prepared as described by Hawlitschek *et al.* (1988). Briefly, 1 mg of Sec23p complex was subjected to preparative SDS-PAGE and transferred to nitocellulose. Protein bands were visualized by staining with Ponceau S, and the p105 band was excised and dried. The dried nitrocellulose was dissolved in dimethylsulfoxide, mixed with Freund's adjuvant, and ~100 μg of antigen was injected into each of two rabbits (9573 and 9569). This primary injection was followed by four boost injections of 25 μg antigen each that occurred at 3-wk intervals. Antisera from each rabbit were tested for p105 binding activity on immunoblots of yeast cell lysate. Antiserum was used at a dilution of 1:1000 in the experiment in Figure 5. IgG fractions and Fab fragments were prepared as described for Sec23p antibodies.

### *In Vitro ER Vesicle Budding Assay*

The vesicle budding assay was performed as previously described (Rexach and Schekman, 1991) with cytosol and freeze-thawed spheroplasts prepared from wild-type cells (RSY255). Reaction mixtures (25 μl) were prepared as for the ER to Golgi transport assay and incubated at 20°C for 30 min. Medium speed supernatant fractions (MSS) were collected by centrifugation of the completed reaction at 13 000 rpm for 37 s in a microcentrifuge. The MSS were treated with 250 μg/ml trypsin for 10 min on ice, followed by the addition of 250 μg/ml trypsin inhibitor. After denaturation with SDS, radiolabeled core-glycosylated pro-α-factor (gpαF) was recovered with Con A-Sepharose, and the radioactivity bound to the Sepharose was quantified. Budding activity is presented as the ratio of Con A-binding pro-α-factor detected in the MSS to that present in the total reaction.

## RESULTS

### *Assay of Functional Sec23p*

The assay used to follow Sec23p activity (Baker *et al.*, 1988) measures the transport of a radiolabeled secretory protein (the mating pheromone precursor prepro-α-factor, pαf) that is synthesized in vitro and introduced into the ER of a yeast lysate by posttranslational translocation. Core-glycosylated α-factor precursor (core gpαf) formed in the ER is converted to a more highly glycosylated form in the Golgi apparatus. This transport event, which is monitored by precipitation of gpαf by

outer chain-specific antibodies, depends on active Sec23p. Membranes and cytosol prepared from *sec23-1* cells grown at a permissive temperature display a temperature-sensitive defect in ER to Golgi transport. Transport at 30°C in *sec23-1* membranes incubated with *sec23-1* cytosol is reduced fivefold relative to transport at 15°C. Incubation of *sec23-1* membranes with cytosol containing wild-type Sec23 protein allows transport in the temperature range of 15–30°C (Baker *et al.*, 1988). Furthermore, transport at 30°C in the mixture of mutant membranes and cytosol is restored by the addition of wild-type Sec23p resolved from other soluble proteins by gel filtration (Hicke and Schekman, 1989). These results provided the basis for the Sec23p activity assay. Purified fractions of the protein were mixed with *sec23-1* cytosol and *sec23-1* membranes containing core gp$\alpha$f and incubated at 30°C. The amount of pro-$\alpha$-factor transported to the Golgi during the incubation was proportional to the amount of wild-type Sec23 protein added to the reaction.

### *Purification of Sec23p Activity*

Preliminary investigations of the association of Sec23p with ion-exchange media indicated the protein had unusual binding properties. Sec23p bound both a cation-exchange support, S-Sepharose in 50 mM KOAc at neutral pH, and the weak anion-exchange medium DEAE Sepharose in 0.5 M KOAc at neutral pH. These chromatography media were used together with gel filtration in the purification scheme developed and shown in Figure 1.

