Cytosolic Sec13p Complex Is Required for Vesicle Formation from the Endoplasmic Reticulum in Vitro

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Abstract. The *SEC13* gene of *Saccharomyces cerevisiae* is required in vesicle biogenesis at a step before or concurrent with the release of transport vesicles from the ER membrane. *SEC13* encodes a 33-kD protein with sequence homology to a series of conserved internal repeat motifs found in β subunits of heterotrimeric G proteins. The product of this gene, Sec13p, is a cytosolic protein peripherally associated with membranes. We developed a cell-free Sec13p-dependent vesicle formation reaction. Sec13p-depleted membranes and cytosol fractions were generated by urea treatment of membranes and affinity depletion of a Sec13p-dihydrofolate reductase fusion protein, respectively. These fractions were unable to support vesicle formation from the ER unless cytosol containing Sec13p was added. Cytosolic Sec13p fractionated by gel filtration as a large complex of about 700 kD. Fractions containing the Sec13p complex restored activity to the Sec13p- dependent vesicle formation reaction. Expression of *SEC13* on a multicopy plasmid resulted in overproduction of a monomeric form of Sec13p, suggesting that another member of the complex becomes limiting when Sec13p is overproduced. Overproduced, monomeric Sec13p was inactive in the Sec13p-dependent vesicle formation assay.

VECTORIAL transport of newly synthesized proteins from the endoplasmic reticulum to the Golgi apparatus is achieved by the formation of transport vesicles from the ER and subsequent transport, targeting and fusion of vesicles with the Golgi membrane (37). In the yeast *Saccharomyces cerevisiae*, at least 22 genes required for vesicle-mediated ER-Golgi transport have been identified genetically (8, 26, 28, 30, 31, 34, 43). *SEC13* is one of these genes, as evidenced by the accumulation of core-glycosylated secretory proteins in *sec13* temperature-sensitive mutants at the restrictive temperature (33). *SEC13* can be further defined as one of a class of genes required for the formation of mature transport vesicles from the ER membrane. At the restrictive temperature, *sec13* cells accumulate ER membrane, but not the intermediate vesicles seen in mutants defective in the later step of vesicle consumption. Furthermore, *sec13* interacts genetically with *sec12*, *sec16*, and *sec23*, which are also defective in vesicle biogenesis (21).

A number of *SEC* gene products have been characterized at the molecular level, and the biochemical functions of Sec proteins are beginning to be elucidated using in vitro assays that reconstitute ER to Golgi transport (2, 3, 39, 52) or the subreaction of vesicle formation and release from the ER (6, 38). The first Sec protein purified by this assay was the product of the *SEC23* gene, which was isolated by biochemical complementation of the *sec23* temperature-sensitive cytosolic defect (16). Sec23p is an 85-kD cytosolic protein that co-purifies with a 105-kD protein also required for ER to Golgi transport and is designated Sec24p (17). A second gene blocked in export from the ER, *SEC12*, encodes a transmembrane glycoprotein resident in ER and Golgi membranes (5, 27). *SAR1*, initially isolated as a multicopy suppressor of the *sec12* mutant phenotype, encodes a 21-kD GTPase with homology to the Ras superfamily of GTPases (28) Sar1p recruitment to membranes is Sec12p-dependent and required for vesicle formation in vitro (6).

Because *SEC13* is required for vesicle formation in vivo and interacts genetically with other *SEC* genes that have been characterized biochemically, we undertook the molecular and biochemical characterization of the *SEC13* gene product. *SEC13* encodes a 33-kD cytosolic protein, peripherally associated with cellular membranes. The sequence of *SEC13* shares homology, within a series of internal repeats, with a number of proteins including the β subunit of heterotrimeric G proteins (7, 12). We developed an in vitro assay for vesicle formation and release from the ER that is dependent on the addition of cytosolic Sec13p. Using this assay, we show that Sec13p is included in a large complex of about 700 kD, which is active in promoting vesicle formation in vitro.

Materials and Methods

Strains, Materials, and General Methods

Yeast strains used in this study are RSY607 (*MATα, ura3-52, leu2-3,112,*

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pep4::URA3), RSY255 (*MATa, ura3-52, leu2-3, 112*; D. Botstein, Stanford University Medical School), RSY265 (*MATα, sec13-1, ura3-52, his4-619*) (21), RSY266 (21); (*MATa, sec13-1, ura3-52, his4-619*), RSY637 (*MAT a/α, ura3-52/ura3-52, leu2-3,112/leu2-3,112, sec13::URA3/SEC13*), RSY742 (pNS1353, *MATα, ura3-52, leu2-3,112, sec13::URA3*), RSY812 (pCK1313; *MATα, leu 2-3, 112*). The protease-deficient wild-type strain RSY607 was constructed by integrative disruption of the *PEP4* gene. Wild-type RSY255 cells were transformed with an Eco RI-Xho I fragment of pTS15 (1), carrying the *PEP4* gene disrupted by *URA3*. The *pep4* deletion phenotype of transformants was verified by a defect in carboxypeptidase Y maturation detected by APNE plate assay (19) and immunoblotting using anti-carboxypeptidase Y antibody (49). Plasmid vectors used were pRS306, 315, or 316 (46), where CEN-containing or integrating plasmids were required. Plasmids YEp352 and YIp352 (18) are a high copy number 2μ and integrating plasmid, respectively. Yeast culture, genetic manipulations and molecular techniques were as described by either Sherman et al. (45) or Sambrook et al. (40). Materials were obtained from Sigma Chemical Company (St. Louis, MO) unless stated otherwise.

Gel electrophoresis was performed according to the Laemmli SDS-PAGE method (23), using 12% or 12.5% polyacrylamide. Electrophoresed proteins were visualized by silver staining (25). Transfer of proteins from SDS-PAGE to nitrocellulose was performed as described (6, 51). Affinity-purified anti-Sec13p antibody was used at 1:300 dilution, affinity purified anti-Sec23p antibody (provided by T. Yoshihisa, this laboratory) at a dilution of 1:2,500 and anti-Sar1p antibodies (provided by C. Barlowe, this laboratory) at 1:1,500 dilution. Secondary antibodies were either goat-anti-rabbit IgG, conjugated to HRP (Amersham Corp., Arlington Heights, IL), at a dilution of 1:5,000 in TBS, or ^{125}I-protein A (Amersham Corp.), at a dilution of 1:2,500. Filter-bound antibodies were then detected by peroxidase-catalyzed chemiluminescence (ECL kit, Amersham Corp.), or by densitometry using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager.

Cloning and Sequence Determination of SEC13 Wild-Type and Mutant Alleles

The *SEC13* gene was isolated from a library of *S. cerevisiae* genomic sequences in the multicopy shuttle vector YEp13 (29). Insert sequences from a plasmid that complemented the temperature sensitive mutation *sec13-1* were subcloned into the 2μ, *URA3* vector YEp352. The smallest complementing subclone contained a 1.9-kb SmaI-SacI genomic fragment (pCK1313). Deletion derivatives were produced from the SacI end of the fragment by digestion of linear plasmid DNA with exonuclease III and S1 (15). Using plasmids with different deletion endpoints as the templates, the minimum complementing region was sequenced by the Sanger method (41) following the protocol for the Sequenase kit (USB, Cleveland, OH). Sequencing of the complementary strand was performed using synthetic oligonucleotide primers derived from the sequence of the first strand. To test for linkage between the cloned sequences and the *SEC13* locus, a 2.0-kb SmaI to HindIII fragment, cleaved by XbaI was used to direct the integration of YIp352 (containing a *URA3* marker gene) to the locus of the cloned DNA by transformation of RSY255 (*α, ura3-52, leu2-3, 112*). Two transformants were crossed to RSY266 (*a, sec13-1, ura3-52, his4-619*) and 12 tetrads were dissected and analyzed for Ts$^+$ and Ura$^+$ phenotypes.

The *sec13* mutations were mapped by marker rescue recombination with plasmid-borne *SEC13*. A *sec13* mutant to be mapped was transformed with each of the plasmids pCK1313, Δ5.7, Δ6.4, Δ6.1, Δ7.1, and Δ8.2 (Fig. 1). Because these plasmids carry incomplete copies of *SEC13* the transformants remain temperature sensitive. To stimulate mitotic recombination, transformant cultures were exposed briefly to shortwave UV such that greater than 50% of the cells survived. Temperature resistant recombinants were scored after plating at 37°C. Each of the mutant alleles was cloned by transformation of the mutant strains with plasmid pCK1313 containing a gap within the *SEC13* sequences produced by cutting with *MscI* (Fig. 2). Expression of the mutant alleles from the high copy number plasmid resulted in less severe Ts$^-$ phenotypes when compared to strains carrying the chromosomal mutant allele. Using this reduced temperature sensitive phenotype as a guide, transformants containing the mutant alleles on the plasmid were identified. The base changes responsible for the mutations were obtained by sequencing the COOH-terminal third of the mutant allele on the plasmid using synthetic oligonucleotide primers and the Sanger method, as described above.

