The EMBO Journal vol.8 no.6 pp.1677 – 1684, 1989

Yeast Sec23p acts in the cytoplasm to promote protein transport from the endoplasmic reticulum to the Golgi complex *in vivo* and *in vitro*

Linda Hicke and Randy Schekman

Department of Biochemistry, University of California, Berkeley, Berkeley, CA 94720, USA

Communicated by G.Schatz

The *SEC23* gene product (Sec23p) is required for transport of secretory, plasma membrane, and vacuolar proteins from the endoplasmic reticulum to the Golgi complex in *Saccharomyces cerevisiae*. Molecular cloning and biochemical characterization demonstrate that Sec23p is an 84 kd unglycosylated protein that resides on the cytoplasmic surface of a large structure, possibly membrane or cytoskeleton. Vigorous homogenization of yeast cells or treatment of yeast lysates with reagents that desorb peripheral membrane proteins releases Sec23p in a soluble form. Protein transport from the endoplasmic reticulum to the Golgi *in vitro* depends upon active Sec23p. Thermosensitive transport in *sec23* mutant lysates is restored to normal when a soluble form of wild-type Sec23p is added, providing a biochemical complementation assay for Sec23p function. Gel filtration of yeast cytosol indicates that functional Sec23p is a large oligomer or part of a multicomponent complex.

Key words: endoplasmic reticulum/intracellular transport/secretion mutant/yeast Sec protein

Introduction

In eukaryotic cells, proteins must be accurately transported between a series of membrane-bounded organelles. Travel through these organelles is initiated by translocation of newly synthesized proteins from the cytoplasm into the endoplasmic reticulum (ER). From the ER, secretory proteins travel to the Golgi cisternae where they are packaged into vesicles for transit to the yeast vacuole (or to the lysosome in mammalian cells), the plasma membrane or the extracellular milieu. In this pathway, transport from the ER to Golgi is the first step that requires efficient movement of membrane and lumenal molecules between separate compartments. Studies have been initiated in yeast and in mammalian cells to identify the molecular components and mechanisms responsible for ER to Golgi transport (reviewed by Pfeffer and Rothman, 1987).

ER to Golgi transport in mammalian cells has been reconstituted *in vitro* using extracts of Chinese hamster ovary cells. *In vitro* transport depends upon energy provided by ATP and cytosolic proteins (Balch *et al.*, 1987; Beckers *et al.*, 1987). However, specific factors required for this reaction have not yet been identified.

In yeast, the study of ER to Golgi transport was initiated by the isolation of temperature-sensitive mutations in nine *SEC* genes. These mutations block protein transport in strains incubated at the nonpermissive temperature (37°C). Nine *sec* mutants, *sec12*, *sec13*, *sec16*, *sec17*, *sec18*, *sec20*, *sec21*, *sec22* and *sec23* (Novick *et al.*, 1980), and two *bet* mutants, *bet1* and *bet2* (Newman and Ferro-Novick, 1987), accumulate vacuolar, plasma membrane and periplasmic proteins in the ER at 37°C. Morphological analysis of these mutant cells revealed that they also accumulate an extensive network of ER membrane at the nonpermissive temperature. Recently, an *in vitro* assay was developed as an additional tool for investigating ER to Golgi transport in yeast (Baker *et al.*, 1988). This assay measures the transport of a radiolabeled secretory protein (the mating pheromone precursor, prepro-α-factor), which is synthesized *in vitro* and introduced into the ER of yeast lysates by post-translational translocation. Protein which is transported to the Golgi acquires outer chain carbohydrate and is identified by immune precipitation with antiserum that uniquely binds this carbohydrate. *In vitro* transport requires intact membranes, ATP, and cytosolic proteins, and is inhibited by *N*-ethylmaleimide and the nonhydrolyzable GTP analogue, GTPγS. Lysates of several *sec* mutants, including *sec12*, *sec18* and *sec23*, are defective for transport in the *in vitro* reaction, implying that these *SEC* gene products are required for transport *in vitro* as well as *in vivo*.

The Sec12 and Sec18 proteins have been characterized at the molecular level. Sec18p is an 84 kd hydrophilic, cytoplasmic protein. Differential centrifugation of yeast lysates suggests that it may exist in two forms, one soluble and one associated with a sedimentable, intracellular structure (Eakle *et al.*, 1988). Sec12p, in contrast, resides in the ER and Golgi membranes as an ~70 kd integral membrane glycoprotein (Nakano *et al.*, 1988).

In this report we describe physical and functional characteristics of the *SEC23* gene product. DNA sequence analysis of the cloned *SEC23* gene and immunologic detection of the gene product both identify an 84 kd protein. Evidence from cell fractionation and biochemical complementation experiments indicates that active Sec23p resides within a complex that is loosely bound to a large intracellular structure.

Results

Isolation and sequence of SEC23 DNA

To begin investigation of Sec23p we isolated the *SEC23* gene by complementation of the temperature-sensitive *sec23-1* mutation. A *sec23-1* strain (MBY8-20C) was transformed with a genomic yeast DNA library contained in the centromere vector YCp50 (Rose *et al.*, 1987). Only one plasmid was found that allowed growth of this *sec23* strain at the nonpermissive temperature (37°C). This plasmid, YCp1142, is represented in Figure 1. Within its 16–20 kb yeast DNA insert, YCp1142 carries a 3.5 kb *Hin*dIII fragment that complemented the *sec23-1* mutation when

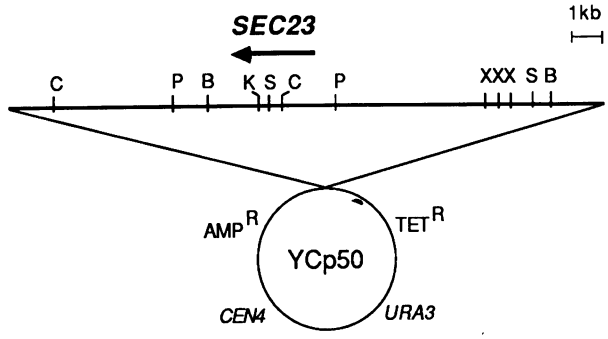


Fig. 1. Restriction map of plasmid YCp1142. YCp1142 contains the *SEC23* gene and was isolated from a genomic yeast DNA library by its ability to complement the *sec23-1* mutation. This plasmid carries a 16–20 kb insert in the centromere vector YCp50. The arrow indicates the direction of transcription and the approximate boundaries of the 2.5 kb *SEC23* mRNA. Hybridization of strand-specific *SEC23* probes to yeast polyadenylated RNA was used to determine the direction of transcription. Abbreviations: B, *Bam*HI; C, *Cla*I; K, *Kpn*I; P, *Pvu*II; S, *Sph*I; X, *Xho*I.