Experiments with cytosol prepared from yeast bearing the *SEC23* gene on a multicopy plasmid indicated that these cells overproduce Sec23p activity; therefore, a soluble fraction of Sec23p overproducing yeast was used as the starting source for purification. Lysates of wild-type yeast cells carrying the *SEC23* plasmid pCF23 were prepared by vigorous homogenization with glass beads. The soluble fraction was isolated by several centrifugation steps, and the salt concentration of the resulting cytosol was adjusted to 0.5 M KOAc. Cytosol was applied to DEAE Sepharose equilibrated in 0.5 M KOAc and a linear gradient of 0.5–0.75 M KOAc was used to develop the column. A profile of Sec23p activity, total protein, and salt concentration of fractions collected from this column is shown in Figure 2A. Recovery of Sec23p activity from this column was typically 50–75%. Fractions containing the peak of activity were pooled and dialyzed overnight. Dialysis at this step not only reduced the salt concentration but also resulted in a twofold increase in activity, perhaps due to the removal of a low molecular weight inhibitor of the transport reaction. After dialysis of the protein into 50 mM KOAc, the sample was applied to S-Sepharose equilibrated in the same buffer. Protein was released from the column with a 50 mM to 0.5 M KOAc gradient. Figure 2B shows the elution pattern of transport activity, protein, and salt. Sec23p activity eluted in two peaks that were collected and pooled separately. The separation of these peaks was reproducible in several preparations, though occasionally the first peak appeared as a shoulder of the second. In a number of preparations, the loss of activity on S-Sepharose was significant (see Table 1). High flow rates reduced binding of Sec23p activity to S-Sepharose; however, slower flow rates that allowed complete binding of Sec23p to the column caused unacceptable loss (>90%) of activity. Sec23p was stable at the salt concentrations used in this step. It is possible that another protein required for full Sec23p activity was separated from Sec23p during this chromatography step; however, addition of S-Sepharose flowthrough fractions to eluted fractions containing Sec23p did not stimulate activity. Alternatively, loss of activity on the cation-exchange medium may be due to a disruptive interaction between the charged beads and the Sec23 protein.

Pool I collected from the S-Sepharose column contained ~11% of the initial soluble Sec23p activity and consisted of an 84-kDa polypeptide that migrated as expected for Sec23p (see Figure 3A, lane 1). A variable level of a species migrating just below Sec23p was seen in some preparations. This polypeptide reacts with Sec23p antisera and may be a product of Sec23p proteolysis. Pool II contained Sec23p and a mixture of several other proteins (see Figure 3A, lane 2). The Sec23p activity in this pool separated into two peaks on gel filtration over Sephacryl S-300 (Figure 2C), one that migrated as a 250- to 300-kDa species (Pool II-A) and another, containing less activity, that behaved as a small protein of <100 kDa (Pool II-B).

### *Overproduced Sec23p is Purified as a Monomer and as Part of a Multimeric Complex*

Gel filtration of crude cytosol prepared from yeast carrying a single chromosomal copy of *SEC23* indicated that all of the soluble Sec23p activity and immunoreactive Sec23p in these cells was in a large 300- to 400-kDa protein fraction (Hicke and Schekman, 1989). It appeared, however, that several forms of active Sec23p contained in Pools I, II-A, and II-B may be purified from yeast overexpressing the protein. We sought to determine the purity and the relationship between the protein content of these three purified fractions.

Figure 3 shows silver-stained samples of purified Sec23p from a representative preparation. Figure 3A depicts the content of Pools I and II collected from S-Sepharose. The single polypeptide present in Pool I behaved as one peak of monomeric Sec23p on gel filtration. The mixture of Sec23p with four other prominent polypeptides in Pool II resolved into two peaks of Sec23p activity on Sephacryl S-300, and Figure 3B depicts the fractionation of the proteins present in Pool II