SEC13 Disruption

To disrupt the *SEC13* gene, SmaI-BglII fragment and ClaI-HindIII fragments from *SEC13* were inserted in inverted orientation into the integrating vector pRS306 (*URA3*) to produce pCK1316. pCK1316 was cut with HindIII and transformed into a diploid *ura3-52, leu2-3,112* strain. Homologous recombination replaced one of the *SEC13* alleles with a disrupted copy of the gene. This procedure is referred to as the γ transformation method for gene disruption (46). The heterozygous diploid was sporulated and progeny were tested for growth and Ura$^+$ phenotypes.

Construction of SEC13:DHFR Hybrid Plasmids and Strains

To construct a full-length fusion of *SEC13* to the mouse dihydrofolate reductase (DHFR)[1] gene, pCK1313, containing the original clone of *SEC13* in

1. *Abbreviations used in this paper*: B88, Buffer 88; B88CB, Buffer 88 column buffer; DHFR, dihydrofolate reductase; MSS, medium speed supernatant; MTX, methotrexate.

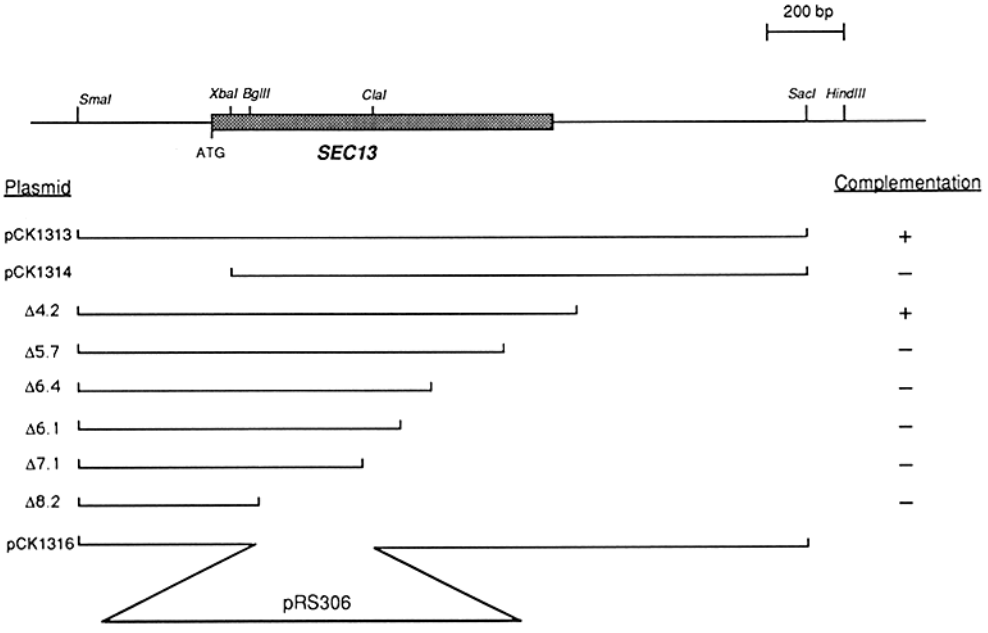

Figure 1. Physical map of *SEC13*. The plasmid subclones of the *SEC13* locus in the high copy vector YEp352 are shown with their ability to complement the *sec13-1* mutation. The hatched region represents the predicted open reading frame. The plasmid pCK1316, used for disruption of the *SEC13* gene, appears in the integrated state. (The size of the pRS306 plasmid sequence is not to scale.)

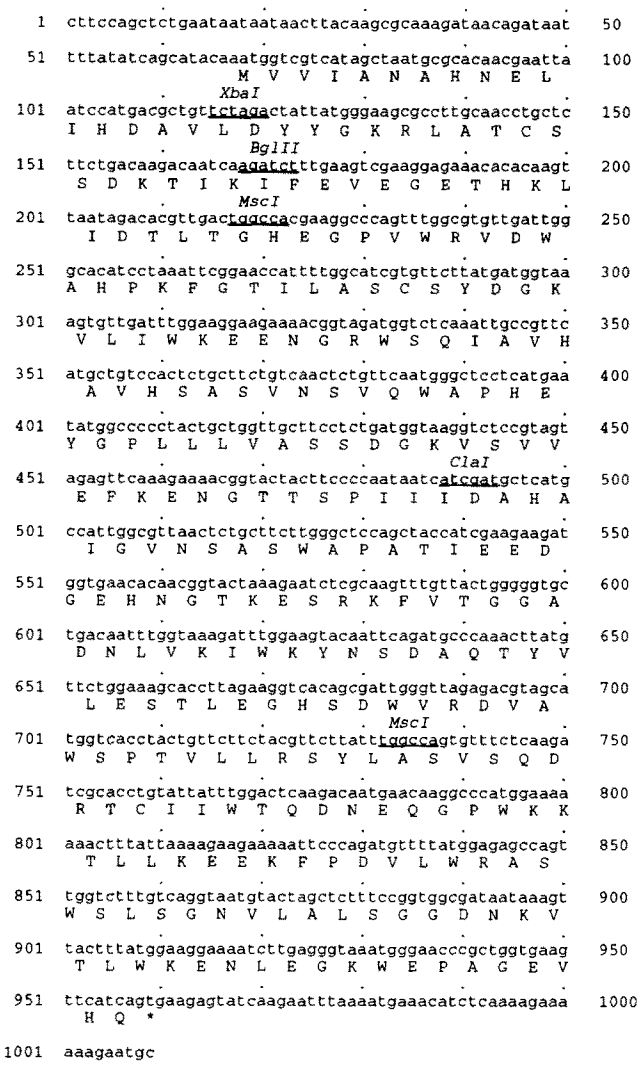

Figure 2. The sequence of the *SEC13* gene. The predicted amino acid sequence is shown in single-letter code. Restriction sites relevant to experiments reported in this paper are underlined. These sequence data are available from EMBL under accession number LO5929.

YEp352 (*URA3*, 2μ) was digested with exonucleaseIII (15) to yield pNS1339. This plasmid contains the entire coding region of *SEC13* with the stop codon replaced by polylinker sequences of the vector. A HindIII fragment containing the entire coding region of mouse *DHFR* from pRFY2 (generously provided by Martin Eilers, Biozentrum, Basel) was subcloned into a HindIII site in the polylinker 3′ of *SEC13* in pNS1339 to generate pNS1350. This plasmid encodes a hybrid protein containing the entire coding region of *SEC13* fused to the entire coding region of *DHFR* connected by a 9-amino acid linker with the following sequence: ACKLGGSGI (single-letter code). The *SEC13:DHFR* fusion was subcloned as an EcoRI-HindIII fragment into YEp351 (*LEU2*, 2μ) to generate pNS1353. Two low copy number vectors containing the fusion gene were generated to test the ability of the hybrid protein to complement a temperature sensitive *sec13* allele and the *sec13* null allele. The EcoRI-HindIII fragment containing the *SEC13:DHFR* fusion was subcloned into pRS316 (*CEN6, URA3*) and pRS315 (*CEN6, LEU2*) to generate pNS1351 and pNS1352, respectively.

To test whether the hybrid protein could restore *SEC13* function to a strain carrying the *sec13-1* temperature sensitive mutations, RSY265 (*sec13-1, ura3-52, his4-619*) was transformed with pNS1350 (2μ, *SEC13:DHFR, URA3*) or pNS1351 (*CEN6, SEC13:DHFR, URA3*). Ura$^+$ transformants were tested for growth at both 30°C and 37°C. The ability of *SEC13:DHFR* to complement a null allele of *SEC13* was tested by transforming RSY637 (*MATa/α*, ura 3-52/ura3-52, leu2-3,112/leu2-3,112, sec13::URA3/SEC13) with pNS1352 (*CEN6, SEC13:DHFR, LEU2*) or pNS1353 (2μ, *SEC13: DHFR, LEU2*). Leu$^+$ transformants were sporulated and Leu$^+$ Ura$^+$ spores were tested for growth at both 30 and 37°C. Transformants of RSY637 with pNS1353 were sporulated and Ura$^+$, Leu$^+$ spores selected to yield RSY742 (pNS1353, *MATa, ura3-52, leu2-3,112, sec13::URA3*).