subcloned into a single-copy or multicopy vector. An internal fragment of the complementing gene hybridized to a 2.5 kb yeast polyadenylated RNA. We confirmed that this DNA contained the *SEC23* gene, rather than a suppressor, by using homologous recombination of a linear fragment of the plasmid with genomic *SEC23* DNA to integrate a selectable marker gene near this locus (as described in Böhni *et al.*, 1988). Also using homologous recombination to direct integration of linear DNA into the genome, we found that a disruption of the chromosomal *SEC23* locus was a lethal event in haploid cells. In the course of these experiments we observed that although diploid yeast cells with one null and one wild-type allele of *SEC23* grew normally, diploid cells did not survive even at the permissive temperature if they carried one null and one mutant (*sec23-1*) allele of the gene. A difference in the abundance of the mutant and wild-type proteins was not observed in a direct comparison of immunoreactive Sec23p in haploid mutant and wild-type cells (unpublished data). Instead, the activity of Sec23p may be a limiting factor for cell growth and the (hypothetical) difference in activity between wild-type and mutant protein may be enough to kill a diploid already crippled by the disruption of one *SEC23* gene.

The nucleotide sequence of *SEC23* contains a 2304 base open reading frame with the potential to encode a protein of 768 amino acids. This protein has a predicted mol. wt of 85.4 kd (Figure 2). The *SEC23* sequence is identical to that of *NUC1*, a *Saccharomyces cerevisiae* gene that has been mapped 20 centimorgans telomere-proximal to a transposable element insertion (Ty1) adjacent to *rad56* on chromosome 16. A temperature-sensitive *nuc1* mutant was isolated by its inability to localize correctly a ribosomal protein-β-galactosidase hybrid protein that is normally transported to the nucleus (J.Teem, personal communication). At this time it is not understood why a mutation in the *SEC23/NUC1* gene blocks nuclear localization of this hybrid protein.

No significant homology of *SEC23* to any other nucleic acid sequence or protein sequence was found in the GENBANK or NBRF data banks. Examination of the amino acid sequence deduced from the *SEC23* sequence revealed six potential sites for asparagine-linked glycosylation (Asn-X-Ser/Thr), but there was no obvious signal sequence and hydropathic analysis of the sequence (Hopp and Woods, 1981) exposed no distinct membrane-spanning domains. These characteristics indicated that Sec23p may be a cytoplasmic protein rather than a secretory or integral membrane protein.

Characterization and localization of Sec23p

To identify and characterize Sec23p we raised polyclonal antisera against a β-galactosidase–Sec23p hybrid protein. The 3′ two-thirds of *SEC23* was fused in-frame to *lacZ* in an *Escherichia coli* expression vector and the protein encoded by this hybrid gene was purified and injected into two rabbits. Antisera produced in both rabbits was used to detect Sec23p by immune precipitation and by immunoblot of yeast lysates. In close agreement with the predicted size (85.4 kd) of the *SEC23* gene product, an 84 kd protein was detected by both antisera, but not by preimmune sera (Figure 3). The following observations established the identity of the 84 kd polypeptide as Sec23p. Each antiserum recognized other polypeptides; however, only the 84 kd species was recognized by both sera and was more abundant in cells carrying the *SEC23* gene on a multicopy plasmid. In addition, the 84 kd protein was no longer recognized when antiserum was incubated with purified β-galactosidase–Sec23p fusion protein prior to incubation with yeast extracts (unpublished data). Finally, antiserum that was affinity-purified by binding to a *trpE–SEC23* hybrid gene product specifically bound to the 84 kd protein (Figure 3, lane 6).

Immunologic detection of Sec23p allowed us to investigate the intracellular localization of the protein, and we found that Sec23p sedimented with the particulate fraction of the cell during differential centrifugation of yeast lysates (Figure 4). In order to assess the recovery of intact ER through the course of this experiment, fractionation was performed with *sec18*, a strain that accumulates secretory proteins in a soluble form in the ER lumen. Fractionation of the secretory form of invertase that accumulates in the ER of *sec18* cells at 37°C, was compared with the fractionation of cytosolic invertase and of the activity of NADPH cytochrome *c* reductase, an integral membrane protein of the ER. (Yeast produces two species of invertase: cytosolic invertase which is constitutively synthesized, unglycosylated and is soluble in the cytoplasm; and secretory invertase which is an inducible protein, produced under conditions of low glucose, and is a glycoprotein that traverses the secretory pathway en route to its location in the cell periplasm.). *sec18* cells induced to synthesize secretory invertase were incubated at a permissive (24°C) or a nonpermissive (37°C) temperature, converted to spheroplasts and lysed. The lysates were centrifuged at 650 *g* for 3 min to remove unbroken cells. This centrifugation also partially removed rapidly sedimenting membrane including some ER. The 650 *g* supernatant fraction was then centrifuged at 100 000 *g* for 1 h to separate the particulate and soluble fractions of the yeast lysate. The lumenal, membrane and cytosolic markers fractionated as expected in both lysates (Figure 4b and c). Cytosolic invertase was recovered quantitatively in the 100 000 *g* supernatant fraction. Secretory invertase, present in small quantities in cells grown at 24°C and accumulated in cells incubated at 37°C, and NADPH