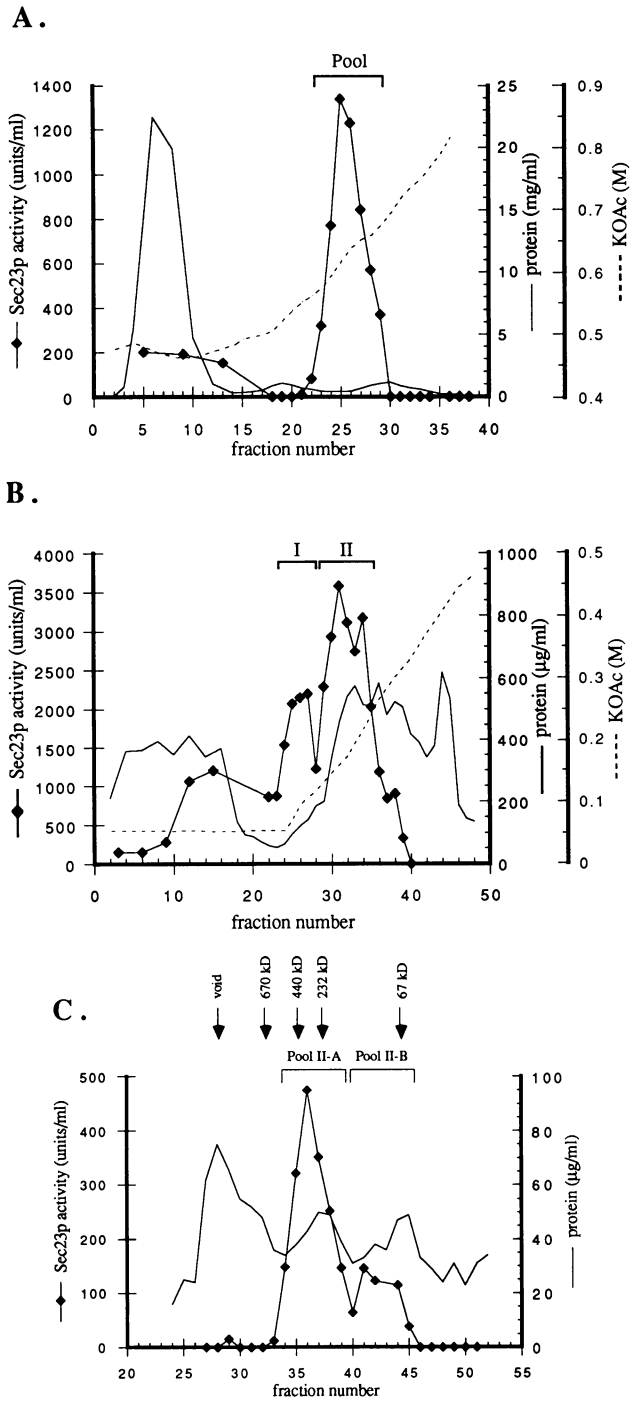


**Figure 2.** Chromatographic profiles of Sec23p purified from an overproducing strain. (A) Anion-exchange chromatography of Sec23p activity on DEAE-Sepharose. Cytosolic yeast proteins bound to DEAE Sepharose in 0.5 M KOAc were eluted with a 0.5 M → 0.75 M KOAc linear gradient. Fractions (9 ml) collected from the column were assayed for activity in a Sec23p-dependent transport assay and for total protein content. Activity assays contained 5 µl DEAE fraction and 150 µg *sec23* mutant cytosolic protein. Fractions 23–29 were pooled for further purification. (B) Sec23p activity recovered from cation-exchange (S-Sepharose) chromatography. The Sec23p activity recovered from DEAE Sepharose was dialyzed against Buffer 88, 50 mM KOAc and loaded onto S-Sepharose in the same buffer. Proteins that flowed through the column were collected in 5-ml fractions (fractions 1–15). Bound proteins were recovered with a 50 mM → 0.5 M KOAc gradient in 1-ml fractions (fractions 16–50). Aliquots of 2.5 ml of each fraction were assayed for total protein and with 150 µg *sec23-1* mutant cytosolic protein for transport activity. Two peaks of activity, Pool I (fractions 24–28) and Pool II (fractions 29–35), were pooled and analyzed separately. (C) Gel filtration of Sec23p on Sephacryl S-300. S-Sepharose Pool II (6 ml) was loaded onto a 350-ml Sephacryl S-300 column and eluted in Buffer 88. Total protein was determined and Sec23p activity in 25 µl of each fraction was assayed. The two peaks of activity were pooled independently. Peaks of protein standards used to calibrate the column are indicated by arrows. The void volume was determined by the elution of blue dextran.

on this column. Two peaks of an 84-kDa polypeptide that cross-reacted with Sec23p antiserum were observed in the gel filtered fractions of Pool II (Figure 3B). The fractions that corresponded to activity collected in Pool II-B (lanes 20–22) contained only Sec23p that migrated as a monomer (apparent molecular mass 70 kDa). The Sec23p in fractions containing Pool II-A activity (lanes 10–15) behaved with an apparent molecular mass of 260-kDa protein on the column and copurified precisely with a 105-kDa polypeptide present in similar amounts. (The apparent molecular mass of the two forms of Sec23p were calculated based on the elution volumes of the following protein standards: ferritin, 670 kDa; catalase, 440 kDa; aldolase, 232 kDa; and ovalbumin, 43 kDa). The specific activity of Pool II-A was approximately threefold greater than that of II-B (Table 1).

Quantitation (Table 1) indicates that 10% of the Sec23p activity from the overproducing strain was obtained in pure form in two chromatographic steps (Pool I). A further 10% of the activity was recovered after gel filtration as Sec23p associated with a 105-kDa protein (Pool II-A). A substantial amount of monomeric Sec23p having lower specific activity than that in Pool I was resolved from the Sec23p complex on the gel filtration column. A loss of activity may occur as a result of dilution of the protein during gel filtration. Alternatively, monomeric Sec23p may be heterogeneous. Protein that binds more tightly to S-Sepharose and is therefore eluted at higher salt may be less active.