Preparation of Antibodies to the Sec13 Protein

Sec13p antiserum was elicited against a hybrid protein composed of a COOH-terminal domain of Sec13p fused to Staphylococcal protein A. A BglII-SacI fragment containing the COOH-terminal 263 amino acids of *SEC13* was inserted into the protein A fusion vector pRIT32 (32). Hybrid protein was prepared from *E. coli* extracts and antibody to this protein produced in rabbits, as previously described (13). Antiserum was affinity purified using a β-galactosidase-Sec13p hybrid protein constructed by fusing the same BglII-SacI fragment of *SEC13* to the *lacZ* gene in the pEX2 vector (48). The hybrid protein was isolated and used for affinity purification of the antibody as described (13).

Yeast Cell Fractionation

Wild-type cells and cells expressing *SEC13* from a multicopy plasmid were grown to exponential phase in YPD (2% yeast extract, 1% peptone, 2% glucose). A culture of 100 ml containing ~2 × 10^9 cells was transferred by centrifugation to 50 ml of 0.1 M Tris sulfate, pH 9.4, 28 mM β-mercaptoethanol and incubated at 25°C for 10 min. The cells were then transferred to 10 ml of spheroplasting buffer (2% yeast extract, 1% peptone, 0.7 M sorbitol, 10 mM Tris, pH 8.0) and 3,700 units of lyticase (44) were added. After incubation at 30°C for 60 min, spheroplasts were diluted into 100 ml of YPD containing 0.7 M sorbitol as an osmotic support and then incubated at 30°C for 60 min with gentle shaking, to regenerate spheroplast metabolic activity. The culture was chilled to 4°C, washed with 0.7 M sorbitol, 0.1 M NaCl, 10 mM Tris, pH 8.0, 5 mM $MgCl_2$, and stored as a cell pellet at −70°C. Acid-washed glass beads (0.3 gm) and 0.5 ml of lysis buffer (20 mM MES, pH 6.5, 0.1 M NaCl, 5 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin) were added to the pellet. Cells were lysed by vortex mixing at high speed for 30 s, four times. This treatment resulted in essentially complete cell lysis as judged by light microscopy of the extract. The extract was exposed to different chemical treatments by mixture of 0.1 ml of extract with an equal volume of lysis buffer containing the reagent. Final reagent concentrations were 1% Triton X-100, 2.5 M urea, 0.1 M sodium carbonate (pH 11.5), 0.5 M NaCl. Incubations were continued for 60 min on ice then the extracts were separated into soluble and particulate fractions by centrifugation at 100,000 × *g* for 30 min at 4°C in the ultracentrifuge (Beckman Instruments, Palo Alto, CA). Pellets were dissolved in 250 μl of sample buffer (80 mM Tris, pH 6.8, 2% SDS, 0.1 M DTT, 10% glycerol, and 0.01% bromphenol blue) and 50 μl of a fivefold concentrate of sample buffer was added to the supernatant fractions. Samples were heated to 95°C for 3 min, further diluted threefold in Laemmli sample buffer, resolved on a 12% polyacrylamide gel, transferred to nitrocellulose, incubated with anti-Sec13p antibody, and visualized by chemiluminescence, as described above.

Preparation of Membrane and Cytosol Fractions

Microsomes were prepared from osmotically lysed RSY607 cells using methods described previously (3).

Cytosols were prepared by liquid nitrogen lysis (9, 47) of RSY607, RSY742, or RSY812. Cells were grown to 10 OD_{600} U/ml, washed twice with distilled water, resuspended as a thick paste (1,500–2,000 OD_{600} units/ml) in Buffer 88 (B88; 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc, 20 mM Hepes, pH 6.8), and frozen as drops in liquid nitrogen. Frozen cells were lysed in the presence of liquid nitrogen in a 1-liter stainless steel blender (Waring, New Hartford, CT) by blending at high speed for 10 min. As the lysate thawed, B88 containing protease inhibitor mix (0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) was added at the proportion of 1 ml B88 per g cells. The lysate was clarified at 18,000 *g* in a Sorvall (Du Pont, Wilmington, DE) SS-34 rotor (12,000 RPM, 15 min). The supernatant from this centrifugation was clarified further at 150,000 × *g* in a Beckman 45Ti rotor (40,000 rpm, 60 min), frozen in liquid nitrogen, and stored at −80°C. Before use, cytosol fractions were thawed and clarified at 300,000 *g* (90,000 rpm, 60 min, Beckman TLA-100.3 rotor). Glycerol, DTT, ATP, and GTP were added so that the final cytosol preparation was suspended in B88 column buffer (B88CB; B88 containing 10% glycerol, protease inhibitor mix, 0.5 mM DTT, 0.25 mM ATP, and 0.25 mM GTP).

To prepare a Sec13p-enriched cytosol fraction, powdered ammonium sulfate was added gradually, to a final concentration of 30%, to cytosol (prepared by liquid nitrogen lysis of RSY607 cells and clarified at 300,000 ×

g), and stirred, on ice, for 30 min. Precipitated protein was collected by centrifugation at 17,000 g (12,000 rpm, 15 min, Sorvall SS-34 rotor) and resuspended in a minimal volume of B88 to a final concentration of 5–10 mg/ml.

Methotrexate-Agarose Column Chromatography

Methotrexate coupled to agarose beads (MTX-agarose), was poured as a 1-ml column and washed with B88. Before the first use, nonspecific binding sites were blocked by circulating 60 mg wild-type cytosol through the column, and then washing extensively (>20 column volumes) with B88 containing 10% glycerol. RSY742 cytosol (150 mg) was applied to the MTX-agarose column at a flow rate of 4 ml/h. The flow-through fraction was "chased" with 1 ml of B88CB, collected, and frozen in liquid nitrogen. The column was then washed with 10 column volumes of B88CB containing 1 M KCl at a flow rate of 10 ml/h. The collected KCl eluate was dialyzed 2 h against B88CB, and concentrated to one-half the original volume using a Centriprep-10 device (Amicon, Danvers, MA), centrifuged at 5,000 rpm in a Sorvall GSA rotor. The MTX-agarose column was washed with 10 column volumes of B88CB. Proteins bound specifically to MTX were eluted by applying 1.5 column volumes of B88CB containing 50 mM MTX (adjusted to pH 7). After incubating 1–3 h, the column was eluted at 10 ml/min and washed with 10 column volumes of B88CB containing 0.5 mM MTX. All fractions were frozen in liquid nitrogen and stored at −80°C.

The MTX-agarose column was stripped and regenerated before the next use by washing with 5 column volumes urea wash (2 M urea, 0.2 M NaCl, 1% Triton X-100, 0.1 M Tris-HCl, pH 7.5, 2 mM NaN_3), followed by extensive washing with B88, incubation with B88 containing 50 mM MTX for 12 h, and further washing with B88.

Gel Filtration of Cytosol

Cytosol for gel filtration chromatography was prepared by liquid nitrogen lysis, as described above. Cytosol (0.5 ml) was applied to a 21-ml Superose 6 FPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ), equilibrated in B88CB. The column was run at a flow rate of 0.25 ml/min and fractions of 0.5 ml were collected. The protein concentration of fractions was determined by the modified Lowry method (42). Fractions were analyzed for Sec13p abundance by immunoblotting using affinity-purified anti-Sec13p antibody followed by ^{125}I-protein A, as described above. Vesicle formation activity of column fractions from RSY607 cytosol was assayed by adding 25 μg protein per 50 μl reaction from alternating fractions across the included volume of the column to the vesicle formation assay, substituting purified Sar1p and a high salt DEAE eluate for the KCl eluate (see below). Activity of fractions from gel filtration of RSY812 cytosol was determined by adding 10 μl of each even numbered fraction (10–15 μg protein) to the same assay.

Vesicle Formation Assay

A Sec13p-dependent assay for vesicle formation and release from the ER was developed by adapting a two-stage reaction that uses an ER-enriched membrane fraction from yeast (6, 52). The first stage reconstitutes translocation of ^{35}S-labeled pre-pro-α factor into ER-enriched microsomes and is marked by core glycosylation of pro-α factor. Each translocation reaction contained 2.5 μg membranes, 1.5 μl ^{35}S-pre-pro-α factor, 1 mM ATP, and an ATP-regenerating system consisting of 40 mM creatine phosphate and 0.2 mg/ml creatine phosphokinase. Translocation reactions were adjusted to 12 μl each with B88, and incubated 30 min at 10°C. After translocation, Sec13p was extracted from membranes by adding an equal volume of 5 M urea in B88 and incubating on ice for 30–45 min. Extracted membranes were washed twice with 2 ml B88, each followed by centrifugation for 10 min at 25,000 g (in 1.5-ml Eppendorf tubes, 15 K rpm, SS-34 rotor) and resuspended in B88.