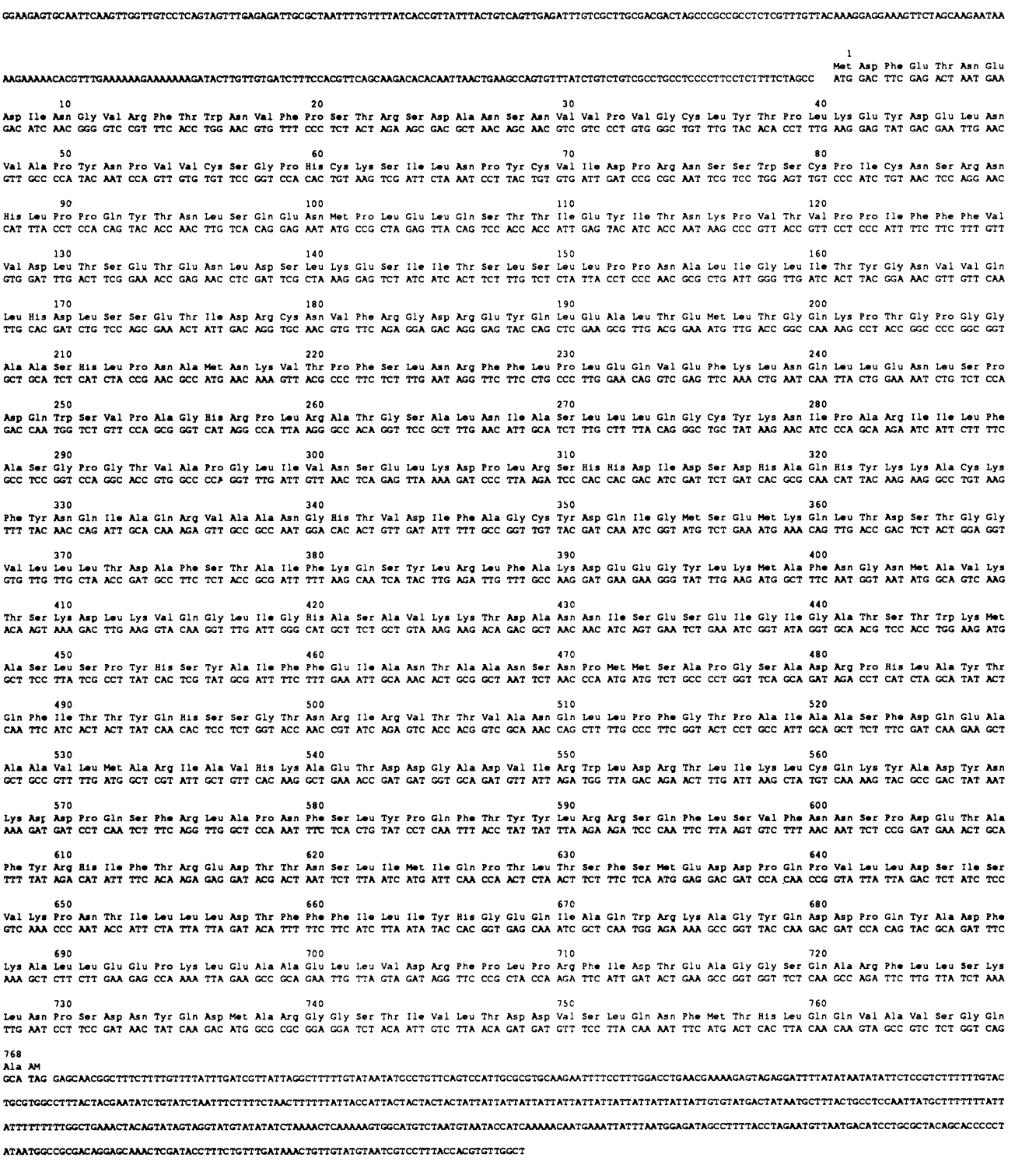


Fig. 2. Nucleotide sequence of *SEC23* and predicted amino acid sequence of the Sec23 protein.

cytochrome *c* reductase activity were recovered in both the 650 *g* and the 100 000 *g* pellet fractions. Secretory invertase detected in the 100 000 *g* supernatant was due to rupture of some ER. As seen in Figure 4a, Sec23p partitioned almost completely into the 100 000 *g* pellet fraction in lysates of cells incubated at 24°C or 37°C. The same fractionation behavior was observed in lysates prepared from wild-type (SEC^+) cells and in *sec23* cells incubated at the nonpermissive temperature (unpublished data). These results indicated that Sec23p is not a soluble protein but is associated with at least one intracellular, sedimentable structure.

Although the *SEC23* sequence did not reveal characteristics expected of a secretory protein, we considered this possibility because of the sedimentation behavior of the protein. Several other observations, however, supported the notion that Sec23p is a cytoplasmic, not a secretory protein. Since the predicted and apparent mol. wts of Sec23p were equivalent, it seemed unlikely that the potential sites of N-linked

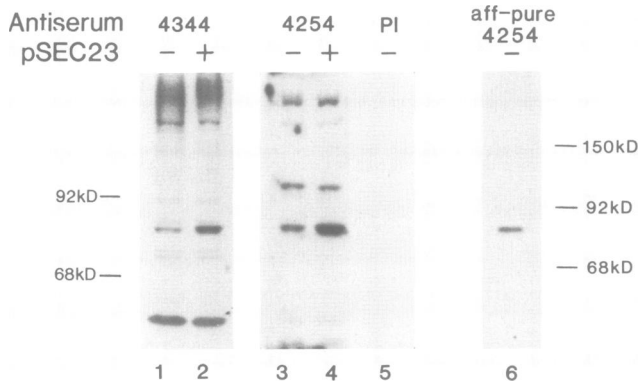
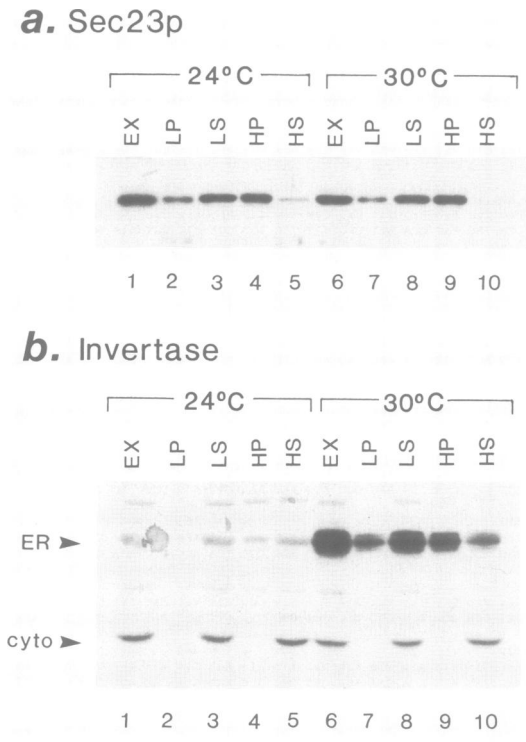


Fig. 3. Sec23p antisera recognize an 84 kd yeast protein. Immunoblots of yeast extracts indicate two different antisera (4254 and 4344) bind to an 84 kd protein (**lanes 1** and **3**) that is overproduced in yeast cells carrying the *SEC23* gene on the multicopy plasmid pSEC23 (**lanes 2** and **4**). This protein is not recognized by preimmune (PI) serum (**lane 5**) and is the only protein recognized by affinity-purified Sec23p antiserum (**lane 6**).