### *Active Sec23p Purified From Cells Carrying One SEC23 Gene Exists Only in the Large Multimeric Form*

To determine what forms of Sec23p are found in yeast expressing a single copy of *SEC23*, Sec23p was purified from nontransformed yeast using minor modifications designed to improve the yield of activity (see MATERIALS AND METHODS). Only one peak of Sec23p activity was recovered from both the S-Sepharose and Sephacryl S-300 columns (Figure 4, A and B), and all of the purified activity behaved as a protein of 250–300 kDa on the gel filtration column (Figure 4B). Coomassie

**Table 1.** Purification of Sec23p activity

| Fraction | Total protein (mg) | Activity (units/ml) | Total activity (units) | Specific activity (units/mg) | Activity yield (%) | Purification |
|---|---|---|---|---|---|---|
| Cytosol | 1 000 | 2 710 ± 390 | 87 800 | 88 | 100 | 1 |
| DEAE Sepharose before dialysis | 37.5 | 878 ± 13 | 55 300 | 1 480 | 63.0 | 17 |
| DEAE Sepharose after dialysis | 33.8 | 1 380 ± 140 | 103 500 | 3 060 | 118 | 35 |
| S-Sepharose: Pool I | 0.53 | 1 730 ± 160 | 9 510 | 17 800 | 10.8 | 202 |
| S-Sepharose: Pool II | 3.3 | 2 250 ± 330 | 17 300 | 5 260 | 19.7 | 60 |
| Sephacryl S-300 Pool II-A | 1.4 | 283 | 9 340 | 6 740 | 10.6 | 76 |
| Sephacryl S-300 Pool II-B | 1.3 | 97 | 3 200 | 2 490 | 3.6 | 28 |

staining of eluate fractions indicated that the 105-kDa polypeptide (p105) copurified with Sec23p and with transport activity across the gel filtration profile (Figure 4C). A protein species that migrated just below p105 varied among Sec23p preparations and was enhanced on storage of the complex. As antibody developed against p105 cross-reacted with the lower molecular weight band, we believe this species is a proteolytic fragment of p105. We conclude from this purification that the large species of Sec23p associated with p105 is the active form of the protein present in wild-type yeast cells.

The coelution of p105 and Sec23p from Sephacryl S-300 implied an association between the two proteins; however, monomer Sec23p appeared to function on its own in complementation of the *sec23* transport defect. One explanation for this observation is that purified Sec23p monomer may recruit its associated protein from *sec23* cytosol during the course of the transport reaction. Alternatively, coelution of Sec23p and p105 during the last step of Sec23p purification may be fortuitous and indicates no meaningful interaction between the two. To investigate whether a stable association between Sec23p and p105 exists, we prepared antisera against p105 and followed the behavior of p105 throughout the Sec23p purification. Figure 5 demonstrates that p105 copurifies with the large form of Sec23p over each of the three columns employed in the purification. In addition, it appears that all of the p105 present in the cell is associated with Sec23p, as the 105-kDa band that cross-reacts with p105 antiserum, is quantitatively depleted from the flowthrough fractions of these columns.

### *Antibodies Against Sec23p and p105 Inhibit ER Vesicle Budding In Vitro*

As Sec23p is required for transport vesicle budding from the ER to the Golgi in vivo (Kaiser and Schekman, 1990)