The second stage of the assay permits the cytosol- and ATP-dependent release of protease-protected ^{35}S-pro-α factor into a supernatant fraction separated from ER by differential centrifugation. In the second stage, 2.5 μg microsomes were combined with Sec13p-depleted cytosol provided by one of two mixtures. The first type of Sec13p-depleted cytosol was a mixture of flow-through and KCl eluate fractions of RSY742 cytosol fractionated on MTX-agarose, as described above. The MTX flow through and KCl eluate were combined in a ratio reflecting the proportion of each fraction in the original cytosol (about a 10:1 ratio of flow-through: eluate), and 200 μg was used in each reaction. We later found that the KCl eluate fraction could be replaced with 0.5 μg purified Sar1p (3a) and 7 μg of a high-salt DEAE eluate (kindly provided by M. Hosobuchi, this laboratory). The high-salt DEAE eluate was prepared by applying wild-type cytosol (1.7 g; prepared by liquid nitrogen lysis and clarified at 100,000 g) to a 90-ml DEAE Sepharose FF column (Pharmacia LKB Biotechnology) equilibrated in B88 containing 10% glycerol, 0.5 M KOAc, and 0.5 mM PMSF. The column was washed with this buffer until the protein concentration, measured as absorbance at 280 nm, was zero. The column was then washed with B88 containing 10% glycerol, 0.75 M KOAc, and 0.5 mM PMSF, and fractions of 8 ml were collected. The protein concentration of each fraction was measured by the Bradford assay (Bio-Rad, Richmond, CA), and fractions containing more than 0.75 mg/ml protein were combined and dialyzed against 2 liters of B88 containing 10% glycerol and 0.5 mM PMSF. The dialyzed eluate was concentrated twofold by dehydration in dry Aquacide I (Calbiochem-Novabiochem Corp., LaJolla, CA) and frozen in liquid nitrogen. The high-salt DEAE eluate is enriched in Sec23p and Sec24p (17), but devoid of Sec13p, which does not bind to DEAE Sepharose in 0.5 M KOAc. In the reaction using purified Sar1p and the high-salt DEAE eluate, 100 μg of MTX flow-through fraction was used.

Reactions also contained 50 mM GDP-mannose, 0.2 mM GTP, 1 mM ATP, and an ATP-regenerating system consisting of 40 mM creatine phosphate and 0.2 mg/ml creatine phosphokinase. The total reaction volume was adjusted to 50 μl by adding B88. Conditions were adjusted so that the addition of unfractionated cytosol fell within the linear range of the assay. Reactions were incubated 30 min at 30°C and terminated by transfer to ice. An aliquot of total reaction mixture was held on ice while the remaining reactions were centrifuged at 25,000 g (15,000 rpm, Sorvall SS-34 rotor, 10 min) to generate the medium speed supernatant (MSS) fractions. The total and MSS fractions were each treated on ice for 15 min with 0.3 mg/ml trypsin, followed by soybean trypsin inhibitor (1.2 mg/ml for 10 min). Protease-treated reactions were solubilized by adding SDS to 1% and heating at 95°C for 5 min. Core-glycosylated ^{35}S-pro-α factor was quantified in the total and MSS fractions by precipitation with concanavalin A (ConA)–Sepharose, as described (2). The efficiency of vesicle formation was expressed as the percentage of protease protected ConA-precipitable radioactivity (cpm) in the MSS relative to protease-protected ConA-precipitable radioactivity (cpm) in the total fraction. The efficiency of vesicle formation in a reaction using extracted membranes and unfractionated (Sec13p-containing) cytosol was 25–30%.

Results

Cloning and Sequencing of the SEC13 Gene

The *SEC13* gene was isolated by screening a genomic library of *S. cerevisiae* in YEp13 for complementation of the temperature sensitive growth defect of *sec13-1*. A complementing plasmid was subcloned and a 2.0-kbp HindIII to SmaI fragment that on a centromere plasmid complemented the *sec13-1* mutation was identified. To test whether this fragment contained the authentic *SEC13* gene, the fragment was inserted into the nonreplicating plasmid YIp352 and was directed to integrate into the homologous chromosomal locus in *S. cerevisiae* by cleavage within the insert sequence before to transformation. Transformants were crossed with *sec13-1* cells and sporulated. 12 tetrads were dissected and each dissection resulted in two Ts^+,Ura^+ and two Ts^-,Ura^- spore clones showing linkage of the integrated plasmid sequences to the *SEC13* locus.

The *SEC13* locus was defined further within the HindIII-SmaI fragment by subcloning and deletion analysis to identify the minimal fragment necessary for complementation of the Ts^- growth defect (Fig. 1). The DNA sequence of this segment revealed a single open reading frame encoding 297 amino acid residues (Fig. 2) and predicting a hydrophilic protein with a molecular weight of 33 kD. The peptide sequence of *SEC13* was used to search the PIR protein data base using the FASTA algorithm and no significant similarities to known proteins were found. However, examination of the *SEC13* sequence revealed an internal repeated structure

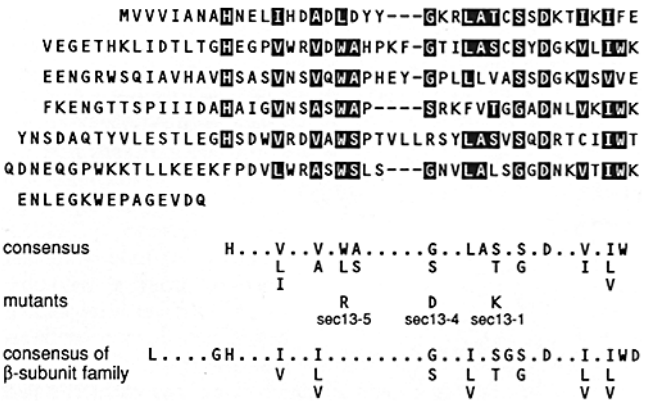

Figure 3. SEC13 contains a repeated, conserved motif. The *SEC13* sequence is displayed to align the internal repeated sequence elements. The consensus derived from this alignment is compared to a consensus sequence derived from a number of proteins related to the β subunit of transducin (7). The positions of the amino acid substitutions in *SEC13* are shown relative to this alignment. The *sec13-1* mutation is in the second to last repeated element, and the *sec13-4* and *sec13-5* mutations are in the last element.

(Fig. 3). This same peptide motif is tandemly repeated in a number of different proteins, the best known of which is the β subunit of transducin (10, 12). Other proteins containing this repeated motif are divergent in function and thus, the structural or functional significance of the repeated motif is not yet clear.

Mapping and Sequencing of sec13 Alleles

To investigate the relationship between the repeated peptide motif and the function of *SEC13*, the mutations responsible for the four temperature-sensitive alleles of *SEC13* were identified. The alleles *sec13-1, sec13-3,* and *sec13-4* were obtained by nitrous acid mutagenesis (34) and *sec13-5* was obtained by UV mutagenesis (Wuestehube, L., personal communication). *sec13-1, sec13-3,* and *sec13-5* are the most restrictive alleles and do not permit growth above 30°C. The *sec13-4* allele is less restrictive and will allow growth at temperatures up to 34°C. Each of the mutations was mapped by marker rescue (11), evaluating the ability of a set of plasmids containing noncomplementing fragments of the *SEC13* gene to recombine with a given chromosomal *sec13* mutation to produce a wild-type phenotype. *sec13-1* and *sec13-3* recombined with pCK1314 and Δ5.7 (Fig. 1) but not with the other deletions. Mutations in *sec13-4* and *sec13-5* recombined with pCK1314 but not with the COOH-terminal deletion plasmids. Thus, these mutations map to the most distal COOH-terminal segment. The mutant alleles were recovered from the chromosome by repair of gapped plasmid sequences with chromosomal information (36). The sequence of the mutation bearing plasmids revealed a single base substitution corresponding to each mutant allele: *sec13-1* and *sec13-3* have the same G-to-A change at position 738 changing Ser (224) to Lys (these mutants are probably siblings because they were obtained from the same mutagenized culture), *sec13-4* has a G-to-A change at position 864 changing Gly(266) to Asp, and *sec13-5* has a T-to-A change at position 851 changing Trp (262) to Arg. These three mutations alter conserved positions of the repeated motif emphasizing the importance of these amino acid sequence elements for *SEC13* function (Fig. 3).