glycoslyation in the protein were exposed to the ER lumen. In accord with this, treatment of Sec23p with endoglycosidase H, an enzyme that cleaves N-linked core oligosaccharide from peptide backbones, had no effect on the electrophoretic mobility of Sec23p (unpublished data). In another experiment, treatment of *sec18* lysates with proteinase K was used to evaluate the exposure of Sec23p to the cytosol (Figure 5). α-Factor precursor, accumulated within the ER lumen of *sec18* cells, was used to assess the proteolytic degradation of ER lumenal proteins in this lysate. Since α-factor precursor synthesis or stability is greatly reduced at 37°C and ER to Golgi transport in *sec18* strains is completely blocked even at 30°C, we used 30°C as the nonpermissive temperature in this experiment. Following incubation at a permissive (24°C) or a nonpermissive temperature (30°C), *sec18* cells were converted to spheroplasts and lysed gently under conditions that retained intact ER (Feldman *et al.*, 1987). The lysates were treated with proteinase K at 0°C in the presence or absence of nonionic detergent. Sec23p was degraded very rapidly in lysates prepared from cells incubated at either 24°C (Figure 5a) or 30°C (unpublished data) whether or not detergent was present. The amount of degradation that was observed at the zero timepoint was due to proteolysis that occurred during the time it took to process the sample (~15 s). In contrast, pro-α-factor present in the ER lumen was protected from proteolysis (Figure 5b). Pro-α-factor was not inherently protease insensitive as it was degraded when detergent was used to permeabilize the ER membrane. Some pro-α-factor was released by rupture of the ER (Figure 5b, lanes 13 and 14) which probably accounted for the small amount of proteolysis observed in the absence of detergent. The large fraction of Sec23p observed in a soluble form (Figure 5a, lanes 13 and 14) can be explained by the difference in pH of the buffer used in this experiment as compared to the buffer used in the experiments presented in Figure 4 and 6 (unpublished data, see below). These data eliminate the possibility that Sec23p is a lumenal protein and suggest that the protein is fully exposed to the cytoplasm.

The nature of the association between Sec23p and a sedimentable structure was examined by treatment of a lysate with agents that disrupt membranes or release peripheral



c. NADPH cytochrome *c* reductase

	fraction	activity in extract	% activity
24°C	EX	12.7	100
	LP	5.1	40
	LS	6.5	51
	HP	7.1	56
	HS	0.2	2
30°C	EX	9.1	100
	LP	3.8	41
	LS	5.0	55
	HP	5.6	61
	HS	0.2	2

Fig. 4. Sec23p sediments with the particulate fraction of yeast lysates. Synthesis of secretory invertase in *sec18* cells growing at the permissive temperature (24°C) was derepressed concomitant with either a shift to the restrictive temperature (37°C) or continued incubation at 24°C. Extracts from each of these *sec18* cultures were prepared by gentle lysis of spheroplasts and subjected to differential centrifugation. The extracts (EX) were first centrifuged at 650 *g* for 3 min to remove unbroken cells and large debris in the pellet (LP). The supernatant (LS) from this spin was further centrifuged at 100 000 *g* for 1 h to separate the particulate (HP) and soluble (HS) fractions of the yeast cell. (**a**) Immunoblot of fractionation samples with Sec23p antiserum. (**b**) Immunoblot of fractionation samples with invertase antiserum. Arrows indicate the position of cytoplasmic invertase and the position of core-glycosylated invertase accumulated in the ER lumen of *sec18* cells at the nonpermissive temperature. Core-glycosylated invertase present in *sec18* cells incubated at 24°C represents proteins in transit through the ER. (**c**) Fractionation behavior of an ER membrane protein, NADPH cytochrome *c* reductase. Activity is expressed as ΔA_{544nm} per minute per microliter.

membrane proteins (Figure 6). A lysate of *sec18* cells was prepared as for differential centrifugation and aliquots were diluted into fractionation buffer containing various reagents,

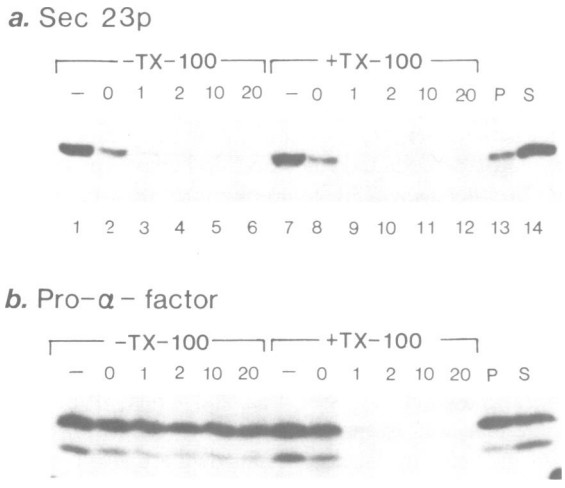


Fig. 5. Sec23p is degraded by protease added to yeast lysates under conditions in which lumenal proteins are protected. Lysates were gently prepared from *sec18* yeast incubated at either 24 or 30°C. The lysates were incubated with 0.25 mg/ml proteinase K in the presence or absence of 0.1% Triton X-100 and aliquots were withdrawn and precipitated with trichloroacetic acid before the addition of protease (**lanes 1** and **7**) or at various times after protease addition, 0, 1, 2, 10 or 20 min (**lanes 2–6** and **lanes 8–12**). A separate aliquot of each lysate was centrifuged in a microcentrifuge for 10 min to separate pro-α-factor associated with intact ER (P) from that released by lysis of the ER during the experiment (S). These samples were also analyzed for the distribution of Sec23p (**lanes 13** and **14**). Degradation of Sec23p was assessed in samples from lysates of cells incubated at 24 or 30°C and was the same in both cases. Data is shown for 24°C samples. Degradation of pro-α-factor was determined only in lysates of cells incubated at 30°C. (**a**) Immunoblot incubated with Sec23p antiserum. (**b**) Immunoblot incubated with α-factor antiserum. The upper band is pro-α-factor modified by three core oligosaccharides; the lower band is pro-α-factor carrying only two core oligosaccharides.