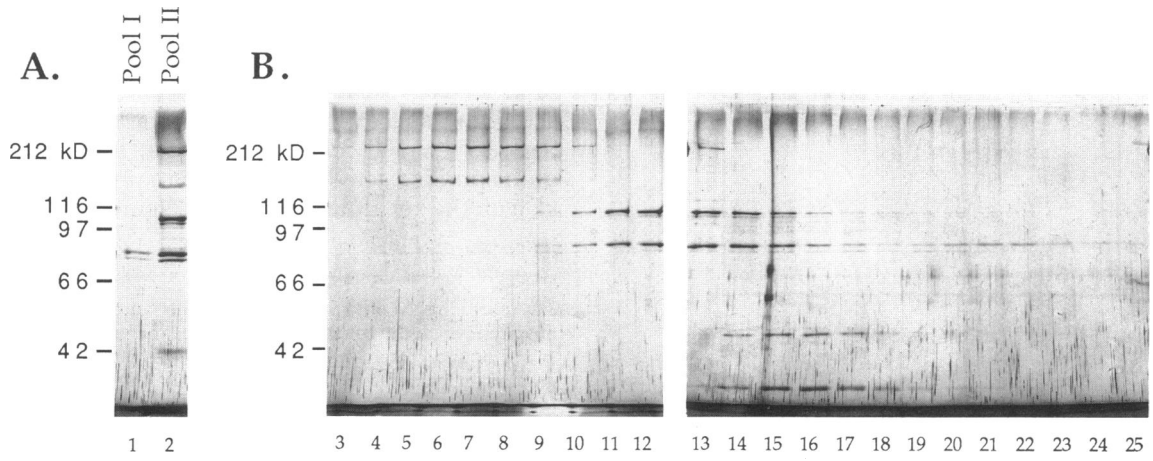


**Figure 3.** Overproduced Sec23p is purified in two forms; as a monomer and as a large multimeric complex. Aliquots of Pools I and II and fractions of Pool II that had been further separated on Sephacryl S-300 were electrophoresed on 7.5% SDS-polyacrylamide gels and stained with silver to visualize the proteins contained in these fractions. (A) Proteins present in Pools I and II. Pool I contained an 84-kDa polypeptide that corresponded to Sec23p. Pool II contained the 84-kDa Sec23p in addition to several other prominent bands. (B) Proteins resolved by chromatography of Pool II on Sephacryl S-300. Two peaks of Sec23 protein were resolved from other proteins present in Pool II. The first peak (lanes 11–15) filtered as a protein of 250–300 kDa and contained a 105-kDa polypeptide that copurified with this form of the Sec23 protein. The second peak (lanes 20–22) behaved as a protein of <100 kDa and contained only the Sec23 polypeptide. The S-300 fractions were resolved on two gels (fractions 3–12 and 13–25), each calibrated with adjacent molecular weight standards.

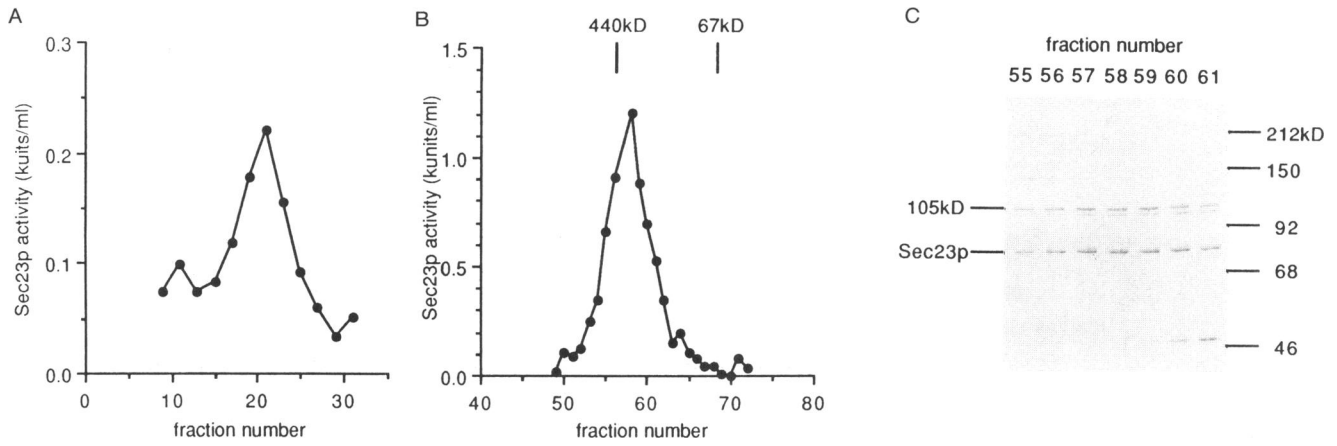


**Figure 4.** Sec23p purified from yeast containing one *SEC23* gene exists only as part of a multimeric complex. Sec23p was purified from RSY607 cells that contained only one chromosomal copy of *SEC23*. (A) Sec23p activity contained in these cells eluted in a single peak from S-Sepharose. (B) Further purification of Sec23p on Sephacryl S-300 again resulted in the elution of a single peak of activity that chromatographed as a large (300-kDa) species. (C) Aliquots of Sephacryl S-300 fractions that contained Sec23p activity were analyzed by SDS-PAGE and stained with Coomassie blue. The 84-kDa Sec23p present in these fractions coeluted with a 105-kDa polypeptide.

and in vitro (Rexach and Schekman, 1991), the stable association of p105 with Sec23p implied a role for p105 in vesicle budding. p105 is not encoded by any of the previously identified *SEC* or *BET* genes (Yoshihisa and Schekman, unpublished data); therefore, to determine if it is required for ER to Golgi transport, we resolved purified p105 from Sec23p by SDS-PAGE, prepared antibodies against the protein, and tested their ability to inhibit ER to Golgi transport in vitro. Antibodies against p105 inhibited ER to Golgi transport as well as antisera that recognize native Sec23p (L. Hicke, T. Yoshihisa, and R. Schekman, unpublished data).We tested the effect of these antibodies on an early stage of ER to Golgi transport in an ER vesicle budding assay. As shown in Figure 6, 10 $\mu$g of the anti-native Sec23p IgG (9188) and 15 $\mu$g of the anti-p105 IgG (9573) strongly inhibited the budding reaction, whereas the equivalent amount of preimmune IgG had no effect. Inhibition was also observed in reactions containing Fab fragments prepared from the immune IgGs (Figure 6, Fab). The inhibition by Fab fragments suggests that the antisera inhibit Sec23p and p105 activities directly and not by merely reducing the amount of free Sec23p complex.