SEC13 Null Is Inviable

The existence of recessive temperature sensitive alleles of *SEC13* implies that this gene is essential. The remaining possibility that *SEC13* was required for growth only at high temperature was eliminated by the demonstration that a disruption allele of *SEC13* was inviable at 24°C. A 320-bp fragment (BglII-ClaI) internal to the *SEC13* coding sequence was replaced with the nonreplicating plasmid pRS306 to generate pCK1316 (Fig. 1). The *SEC13* locus was replaced with this disruption allele by the γ-transformation method, selecting for Ura$^+$ transformants of a *ura3-52* diploid (46). Upon sporulation, the transformed diploids carrying a disrupted allele of *SEC13* gave rise to two viable Ura$^-$ spore clones and spores that remained as single cells at 24°C (20 tetrads dissected). *SEC13* is therefore required for germination and/or growth at 24°C.

Sec13p Is a Peripheral Membrane Protein

Antibodies reactive with Sec13p were produced using a hybrid protein composed of *Staphylococcus* protein A fused to most of Sec13p. This serum was affinity purified using a hybrid protein encoded by a fusion of the same segment of the *SEC13* gene linked to *E. coli* LacZ. On immunoblots, the purified antibodies recognized a prominent band with the expected apparent molecular weight of ~33 kD (Fig. 4, lane *4*). This protein band was of greater intensity in extracts of cells carrying the *SEC13* gene on the high copy number plasmid (pCK1313) showing that the 33-kD band was indeed the *SEC13* gene product (Fig. 4, lane *1*).

Sec13p behaved as a peripheral membrane protein in cell fractionation studies. In wild-type cells, most Sec13p sedimented in a particulate fraction (Fig. 4, lanes *5* and *6*). Treatment of the extract with the nonionic detergent Triton X-100 released most of the Sec13p into the soluble fraction (Fig. 4, lanes *7* and *8*) suggesting that, in untreated extracts, Sec13p partitions into the particulate fraction by association with intracellular membranes. Some of the Sec13p was also released into the soluble fraction by agents that disrupt protein interactions. Treatment of the extract with carbonate buffer, pH 11.5, released most of Sec13p into the soluble fraction (Fig. 4, lanes *9* and *10*) and either 2.5 M urea or 0.5 M NaCl solubilized some of the protein (Fig. 4, lanes *11–14*). These data indicate that Sec13p is not an integral membrane protein. Given that Sec13p has no signal sequence, we infer that it is a cytosolic, peripheral membrane protein. In extracts from cells overproducing Sec13p, much of the excess protein was in the soluble rather than particulate fraction suggesting that the putative membrane association sites for Sec13p are saturated by overproduction of the *SEC13* gene product (Fig. 4, lanes *2* and *3*).

Affinity Depletion of a Sec13p-Dihydrofolate Reductase Hybrid Protein

To study the role of Sec13p in vesicle formation, we wished to develop a Sec13p-dependent in vitro reaction reconstituting vesicle formation and release from the ER. One approach that was developed for the assay of Sec23p relied on biochemical complementation of *sec23-1* mutant membrane and cytosolic components incubated at a restrictive temperature (16). A similar approach using *sec13* mutant membranes and/or cytosol was unsuccessful: *sec13* mutant components were no more thermolabile than wild-type components. We

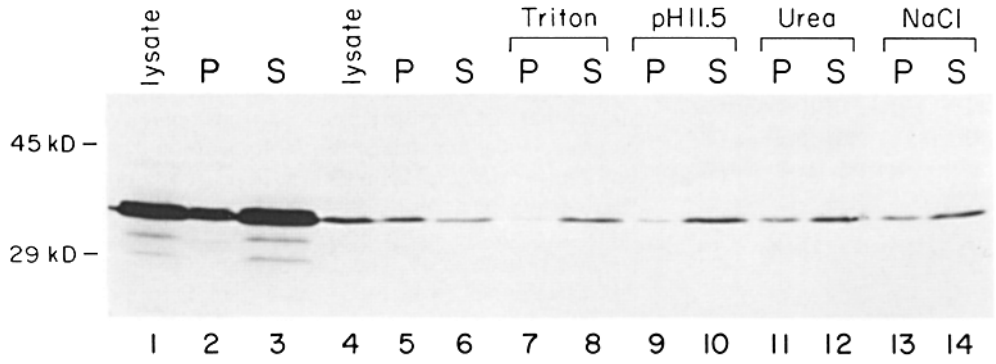

Figure 4. The intracellular distribution of Sec13p. Cell lysates were divided into supernatant (*S*) and pellet (*P*) fractions after centrifugation at 100,000 *g*. (Lanes *1–3*) Total lysate and fractions of a strain carrying the high copy *SEC13* plasmid pCK1313. (Lanes *4–14*) Total lysate and fractions from a wild-type strain. Extracts were treated before centrifugation with either 1% Triton X-100, 0.1 M sodium carbonate (pH 11.5), 2.5 M urea, or 0.5 M NaCl. Proteins were resolved by SDS-PAGE, and immunoblot analysis was performed using affinity purified anti-Sec13p antibody and chemiluminescence detection.

then developed an alternative approach involving the physical depletion of Sec13p from membrane and cytosol fractions. Sec13p was extracted from membranes by treatment with urea, and removed from cytosol by affinity depletion of a Sec13p-DHFR fusion protein.

SEC13 was fused to the mouse dihydrofolate reductase (*DHFR*) gene to exploit the high-affinity binding of DHFR protein to an inhibitor, methotrexate (14, 22). A *SEC13:DHFR* fusion was constructed by subcloning the entire coding region of mouse *DHFR* into a plasmid containing *SEC13* lacking only the stop codon. This *SEC13:DHFR* fusion contained on either a high copy number plasmid (pNS1352) or a centromere-containing, low copy number plasmid (pNS1353) complemented the temperature-sensitive growth defect of *sec13-1* at 37°C. To test whether the *SEC13:DHFR* fusion was able to complement a *sec13* null defect, diploid strain RSY 637, heterozygous for a *SEC13* disruption (*MAT α/a, ura 3-52/ura3-52, leu2-3,112/leu2-3,112, sec13::URA3/SEC13*) was transformed with pNS1352 or pNS1353. Upon sporulation, viable, *SEC13:DHFR*-containing (Ura^+) spores were obtained only from the diploid transformed with the high copy number plasmid. The strain RSY742 (pNS1353, *MATα, ura3-52, leu2-3,112, sec13::URA3*), was isolated from these progeny and expressed Sec13p only in the form of a Sec13p-DHFR hybrid, as described below. RSY742 cells grew best at temperatures from 24 to 34°C, but grew slowly at 37°C.

SDS-PAGE and immunoblot analysis of a cell lysate from RSY742 cells using affinity-purified anti-Sec13p antibody detected the presence of a single Sec13p-reactive species that migrated more slowly than native Sec13p (not shown). This protein of approximately 55 kD is the size expected for a hybrid protein of Sec13p (33 kD) with DHFR (21.6 kD). Cytosol (300,000 *g* supernatant fraction) prepared from RSY742 cells and fractionated on a gel filtration column revealed a high molecular weight complex containing Sec13p-DHFR and an abundant monomeric form of Sec13p-DHFR. A similar distribution was seen with cytosol from a strain carrying *SEC13* on a 2μ plasmid (see Fig. 7 *b* below).

To remove cytosolic Sec13p from other proteins, a 300,000 *g* supernatant prepared from RSY742 cells was applied to a column of methotrexate coupled to agarose (MTX-agarose). The majority of protein (70–75%) did not bind to MTX-agarose, but instead appeared in the column flow-through (Fig. 5 *a*, lane *2*). Additional proteins that were bound nonspecifically to MTX-agarose were eluted by washing the column with B88 containing 1M KCL (Fig. 5 *a*, lane *3*). This treatment released an additional 12–20% of the total applied protein. After a wash with B88 alone (Fig. 5 *a*, lane *4*), the remaining proteins bound to MTX-agarose were eluted by competition with a 10-fold molar excess of MTX (Fig. 5 *a*, lane *5*) followed by a wash with a low concentration of MTX (Fig. 5 *a*, lane *6*). The MTX-eluted material contains one abundant protein of approximately 55 kD, and a number of less-abundant proteins. In a parallel MTX-agarose column fractionation of wild-type cytosol, this abundant protein was not seen in the MTX eluate (not shown).