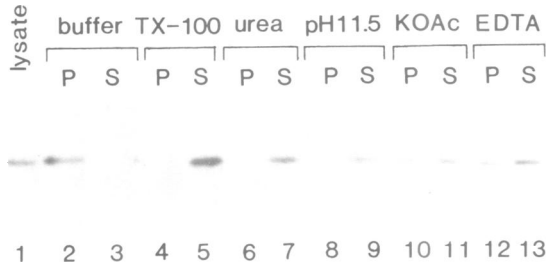


Fig. 6. Sec23p is solubilized by treatment of gently lysed yeast with reagents that release peripheral membrane proteins. Aliquots of a yeast lysate were diluted into fractionation buffer containing various reagents, incubated on ice for 30 min and centrifuged for 10 min in a microcentrifuge. The pelleted material (P) and supernatants (S) were separated. An immunoblot of these samples was incubated with Sec23p antiserum. Samples treated with the following reagents are shown: **lane 1**, untreated lysate; **lanes 2** and **3**, fractionation buffer; **lanes 4** and **5**, 0.5% Triton X-100; **lanes 6** and **7**, 2.5 M urea; **lanes 8** and **9**, 0.1 M Na_2CO_3, pH 11.5; **lanes 10** and **11**, 0.5 M KOAc; **lanes 12** and **13**, 25 mM EDTA.

incubated at 0°C, and centrifuged to obtain the soluble and particulate fractions. Dilution of the lysate into buffer had no effect on the solubility of Sec23p since it remained in the particulate fraction. Treatment of the lysate with 0.5% Triton X-100, 2.5 M urea, or 0.1 M sodium carbonate, pH 11.5, converted all of Sec23p to a soluble form and treatment

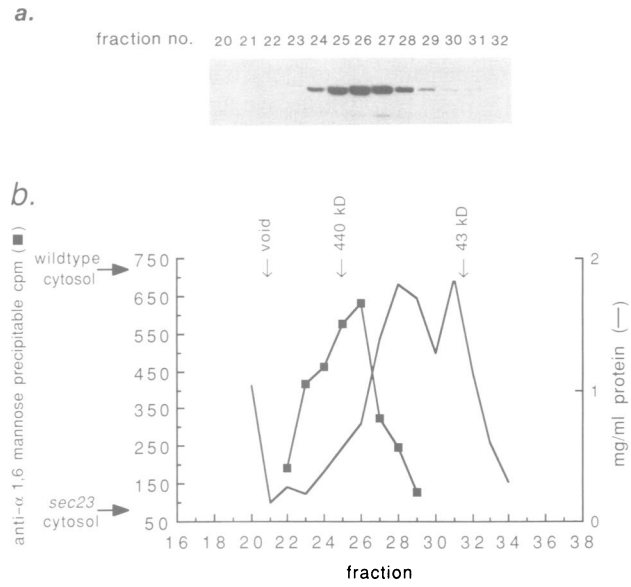


Fig. 7. Fractions of wild-type yeast cytosol enriched in Sec23p by gel filtration complement *sec23* mutant lysates in an *in vitro* assay for ER to Golgi transport. Cytosol was prepared from yeast strain GPY60 (SEC^+) and loaded onto a Sephacryl HR 300 gel filtration column. Fractions were collected, assayed for total protein and immunoblotted with Sec23p antiserum. Eight fractions that included the peak of Sec23 protein were each concentrated ~10-fold and tested for the ability to restore ER to Golgi transport in *sec23* lysates at 30°C. (**a**) Immunoblot of fractions 20–32 with Sec23p antiserum. (**b**) Sec23p activity and total protein content of fractions. Sec23p activity in fractions 22–29 was measured by the addition of each fraction to an *in vitro* transport reaction containing *sec23* membranes and *sec23* cytosol. Transport in the presence of each fraction is measured by the amount of anti α1→6 mannose precipitable cpm generated in the reaction. The horizontal arrows indicate the levels of transport that occurred in *sec23* membranes in the presence of *sec23* cytosol alone or in *sec23* membranes mixed with wild-type cytosol. Fractions have no transport activity above background if *sec23* cytosol is omitted from the reaction mixture. Vertical arrows denote the elution of standards used to calibrate the Sephacryl HR 300 column. The void volume was determined by the elution of blue dextran.

with 0.5 M potassium acetate or 25 mM EDTA solubilized ~50% of the protein. ATP (500 mM), GTP (100 mM) and Ca^{+2} (2 mM) were without effect (unpublished data). These solubilization experiments were carried out with cells gently lysed in a buffer of pH 6.5. A large fraction of Sec23p was soluble in lysates prepared in pH 7.5 buffer without any other treatment (for example see Figure 5a, lanes 13 and 14), or in extracts prepared by vigorous (glass bead homogenization) lysis of cells (unpublished data). From these results, Sec23p did not behave as an integral membrane protein and instead displayed characteristics of a peripheral membrane protein.

Biochemical complementation assay for Sec23p activity

ER to Golgi transport in yeast has recently been reconstituted *in vitro* (Baker *et al.*, 1988). Transport of secretory glycoproteins is monitored with pro-α-factor which is translated and labeled with [^{35}S]methionine *in vitro*, and introduced into ER of gently lysed yeast by a post-translational translocation reaction. Labeled pro-α-factor that reaches the Golgi is identified by immune precipitation with anti-α1→6 mannose serum, an antibody that recognizes a Golgi-specific carbohydrate modification. ER to Golgi transport measured by this assay depends upon ATP,

organelle integrity and yeast cytosol. In a reaction containing lysates and cytosol prepared from *sec23* cells grown at the permissive temperature, ER to Golgi transport is temperature sensitive (15°C, *in vitro* permissive temperature; 30°C, *in vitro* nonpermissive temperature). The temperature-sensitive defect is abolished when wild-type cytosol rather than *sec23* cytosol is included in the reaction (Baker *et al.*, 1988).

To establish the *in vitro* reaction as a reliable assay for identifying Sec23p activity during purification of the protein, cytosol prepared from wild-type cells lysed by vigorous homogenization was resolved by gel filtration on Sephacryl HR 300 (Figure 7). Column fractions were analyzed by immunoblot with Sec23p antiserum, and separate aliquots were assayed for the ability to restore transport at 30°C in lysates that contained *sec23* membranes and *sec23* cytosol. Figure 7a shows that fraction 26 contained the peak of Sec23p immunoreactive material. The same fraction contained the peak of Sec23p complementing activity (Figure 7b). The peak was one fraction displaced from a marker protein, ferritin (440 kd), suggesting Sec23p was part of a large (~400 kd) complex or oligomer.