To confirm that the antibody inhibition was a result of inactivating Sec23p or p105 rather than an indirect effect on other proteins that may interact with the complex during the budding process, we demonstrated that the inhibition could be overcome by the addition of purified Sec23p-p105 complex. Figure 7 shows the percent of pro-$\alpha$-factor found in a vesicle fraction in reactions that included anti-Sec23p, anti-p105, or no antisera and were supplemented with either Sec23p complex or monomer. The concentration of IgG used to inhibit these reactions was the minimum amount that was necessary to reduce budding >90%. The decrease in budding activity caused by either antibody can be restored partially by the addition of 1.5 $\mu$g Sec23p-p105 complex (Figure 7A). Addition of up to fivefold more complex did not further alleviate the inhibition. In contrast, 1.5 $\mu$g pure Sec23p monomer had no effect on a reaction inhibited by anti-p105, though it effectively restored budding in a reaction containing anti-Sec23p (Figure 7B). Surprisingly, Sec23p monomer was significantly more efficient than Sec23p-p105 complex in relieving anti-Sec23p inhibition. This effect is more than the difference expected from the higher specific immunoreactivity of the monomer for the antibody. Because the polyclonal Sec23p antiserum was raised using the Sec23p monomer as an antigen and hence may inhibit by binding Sec23p epitopes that are masked by Sec23p-p105 interaction, the monomeric Sec23p may neutralize inhibiting antibodies much more efficiently than the complex form. Alternatively, the antibody-Sec23p-p105 may interfere in some way that does not occur with the complex of the antibody and Sec23p monomer.

## DISCUSSION

A reaction that reproduces ER to Golgi transport in vitro provided an assay for purification of an active form of the Sec23 protein. Sec23p was purified from cells containing a multicopy form of *SEC23* because cytosol from these cells had significantly more activity than an equal amount of cytosol prepared from untransformed cells. The protein exists in two active forms: a monomer and a 260-kDa multimer with a 105-kDa polypeptide (p105) subunit. In contrast, Sec23p exists solely in the multimeric form in cells carrying one copy of *SEC23*.

Sec23p exhibits unusual properties that facilitate its purification, binding both cation- and anion-exchange

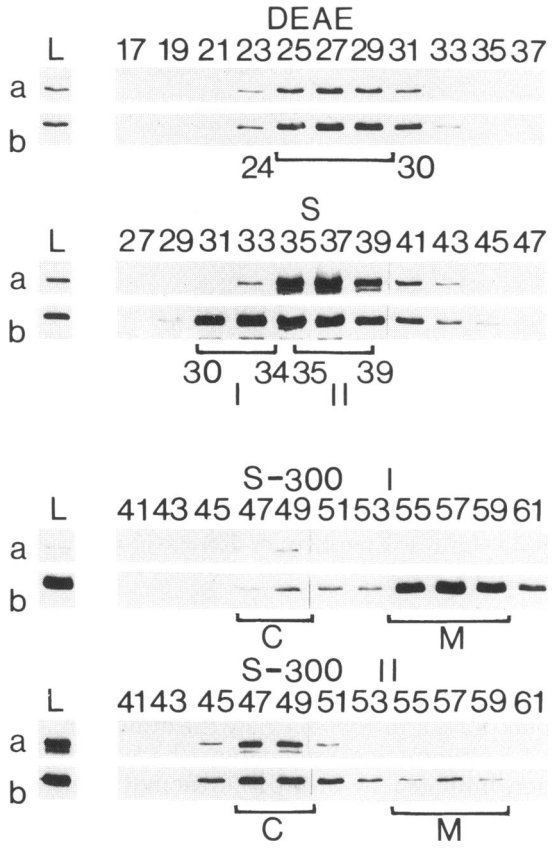


**Figure 5.** p105 cofractionates with the large form of Sec23 protein throughout the entire purification procedure. Sec23 complex and monomer were purified from the Sec23p overproducing strain RSY255/pCF23. Protein loaded onto a column (load, lane L) and fractions that contained Sec23p activity (numbered lanes) from each chromatography step throughout the purification were electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with affinity-purified p105 antiserum (a) or Sec23p antibody (b). DEAE, load and fractions collected from DEAE-Sepharose chromatography. Fractions 24–30 were pooled and chromatographed on S-Sepharose. S, S-Sepharose fractions. Fractions 30–34 (Pool I) and fractions 35–39 (Pool II) were combined separately, and each pool was individually filtered on Sephacryl S-300. S-300 I, fractions of Pool I resolved on Sephacryl S-300. S-300 II, fractions of Pool II resolved on Sephacryl S-300. The brackets labeled C correspond to the position at which Sec23p complex elutes from the gel filtration column and brackets labeled M mark the elution position of monomeric Sec23p.

media at neutral pH. The anion-exchange binding is particularly tight, occurring even in 0.5 M KOAc. There is no obvious feature of the predicted amino acid sequence that would explain this behavior nor is the interaction mediated by p105 because the overexpressed monomeric Sec23p also binds to DEAE-Sepharose under these conditions.