Immunoblot analysis of Sec13p distribution in MTX-agarose column fractions is shown in Fig. 5 *b* (RSY742 cytosol) and Fig. 5 *c* (RSY607 cytosol). Sec13p-DHFR was not detected in the flow-through fraction (Fig. 5 *b*, lane *2*) and trace amounts were present in the KCl eluate (Fig. 5 *b*, lane *3*). The majority of Sec13p-DHFR was eluted from MTX-agarose by competition with excess MTX (Fig. 5 *b*, lane *5*). A parallel MTX-agarose fractionation of wild-type cytosol is shown in Fig. 5 *c*. Native Sec13p did not bind to MTX-agarose, appearing entirely in the flow-through fraction (Fig. 5 *b*, lane *2*).

The distribution of two additional proteins known to be required for vesicle formation in vitro also were determined by immunoblotting. Sec23p appeared to bind tightly to MTX-agarose, and was not detected in the flow-through (Fig. 5 *b*, lane *2*). Sec23p was partially eluted by 1 M KCl (Fig. 5 *b*, lane *4*), but a portion remained bound and was eluted by MTX (Fig. 5 *b*, lanes *5* and *6*). The distribution of Sec23p was similar in wild-type cytosol fractionated by MTX-agarose (Fig. 5 *c*). However, more Sec23p was eluted by MTX treatment in the Sec13p-DHFR-containing cytosol than in wild-type cytosol (Fig. 5, *b* and *c*). Sar1p also bound to MTX-agarose (Fig. 5 *b* and *c*), and the great majority eluted with the KCl wash in both wild-type and Sec13p-DHFR cytosol. As for Sec23p, a small amount of Sar1p was tightly bound to MTX-agarose in the Sec13p-DHFR cytosol, eluting with MTX treatment (Fig. 5 *b*). The partial retention of Sec23p and Sar1p by MTX-agarose in the presence of the Sec13p-DHFR hybrid protein might reflect interactions between these proteins.

Sec13p-Dependent Vesicle Formation Reaction

Vesicle formation and release from an ER-enriched membrane fraction has been reconstituted in a two stage reaction

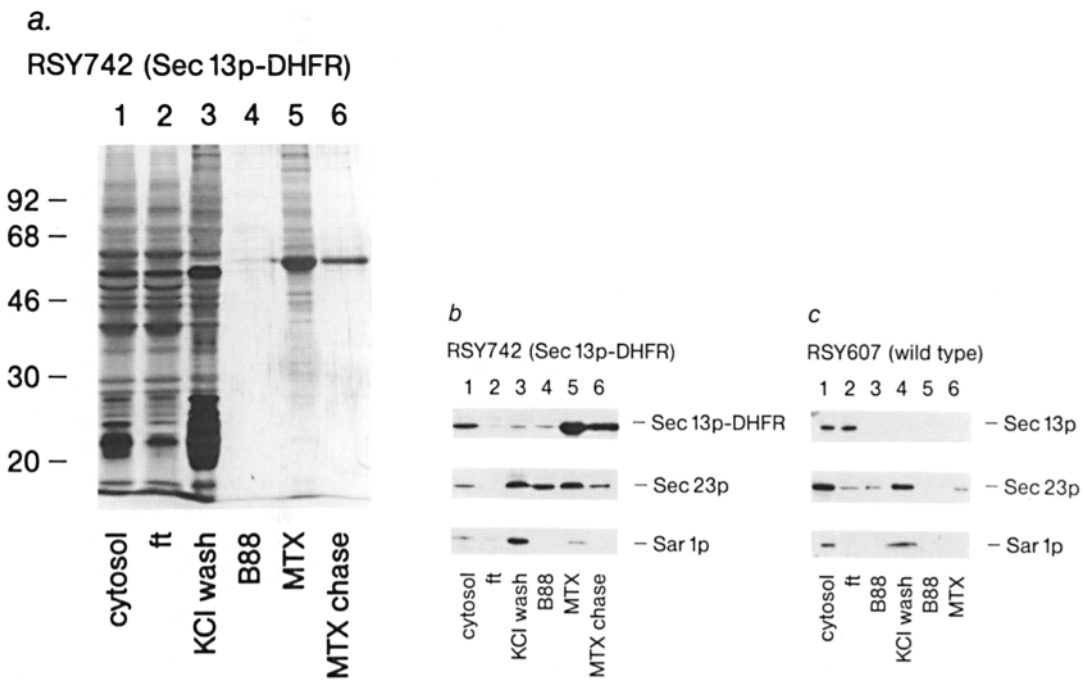

Figure 5. Methotrexate-agarose chromatography of RSY742 (Sec13p-DHFR) cytosol and wild-type cytosol. (*a*) RSY742 cytosol applied to a column of MTX-agarose. The column flow-through was collected (lane *2*), and the column was washed with 1 M KCl in B88CB (lane *3*). Following a B88CB wash (lane *4*), bound proteins were eluted by 50 mM MTX in B88CB (lane *5*) and washed through the column with 5 mM MTX in B88CB (lane *6*). Protein fractions were resolved by SDS-PAGE (10 μg/lane) and visualized by silver staining. M_r marker values are given in kD. (*b*) Immunoblot analysis of Sec13p-DHFR, Sec23p, and Sar1p distributions in fractions from MTX-agarose fractionation of RSY742 (expressing Sec13p-DHFR) cytosol: lane *1*, cytosol; lane *2*, flow through; lane *3*, 1 M KCl eluate; lane *4*, B88 wash; lane *5*, 50 mM MTX eluate; lane *6*, 5 mM MTX "chase." Fractions were resolved by SDS-PAGE (10 μg/lane), and immunoblot analysis was performed and developed by chemiluminescence. (*c*) Immunoblot analysis of Sec13p, Sec23p, and Sar1p distributions in fractions from MTX-agarose fractionation of RSY607 cytosol: lane *1*, cytosol; lane *2*, flow through; lane *3*, B88 wash; lane *4*, 1 M KCl eluate; lane *5*, B88 wash; lane *6*, 50 mM MTX eluate. Note that the RSY607 column had an additional B88 wash and that the 5 mM MTX "chase" is not shown because no protein was detected in this fraction. Protein fractions were resolved by SDS-PAGE, immunoblot analysis was performed, and developed by chemiluminescence.

using unextracted membranes and cytosol (6). In the first stage, ^{35}S-labeled pre-pro-α factor was translocated into an ER-enriched membrane fraction to serve as a secretory protein marker (2, 20). Translocation into the ER was marked by the addition of core oligosaccharides to ^{35}S-pro-α factor. In the second stage, cytosol and ATP were added to membranes and the reaction was incubated at 30°C. Under these conditions, protease-protected, core glycosylated ^{35}S-pro-α factor was released as a slowly sedimenting compartment separable from the ER by differential centrifugation.

Sec 13p-dependency in the vesicle formation assay was generated by depleting Sec13p from membrane and cytosol fractions. Membrane-bound Sec13p was extracted by treating membranes with 2.5 M urea. Urea extraction of microsomes removed Sec13p more efficiently than shown in Fig. 4 for extraction of crude cell lysates. Sec13p was removed from the cytosol by affinity depletion of Sec13p-DHFR, as described above. To provide all cytosolic proteins except Sec13p, we pooled the MTX-agarose KCl eluate, enriched in Sec23p and Sar1p, with the MTX-agarose flow-through to serve as the Sec13p-depleted cytosol. We later found that the KCl eluate could be replaced by a Sec23p-Sec24p complex-enriched fraction (high salt DEAE eluate; see Methods) and purified Sar1p.

When Sec13p depleted membranes were combined with Sec13p-depleted MTX-agarose flow-through and KCl eluate fractions, vesicle release efficiency was only 20% of the efficiency of a complete cytosol fraction (Fig. 6, bar *2*). This level of efficiency was about twice the background level measured in a reaction incubated without cytosol. Activity was restored synergistically to the combined Sec13p-depleted flow-through and KCl eluate fractions by adding 50 μg of unfractionated cytosol (Fig. 6, bar *3*). This same amount of unfractionated cytosol alone stimulated minimal vesicle release (Fig. 6, bar *4*). A Sec13p-enriched fraction was produced by precipitation of wild-type cytosol with 30% ammonium sulfate, which precipitates about 5% of cytosolic proteins, including all of the Sec13p immunoreactivity. Addition of 10 μg of this enriched fraction restored vesicle formation with a higher specific activity than unfractionated cytosol (Fig. 6, bar *5*). The reduction in activity of the MTX flow-through and KCl wash fractions was due to the removal of Sec13p-DHFR, because comparable flow-through and KCl eluate fractions derived from MTX-agarose chromatography of wild-type cytosol were not defective (Fig. 6, bar *7*). Unfractionated cytosol derived from RSY742 (expressing Sec13p-DHFR) was as active as wild-type cytosol in the vesicle release assay (Fig. 6, bar *1*).