The ratio of transport complementing activity to Sec23p immunoreactivity was considerably higher on the leading edge of the column peak. This may be due to the presence of a transport inhibitor coinciding with the trailing edge of the activity peak, or to the presence of inactive, possibly lower oligomeric forms of Sec23p that chromatograph behind the peak of complementing activity. Further purification of the complementing activity will distinguish between these possibilities. Complementation activity required both *sec23* cytosol and an aliquot of the active column fractions. No activity was detected with column fractions only mixed with *sec23* membranes. Thus, wild-type Sec23p was the soluble molecule responsible for restoring transport, at the nonpermissive temperature, to a reaction containing *sec23* membranes and *sec23* cytosol.

Discussion

We have cloned and determined the nucleotide sequence of the yeast *SEC23* gene and characterized its gene product, Sec23p. Though Sec23p is required for protein transport from the ER to the Golgi, the cloned gene encodes an 84 kd protein with no obvious sequence characteristics of a protein which enters the secretory pathway. Further characterization of the protein using Sec23p antiserum and a biochemical complementation assay suggests that it functions in intracellular transport as part of a complex peripherally associated with the cytoplasmic face of an intracellular membrane or cytoskeletal structure. Several lines of evidence substantiate this conclusion. First, Sec23p sediments quantitatively with the particulate fraction of a gently prepared yeast lysate. Second, Sec23p is degraded rapidly by protease added to yeast lysates and is therefore not sequestered in the lumen of an organelle. Supporting this notion, asparagine-linked carbohydrate is absent on Sec23p, though the protein has the appropriate glycoslyation sites. Finally, Sec23p is released from the particulate fraction by reagents, e.g. urea and a high concentration of salt, that disrupt protein−protein interactions. Sec23p is also released by 0.1 M sodium carbonate, pH 11.5, a reagent that transforms membranes into sheets and releases peripheral membrane proteins (Fujiki *et al.*, 1982). A low concentration of the nonionic detergent Triton X-100 converts the protein to a soluble form, perhaps by disrupting its interaction with a membrane or by releasing it and a hypothetical membrane anchor from a membrane. Gel filtration of cytosol prepared from wild-type cells indicates Sec23p behaves as a 400-kd protein, either a Sec23p homo-oligomer or a complex of different proteins. This complex may act in ER to Golgi transport by a loose association with an intracellular structure, an association mediated by electrostatic interactions.

Cytoskeletal elements, particularly microtubules, have recently been implicated in ER biogenesis and function (Dabora and Sheetz, 1988; Lee and Chen, 1988). It is possible that Sec23p functions in ER to Golgi transport as part of the yeast cytoskeleton. Our data are also consistent with the localization of Sec23p to the cytoplasmic surface of the ER, the Golgi or a transport intermediate. A reliable fractionation scheme for resolving these membranes will be useful in determining the cellular location of Sec23p. Unfortunately, attempts to localize Sec23p by immunofluorescence microscopy have failed, perhaps because our Sec23p antiserum does not recognize the native conformation of the protein.

Sec23p exhibits characteristics similar to those described for several other proteins involved in intracellular transport. Its fractionation and solubility properties resemble those described for the *SEC15* gene product, a yeast protein involved in the transport of secretory vesicles from the Golgi to the cell surface (Novick *et al.*, 1980). The Sec15 protein is a 115 kd hydrophilic protein that sediments with the particulate fraction of a yeast extract. The association of Sec15p with a sedimentable structure is pH dependent and is disrupted by urea and a high concentration of salt (A.Salminen and P.Novick, personal communication). These properties are similar to those of Sec23p, though the proteins behave differently upon treatment with Triton X-100. Perhaps these proteins are associated with the same intracellular structure and serve similar functions in different parts of the secretory pathway.

Another protein required for intracellular transport, *N*-ethylmaleimide (NEM) sensitive factor (NSF), has been identified and purified using an *in vitro* assay for protein transport between Golgi cisternae in Chinese hamster ovary cells (Block *et al.*, 1988). Treatment with NEM inactivates Golgi membranes in this assay. Activity is restored to NEM-treated membranes by the addition of an ATP-extract from untreated membranes (Glick and Rothman, 1987). This NEM-sensitive activity, associated with Golgi membranes and released by incubation of membranes with ATP, was purified and found to reside in a single protein, a tetramer of 76 kd subunits. This protein is thought to act in transport through multiple levels of the Golgi cisternae and may also be required for ER to Golgi transport. The yeast *in vitro* ER to Golgi transport reaction is also sensitive to treatment with NEM suggesting there may be an NSF homolog in yeast (Baker *et al.*, 1988). We have considered the possibility that Sec23p is functionally related to NSF, as both proteins behave as peripheral membrane proteins, have relatively high cysteine content and are of similar size. However, Sec23p antiserum does not recognize purified NSF (M.Block and J.Rothman, unpublished data), and instead the NSF amino acid sequence is highly homologous to that of the *SEC18*

gene product (J.Rothman, personal communication), implying that NSF is the mammalian homologue of Sec18p.

The 400 kd form of Sec23p complements defective *sec23* lysates in an *in vitro* transport reaction. Genetic analyses suggest an interaction of Sec23p with the Sec16 and Sec13 proteins (C.Kaiser and R.Schekman, unpublished data). The *SEC16* and *SEC13* gene products remain uncharacterized and are candidates for other members of the 400 kd complex. We are investigating the effect of the *sec16* and *sec13* mutations on the fractionation properties and gel filtration behavior of Sec23p.

Another laboratory has recently described *in vitro* reconstitution of ER to Golgi transport in yeast and reported a defect in *sec23* mutant lysates (Ruohola *et al.*, 1988). Ruohola *et al.* describe a transport reaction similar to ours but which can be separated into distinct donor and acceptor membrane fractions. They observe an apparent transport defect in *sec23* lysates *in vitro* that is attributed to the acceptor compartment. Transport is observed with wild-type donor and acceptor membranes incubated with a wild-type or *sec23* soluble fraction, whereas no transport occurs in lysates that contain the wild-type donor and *sec23* acceptor compartments incubated with wild-type cytosol. These results are not inconsistent with our observations. Sec23p may not be released in a soluble form under the conditions in which the lysates are prepared for their assay, and it is quite possible that Sec23p is partially or completely localized to the Golgi. However, another interpretation of these results is that the Golgi apparatus in *sec23* cells may be crippled by indirect effects associated with mutant cells, not as a direct result of inactivating the Sec23 protein. *sec23* fractions used in the experiments of Ruohola *et al.* were prepared from cells incubated for 2 h at the nonpermissive temperature prior to lysis. To address the indirect effects that a long nonpermissive incubation *in vivo* may cause, it was mentioned that *sec23* fractions prepared from mutant cells incubated at the permissive temperature show the same acceptor compartment defect (all *in vitro* reactions are performed at 20°C). However, since the acceptor defect observed in their experiments is apparently not correlated with the temperature-sensitive nature of the *sec23* mutation, it cannot be ascribed directly to an effect of this mutation. Sec23p is certainly associated with some structure found in either the donor or acceptor compartment fractions or both, yet its function cannot be assigned to either at this time.