Sec23p elutes from S-Sepharose in two peaks of activity. Silver staining of the proteins present in these peaks indicates that the first peak (Pool I) represents pure Sec23p and the second peak (Pool II) contains Sec23p in addition to four or five other prominent proteins. Further fractionation of Pool II by gel filtration resolved two forms of Sec23p. One form chromatographed as expected for monomeric protein and a second peak of Sec23p along with a 105-kDa protein behaved as a much larger species. This large form filtered at a similar position, corresponding to a 250- to 300-kDa protein, as reported previously (Hicke and Schekman, 1989). A complex of two 85-kDa subunits and one 105-kDa subunit would have a predicted molecular mass of 275 kDa, a size consistent with the gel filtration behavior of the Sec23p-p105 multimer.

In addition to the copurification of Sec23p and p105 in similar amounts, several additional lines of evidence indicate the two polypeptides are associated in a heterooligomeric complex. First, Sec23p purified from yeast with one *SEC23* gene is present solely in the 260-kDa form, whereas the overexpression of Sec23p results in

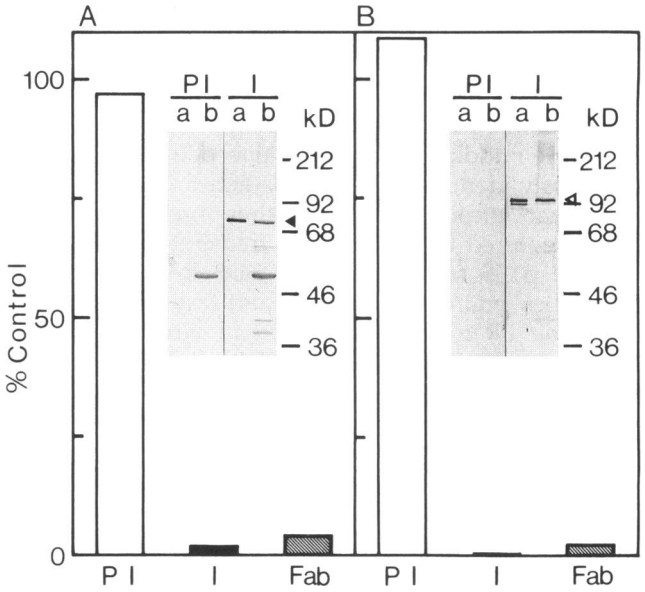


**Figure 6.** Antibodies against native Sec23p and p105 inhibit vesicle budding in vitro. The effects of antisera raised against native Sec23p and p105 on vesicle budding were tested in an in vitro assay that measures ER vesicle budding. IgG prepared from preimmune serum or from immune serum, or Fab fragments prepared from the immune IgG, was added to a 25-$\mu$l budding reaction. After incubation on ice to promote antibody-antigen binding, the reaction mix was incubated at 20°C to allow vesicle budding, and trypin-protected gp$\alpha$F was measured. Each column describes the budding activity relative to a control reaction containing no IgG. The insets show immunoblots of purified Sec23p complex (60 ng, lane a) and cell lysate (10 $\mu$g, lane b) probed with 2.5 $\mu$g/ml preimmune (PI) or immune (I) IgG. (A) Inhibition by anti-native Sec23p antibody. The reaction mixture contained either 10 $\mu$g of 9188 preimmune IgG (PI), 10 $\mu$g 9188 immune IgG (I), or 1.9 $\mu$g immune Fab fragment (Fab). The inset immunoblot was probed with 9188 IgG. The closed triangle indicates the position of Sec23p. (B) Inhibition by p105 antibody. These budding reactions contained 15 $\mu$g 9573 preimmune IgG (PI), 15 $\mu$g 9573 immune IgG (I), or 4.2 $\mu$g Fab fragment prepared from 9573 immune IgG (Fab). The inset immunoblot was incubated with 9573 IgG, and the position of p105 is shown by the open triangle.