Additional controls showed that removal of Sec13p from both membrane and cytosol fractions was necessary to optimize the assay. Extracted membranes combined with unfractionated cytosol containing Sec13p supported vesicle re-

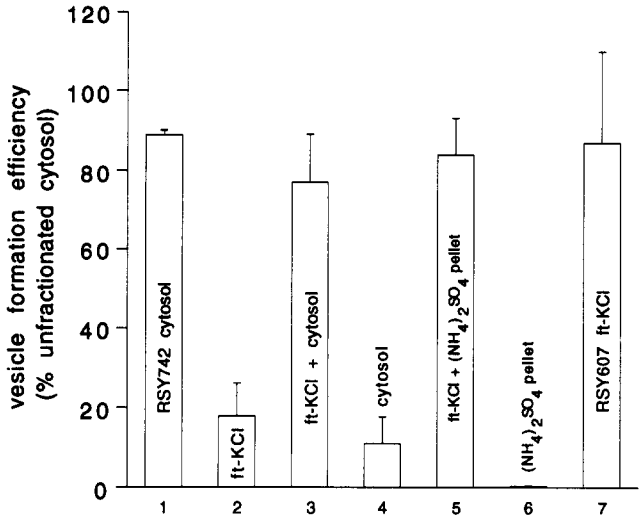

Figure 6. Vesicle release in vitro is deficient in a Sec13p-depleted reaction and restored by cytosolic fractions containing Sec13p. Vesicle release was measured as protease-protected pro-α factor released into the MSS, relative to total translocated pro-α factor. Vesicle release is indicated as a function of the efficiency of a reaction combining complete wild-type cytosol with urea-extracted microsomes (the absolute efficiency of this reaction was 25–30%). Using this complete reaction as a standard, vesicle formation efficiency values from different reactions were pooled and averaged. Error bars represent the standard deviation within these averaged values. Background levels in the assay, measured as a no cytosol reaction, were typically 5% of the complete reaction. (*1*) Unfractionated RSY742 (expressing Sec13p-DHFR) cytosol (n = 2); (*2*) combined RSY742 flow-through and KCl eluate from MTX-agarose chromatography, depleted of Sec13p-DHFR (n = 8); (*3*) addition of 50 μg complete cytosol to RSY742 combined flow-through and KCl eluate (n = 7); (*4*) 50 μg complete cytosol alone (n = 8); (*5*) addition of 10 μg Sec13p-enriched 30% $(NH_4)_2SO_4$ precipitate of wild-type cytosol to RSY742 combined flow-through and KCl eluate (n = 5); (*6*) 10 μg 30% $(NH_4)_2SO_4$ precipitate alone (n = 2); (*7*) combined flow-through and KCl eluate from wild-type cytosol applied to MTX-agarose (n = 6).

lease in vitro with the same efficiency as unextracted membranes (not shown). Urea extraction of membranes reduced background twofold in the presence of Sec13p depleted cytosol and rendered the vesicle formation assay more dependent on the addition of cytosolic Sec13p, as compared to unextracted membranes.

Sec13p Fractionates as a High Molecular Weight Complex

The position at which Sec13p eluted from gel filtration columns suggested that Sec13p participates in a high molecular weight protein complex. Fractionation of wild-type cytosol by gel filtration, followed by immunoblot analysis showed that Sec13p eluted at the position expected for a protein of about 700 kD (Fig. 7 *a*). Depending on the cytosol preparation, a much smaller signal of immunoreactivity was usually detected at the position expected for a monomeric form of Sec13p.

Results of fractionation of cytosol prepared from cells overexpressing Sec13p were consistent with the hypothesis that Sec13p is assembled into a large protein complex. Expression of *SEC13* by a multicopy plasmid in a wild-type strain background yielded a nearly 40-fold increase in the level of Sec13p. Gel filtration of cytosol prepared from this strain (RSY812) showed that the amount of Sec13p complex form was about the same as in wild-type cytosol (Fig. 7 *b*; note the difference in scale of arbitrary immunoreactivity units in Fig. 7, *a* and *b*) and that the majority of overproduced Sec13p eluted in the position expected of a monomer (Fig. 7 *b*). This result suggested that the assembly of Sec13p into a larger complex form is limited by a factor distinct from Sec13p.

The activity of gel filtration column fractions in the vesicle formation assay suggested that the Sec13p complex and not the small, probably monomeric form, is the active species. Gel filtration fractions of wild type and Sec13p overproducer cytosol were assayed and the peak of activity in both coincided with the large complex form of Sec13p (fractions 24 and 25; see Fig. 7 *a*). Vesicle formation activity was not detected for the low molecular weight form of Sec13p, even in cytosol prepared from the Sec13p overproducer strain, in which monomeric Sec13p is much more abundant than normal (Fig. 7 *b*).

Discussion

Genetic and morphological data indicate that the *sec13* mutant defect in secretion is imposed in a step before or concurrent with the release of mature transport vesicles from the endoplasmic reticulum membrane (21, 33). In an effort to understand the biochemical function of Sec13p in the molecular processes contributing to vesicle assembly, maturation and release, we have characterized the *SEC13* gene product and developed a Sec13p-dependent in vitro reaction reconstituting vesicle formation and release from the ER. In this paper, we report that *SEC13* encodes a 33-kD protein containing a series of internal repeated sequence motifs with homology to a functionally divergent group of proteins, including β-transducin, that also contain this repeat. Sec13p is a cytosolic protein that associates peripherally with membranes. Cytosolic Sec13p participates in a large protein complex that stimulates vesicle release from the ER in vitro.

The identification of a known peptide motif in the sequence of *SEC13* may provide a clue to *SEC13* function. In fact, the repeat structure appears to contribute to the role of *SEC13* function in protein transport because we find that the secretion defective mutations alter residues that are conserved in this motif. The repeated sequence element is recognized by a regular spacing between a histidine, an aspartate, and a tryptophan residue within a repeat unit of ~40 amino acid residues and was first seen in the β subunit of transducin (12). Approximately 10 other positions within the repeat are conserved to the extent that they are occupied by any one of a number of chemically similar amino acids. More than 10 proteins that contain this repeated motif have now been recognized (reviewed in refs. 7 and 10). These repeat-containing proteins are involved in diverse cell processes including transcriptional regulation (*TUP1*), RNA processing (*PRP4* and *PRP17*), cell cycle progression (*CDC4* and *CDC20*), and growth regulation (G protein β subunits and *MSI1*). Although *SEC13* and the β subunit of transducin are composed almost entirely of the repeated segments, other proteins such as *CDC4* and *PRP4* contain additional sequences that could impart novel functions beyond those attributable to the repeat motif.