The *in vitro* ER to Golgi transport reaction provides an assay for purification of the 400 kd functional form of Sec23p. Purification of this complex will determine whether it is a Sec23p homo-oligomer or a complex of different proteins, some of which may be other *SEC* gene products. In addition, purification and further characterization of Sec23p will lead to information on its role in intracellular transport, where it functions and how it interacts with other components of the secretion machinery.

Materials and methods

Materials

α-Factor antiserum was provided by J.Rothblatt (this laboratory). Invertase antiserum was prepared by I.Schauer (Schauer *et al.*, 1985) and α1−6 mannose antiserum was prepared by P.Esmon as described (Ballou, 1970). [^{125}I]NaI was purchased from Amersham and ^{125}I-labeled *Staphylococcus aureus* protein A was prepared as described (Hunter and Greenwood, 1962).

Plasmids

Plasmid pSEC23 was constructed by inserting a 3.5 kb *Hin*dIII fragment containing the entire *SEC23* gene into the multicopy *E.coli*−yeast shuttle plasmid pCF35 (from the collection of this laboratory).

Cloning and DNA sequence determination

To isolate the *SEC23* gene, a *sec23* strain, MBY8-20C (*sec23-1, leu2-3,112, trp1-289, his, MAT*α), was converted to spheroplasts and transformed with genomic DNA libraries contained in the centromere vector YCp50 (Rose *et al.*, 1987) or in the multicopy vector YEp24 (Carlson and Botstein, 1982). Plasmids that complemented the *sec23* mutation were recovered only from the YCp50 library and were isolated and purified as described previously (Bernstein *et al.*, 1985).

A sequential series of overlapping deletions in the *SEC23* gene was created by treating linearized pSEC23 with *Bal*31 exonuclease (Fast Form; International Biotechnologies, Inc.). The deleted gene fragments were subcloned into pUC118 and pUC119 (gifts from J.Viera, University of Minnesota) and sequenced by the dideoxy-chain termination method (Sanger *et al.*, 1977). The nucleotide sequence of both DNA strands was determined.

Sec23p antiserum

A 2.1 kb *Hpa*I fragment, containing two-thirds of the *SEC23* gene, was fused in-frame to *lacZ* in the plasmid pUR291 (Ruther and Muller-Hill, 1983). The resultant plasmid, pLACZ23P, was transformed into *E.coli* strain BMH71-18 [Δ(*lac pro*) (F′: *lacI^{q}Z ΔM15 pro*$^+$)]. Transformants were induced to express β-galactosidase−Sec23p hybrid protein by adding 1 mM isopropylthio-β-D-galactoside to exponentially growing cultures; the induced fusion protein of ~155 kd was purified as described (Nakano *et al.*, 1988).

Two rabbits were each injected with ~100 μg purified hybrid protein emulsified in Freund's complete adjuvant. One month after this primary injection boost injections were repeated every 10−14 days with ~50 μg protein emulsified in incomplete Freund's adjuvant. Yeast extracts used for immunoblots incubated with Sec23p antisera were prepared by glass bead lysis of X2180-1A cells (*MAT*a, *gal2*) as described (Ferro-Novick *et al.*, 1984).

Sec23p antiserum was affinity-purified by binding to a CNBr-activated Sepharose 4B (Pharmacia) column derivatized with protein encoded by a hybrid *trpE-SEC23* gene. This gene was constructed by fusing the 2.1 kb *Hpa*I *SEC23* fragment in-frame to a truncated *trpE* gene contained on plasmid pATH3 (obtained from T.Koerner and A.Tzagaloff, Department of Biological Sciences, Columbia University). The plasmid containing this gene fusion, pTRPESEC23, was transformed into *E.coli* strain MC1061 [F$^-$ *araD139* Δ(*araABOIC-leu*)7679 Δ*lac*$_X$*74 galU galK rpsL hsdR*] and induced to express hybrid protein by adding 10 μg/ml indoleacrylic acid to exponentially growing cells. The induced hybrid protein was purified by the same method used to purify the β-galactosidase−Sec23p protein.

Approximately 1 mg purified hybrid protein in 1 ml coupling buffer (0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl) was incubated with 1 ml packed CNBr-activated Sepharose 4B beads overnight at 4°C. Derivatized beads were washed three times with 1 M ethanolamine−HCl, four times with PBS (4 mM KH_2PO_4, 16 mM $NaHPO_4$, 115 mM NaCl), twice with 0.1 M glycine−HCl, pH 3.0, and twice with PBS. Beads were loaded into a 1-ml column and stored at 4°C until use. Another affinity column was prepared by coupling cell extract from *E.coli* strain BMH71-18 (8 mg protein) to 8 ml packed CNBr-activated Sepharose 4B in the same way. Sec23p antiserum (2−12 ml) was loaded onto the BMH71-18 extract affinity column at 0.2 ml/h. The flowthrough was collected and loaded onto the *trpE−SEC23* fusion protein affinity column at the same rate. The flowthrough fraction from this column was collected and recycled onto the column which was then washed with ~10 column volumes of PBS. Bound protein was eluted with 0.2 M glycine−HCl, pH 2.5, and samples were neutralized with 1.5 M Tris−Cl, pH 8.8. Fractions were assayed for their ability to recognize Sec23p on immunoblots of yeast lysates and those containing this activity were pooled and concentrated in Centricon 30 Microconcentrators (Amicon).

Fractionation and proteolysis of yeast lysates

Preparation and fractionation of *sec18* lysates by differential centrifugation was performed in fractionation buffer (0.3 M mannitol, 0.1 M KCl, 20 mM MES, pH 6.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3) exactly as described (Bernstein *et al.*, 1985) except the yeast strain used was RSY17 (*sec18-1, pep4-3 MAT*α). Solubilization of Sec23p by chemical reagents was performed with RSY17 lysates prepared as for fractionation. Aliquots of lysate were diluted 1:5 in fractionation buffer containing various reagents, incubated on ice for 30 min, and centrifuged in a microcentrifuge for 10 min at 4°C (conditions sufficient to sediment Sec23p in an untreated lysate).