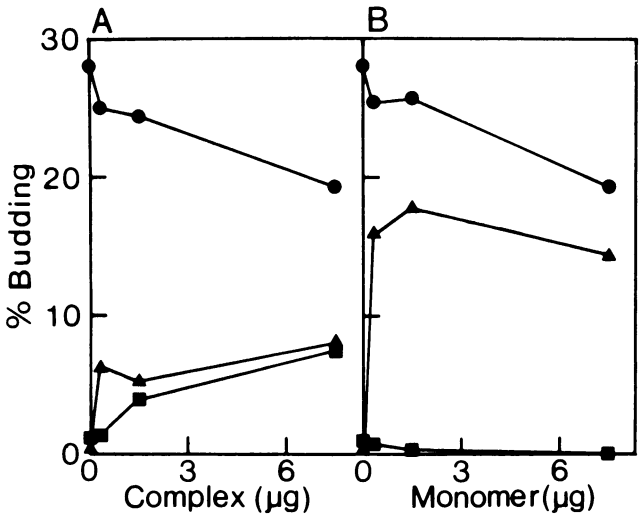


**Figure 7.** Inhibition of vesicle budding by Sec23p and p105 antibodies is overcome by the addition of Sec23p complex. Purified Sec23p (0, 0.3, 1.5, or 7.5 μg) was added to a 25-μl budding reaction containing 5 μg anti-Sec23p IgG (▲), 8 μg anti-p105 IgG (■), or no IgG (●). After 5-min incubation on ice, vesicle budding was allowed to proceed at 20°C for 30 min, and the percent pro-α-factor that bound Con A and was protected from trypsin was measured. (A) Addition of purified Sec23p-p105 complex. (B) Addition of purified Sec23p monomer.

a large fraction of pure Sec23p monomer. Thus, another component present in the Sec23p complex becomes limiting when Sec23p is overexpressed. Second, Sec23p and a 105-kDa protein coprecipitate from crude lysates with either anti-native Sec23p antibodies or anti-p105 antibody (L. Hicke, T. Yoshihisa, and R. Schekman, unpublished data). Though Sec23p and p105 are physically associated, Sec23p need not be added in multimeric form to restore transport in a *sec23-1* lysate because monomeric Sec23p exhibits significant activity. Crude membranes and cytosol from *sec23-1* probably contain wild-type p105. If a Sec23p-p105 association is required to promote ER to Golgi transport, monomer Sec23p may recruit p105 from the *sec23* lysate components added to the transport reaction. Purified Sec23p-p105 complex has a threefold higher specific activity than monomer Sec23p obtained from the same column. Hence, either p105 is present in limiting quantities or recruitment of p105 by Sec23p is rate limiting in the reaction.

We have shown that p105, like Sec23p, is directly involved in budding from the ER membrane by inhibiting a budding reaction with p105 antisera. Budding in these inhibited reactions is restored by the addition of purified Sec23p-p105 complex but not by Sec23p monomer. As p105 is not encoded by any of the previously identified *SEC* genes required for protein transport, copurification of p105 with Sec23p has allowed the biochemical identification of a protein required for ER to Golgi transport that may have been difficult to obtain by genetic screening for conditional lethal mutants (Kaback *et al.*, 1984; Harris and Pringle, 1991). We expect that null and conditional mutants in the gene encoding p105 will demonstrate a requirement for p105 in ER to Golgi transport in vivo.

A specific function for Sec23p or the Sec23p-p105 complex during intracellular protein transport has not yet been determined. Morphological and biochemical evidence indicate a role for the Sec23 protein in vesicle budding. Formation of a yeast ER to Golgi transport intermediate in vitro requires Sec23p (Rexach and Schekman, 1991). In addition, *sec23* mutant cells incubated at the nonpermissive temperature block the accumulation of 50-nm vesicles identified by electron microscopy as an intermediate compartment between the ER and the Golgi (Kaiser and Schekman, 1990). Finally, immunocytochemical analysis of a mammalian Sec23p homologue in pancreatic acinar cells shows specific localization of the protein to tubular extensions of the transitional ER cisternae, as well as to a population of vesicles and cytoplasm present in the zone between ER and Golgi (Orci *et al.*, 1991).

Several alternative possibilities may account for the participation of a cytosolic peripheral membrane protein, such as Sec23p, in the formation of a transport vesicle. These include potential roles 1) as part of a structural coat on the vesicle, 2) in the recognition or establishment of budding competent ER membrane, or 3) in the mechanical action of pinching or releasing a newly formed bud from the ER membrane. Purification and characterization of each of the cytosolic and peripheral membrane proteins required in vesicle budding will allow a more precise determination of the role of the Sec23p-p105 complex. The biochemical complementation assay used here together with more traditional fractionation and reconstitution approaches should lead to a complete resolution of the essential proteins.

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