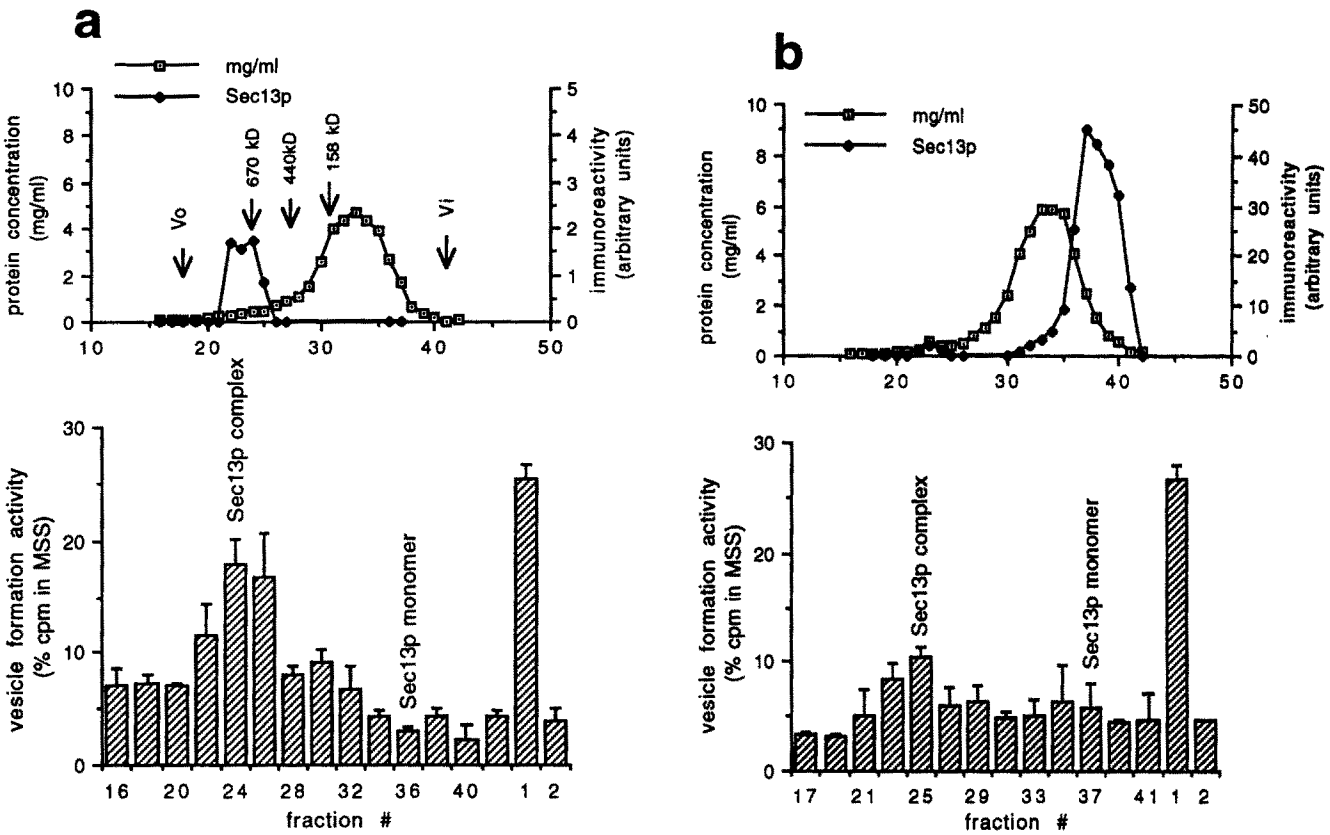

Figure 7. Sec13p fractionates as a high molecular weight complex. Fractions enriched in the complex are active in the Sec13p-dependent in vitro assay for vesicle release. Cytosol was prepared from (*a*) wild type (RSY607) or (*b*) RSY812 (expressing *SEC13* on a multicopy plasmid) cells and applied to a Superose 6 gel filtration column. V_o, V_i, and elution positions of protein standards (in kD) are indicated by arrows in *a* (669 kD, thyroglobulin; 440 kD, ferritin, 158 kD, aldolase). Fractions were assayed for total protein and Sec13p distribution. Sec13p was detected by immunoblot analysis and quantified by ^{125}I-protein A and densitometry. Alternating fractions from the included volume of the column were assayed for activity by combining either (*a*) 25 μg protein (RSY607) or (*b*) 10 μl (10–15 μg protein; RSY812) with Sec13p-depleted MTX agarose flow-through, a high-salt DEAE eluate, and purified Sar1p, as described in the text. The efficiency of vesicle release is expressed as the ratio of protease-protected ^{35}S-pro-α factor released to a medium speed supernatant fraction, relative to total translocated pro-α factor. The last two bars in each plot represent (*1*) complete cytosol reaction and (*2*) Sec13p-depleted cytosol alone. All points are in duplicate; error bars represent standard deviation.

What does the repeated motif say about Sec13p function? One possibility is that Sec13p is a component of a heterotrimeric G protein that acts in protein transport. As yet there is no direct evidence of a role for trimeric G proteins in yeast protein transport from the ER, although an involvement of trimeric G proteins in protein transport in the mammalian Golgi apparatus has been suggested on the basis of inhibition by AlF^-, stimulation by excess βγ subunits and stimulation of transport by overexpression of Gα in vivo (4, 24, 50). The subunit composition of most of the other repeat-containing proteins is not known, and the presence of repeats per se cannot be taken as strong evidence for a functional similarity to the G protein β subunit. A second possibility is that proteins that contain the peptide motif could have similar structures; the motif could define an element of folded structure. In this case, the conserved hydrophobic positions could define buried residues that stabilize a particular polypeptide fold, or that interact with membranes or hydrophobic domains of other proteins. The significance of these repeats may become clearer as we learn more about the proteins that associate with members of this family.

A direct role for Sec13p in vesicle formation from the ER, predicted by the phenotype of *sec13* mutants, has been confirmed biochemically using an in vitro assay that reconstitutes vesicle formation and release. Cell-free transport assays using membranes and cytosol prepared from *sec13* mutants failed to reproduce a temperature-sensitive transport defect. Instead, we devised a Sec13p-dependent assay using membrane and cytosolic fractions that had been depleted of endogenous Sec13p. When such depleted fractions are recombined, vesicle formation becomes dependent on addition of cytosolic Sec13p, as demonstrated by the coincidence of immunoreactive and functional Sec13p fractionated by selective ammonium sulfate precipitation and gel filtration.

Although Sec13p is a 33-kD protein, the majority of cytosolic Sec13p elutes from a gel filtration column at the position expected for a protein of nearly 700 kD. Column fractions containing this complex restore vesicle formation activity to the Sec13p-depleted assay. Overexpression of *SEC13* resulted in abundant overproduction of an apparently monomeric form of Sec13p that does not stimulate vesicle formation. Overproduction of the monomeric form suggests that another subunit of the Sec13p complex is in limited supply when Sec13p is overproduced. Immunoblotting with antibodies against other known Sec proteins required for vesicle formation (Sec23p, Sec24p, Sec21p, Sec16p) failed to

detect an immunoreactive species that co-fractionated with the Sec13p complex, suggesting that other member(s) of the Sec13p complex are proteins not previously identified by mutant selections. We are currently purifying the Sec13p-containing complex and characterizing the other member(s) of the complex.

Sec13p partitions between a sedimentable, membrane-bound form and a soluble, cytosolic pool. The sedimentable portion of Sec13p is peripherally associated with the membrane and can be extracted either by detergent solubilization of the membrane or by treatment with agents that disrupt protein–protein interactions (e.g., urea or salt). While the cell fractionation experiments cannot determine the precise membrane compartment that binds Sec13p, the requirement for Sec13p in vesicle formation from the ER suggests that Sec13p is associated at least with the ER membrane. Sec13p may localize to other cellular membrane compartments as well. Because the addition of cytosolic Sec13p is sufficient to allow vesicle formation in the Sec13p-depleted reaction, either the cytosolic form is active, or the cytosolic form is recruited to the membrane and there stimulates vesicle formation or release. Regulated recruitment of cytosolic Sec13p to sites of active vesicle formation would permit the cell to regulate budding temporally and spatially.

The Sec13p complex is similar in some respects to the Sec 23p–Sec24p complex, which is also required for vesicle formation from the ER (16, 17). Sec23p was identified genetically in the original selection for secretion mutants (34) and Sec24p was identified later upon purification of the complex. Sec23p and Sec24p are both required for protein export from the ER in vivo and in vitro. Both the Sec23p–Sec24p complex and the Sec13p complex partition between membrane and cytosol forms, the complex form predominating in wild-type cytosol. Overexpression of either *SEC13* or *SEC23* results in overproduction of the monomeric form alone (17). Unlike Sec13p, the monomeric form of Sec23p is functional in a biochemical complementation assay.

sec13 exhibits synthetic lethal interactions with *sec23*, *sec12*, and *sec16* (21), in which a double mutant is defective for growth and secretion at a lower temperature than either single mutant alone. Such genetic interaction suggests that proteins function in the same step or pathway, but does not necessarily imply direct protein–protein interaction. Thus the functions of the proteins encoded by genes that interact with *SEC13* may provide a clue to the function of Sec13p. Sec23p recently was found to act as a GTPase-activating protein, stimulating GTP hydrolysis by Sar1p, a Ras-like small GTPase required for vesicle budding from the ER (6, 28, 35). GTP hydrolysis by purified Sar1p in vitro is very slow, but the addition of purified Sec23p monomer stimulates Sar1p GTPase activity 10–15-fold (53). GTP hydrolysis is further stimulated by the cytosolic domain of Sec12p, an integral membrane protein of the ER. Sec12p acts to catalyze GDP release, facilitating its replacement by GTP (Barlowe, C., and R. Schekman, unpublished results). Sec13p might serve as either an upstream regulatory factor or a downstream effector of this system.

Vesicle formation and release from the ER membrane requires the concerted action of a number of proteins. As a membrane-associated protein acting at the cytosolic face of the ER membrane, Sec13p might participate in vesicle budding at a number of different steps. Sec13p may act to specify sites of active, budding-competent ER membrane. Alternatively, Sec13p might contribute to the formation of a vesicle coat, either as a structural component of the coat or an assembly/disassembly factor. Finally, Sec13p could stimulate the ER membrane deformation needed to form a curved vesicle, or aid in severing a newly-formed vesicle from the ER membrane. Reconstitution of vesicle biogenesis in vitro using fully resolved cytosolic and peripheral membrane factors will address the exact roles of Sec13p and other cytosolic and peripheral membrane proteins in vesicle formation and release.

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