The supernatant and pellet fractions were separated and the pellet resuspended in fractionation buffer. Each fraction was precipitated in 20% trichloroacetic acid for 15 min on ice and centrifuged for 10 min in a microcentrifuge. The resulting pellets were washed in −20°C acetone, dried under vacuum, resuspended in Laemmli sample buffer with 2% β-mercaptoethanol (Laemmli, 1970), and stored at −20°C until analyzed by immunoblot.

Proteolysis of RSY17 lysates with proteinase K (Sigma Chemical Co.) was performed in lysis buffer (0.3 M mannitol, 0.1 M KCl, 50 mM Tris−HCl, pH 7.5, 1 mM EGTA) as described (Deshaies and Schekman, 1987).

Immunoblots

Samples (typically an amount equivalent to 0.5−1 OD_{600} cell unit of extract) were electrophoresed on 7.5% (Sec23p and invertase) or 12.5% (pro-α-factor) SDS−polyacrylamide gels. Following electrophoresis proteins were transferred from the gel to nitrocellulose (Schleicher and Schuell) (Burnette, 1981). All incubations and washes were carried out in 2% milk buffer (2% nonfat dry milk, 50 mM Tris−HCl, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 10 mM NaN_3). Nitrocellulose filters were preincubated in 2% milk buffer for 1 h at room temperature to block nonspecific protein-binding sites, then were incubated in 2% milk buffer containing the appropriate antibody for 3 h at 25°C or overnight at 4°C. Filters were washed six times for 5 min each and bound antibody was detected by incubation for 1 h at room temperature with ^{125}I-labeled *S.aureus* protein A. Three further washes for 15 min each were followed by exposure of the filters to Kodak X-OMAT AR film (Eastman Kodak Co.) at −85°C.

Preparation and gel filtration of yeast cytosol

Cytosol was prepared from a SEC^+ yeast strain (GPY60: *leu2-3,112, ura3-52, his4-579, trp1-289, prb1, pep4::URA3, gal2, MATα*) as described (Baker *et al.*, 1988). A 1.5 ml aliquot of cytosol (31 mg protein) in Buffer 88 (20 mM Hepes, pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc) was loaded onto a 75 ml (1.5 × 46 cm) Sephacryl HR 300 (Pharmacia) gel filtration column equilibrated in Buffer 88. The column was eluted in the same buffer at ~15 ml/h. Fractions (1.5 ml) were collected and total protein in each fraction was determined using the Bio-rad protein assay (Bio-Rad Laboratories). Fractions containing Sec23p were identified by immunoblot. Eight fractions, including the peak of immunoreactive Sec23p, were each concentrated ~10-fold in a Centricon 30 Microconcentrator, frozen in liquid N_2, and stored at −85°C before being assayed for *in vitro* transport activity.

In vitro ER to Golgi transport reaction

sec23 membranes were prepared from strain DBY5-3A (*sec23-1, leu2-3,112, ura3-52, pep4::URA3, MATα*) and *sec23* cytosol was prepared from strain LHY3-8C (*sec23-1, leu2-3,112, ura3-52, his3 MATα*). Transport reactions were performed at 30°C for 25 min as described (Baker *et al.*, 1988) with the following modifications. *In vitro* reactions contained *sec23* membranes, 84 μg *sec23* cytosol, and 2 μl concentrated wild-type cytosol fraction (5−35 μg protein). Control reactions contained membranes with no cytosol, with wild-type cytosol, or with *sec23* cytosol alone. Wild-type cytosol fractions in the absence of *sec23* cytosol were also tested for transport activity. Transport efficiency was 28% (normal) in the presence of wild-type cytosol in this experiment.

Acknowledgements

We thank Georjana Barnes for yeast genomic DNA libraries, Lorraine Pillus for advice on antibody affinity-purification and John Teem for communicating unpublished results. Mitch Bernstein, Ray Deshaies and Greg Payne generously provided valuable advice throughout this work, and Martha Cyert, Brian Hicke, Greg Payne, Linda Wuestehube and an anonymous reviewer improved the manuscript with their critical comments. This work was supported by a grant from the National Institutes of Health (GM26755) and L.H. was supported by a National Science Foundation predoctoral fellowship.

References

Baker,D., Hicke,L., Rexach,M., Schleyer,M. and Schekman,R. (1988) *Cell*, **54**, 335−344.

Balch,W.E., Wagner,K.R. and Keller,D.S. (1987) *J. Cell Biol.*, **104**, 749−760.

Ballou,C. (1970) *J. Biol. Chem.*, **245**, 1197−1203.

Beckers,C.J.M., Keller,D.S. and Balch,W.E. (1989) *Cell*, **50**, 523−534.

lysis of X2180-1A cells (*MaATa, gal2*) as described (Ferro-Novick *et al.*, 1984).

Bernstein,M., Hoffman,W., Ammerer,G. and Schekman,R. (1985) *J. Cell Biol.*, **101**, 2374−2382.

Block,M.R., Glick,B.S., Wilcox,C.A., Wieland,F.T. and Rothman,J.E. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7852−7856.

Böhni,P.C., Deshaies,R.J. and Schekman,R.W. (1988) *J. Cell Biol.*, **106**, 1035−1042.

Burnette,W.M. (1981) *Anal. Biochem.*, **112**, 195−203.

Carlson,M. and Botstein,D. (1982) *Cell*, **28**, 145−154.

Dabora,S.L. and Sheetz,M.P. (1988) *Cell*, **54**, 27−35.

Deshaies,R.J. and Schekman,R. (1987) *J. Cell Biol.*, **105**, 633−645.

Eakle,K.A., Bernstein,M. and Emr,S.D. (1988) *Mol. Cell. Biol.*, **8**, 4098−4109.

Feldman,R.I., Bernstein,M. and Schekman,R. (1987) *J. Biol. Chem.*, **262**, 9332−9339.

Ferro-Novick,S., Novick,P., Field,C. and Schekman,R. (1984) *J. Cell Biol.*, **98**, 35−43.

Fujiki,Y., Hubbard,A.L., Fowler,S. and Lazarow,P.B. (1982) *J. Cell Biol.*, **93**, 97−102.

Glick,B.S. and Rothman,J.E. (1987) *Nature*, **326**, 309−312.

Hopp,T. and Woods,K.R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 3824−3828.

Hunter,W.M. and Greenwood,F.C. (1962) *Nature*, **194**, 495−496.

Laemmli,U.K. (1970) *Nature*, **227**, 680−685.

Lee,C. and Chen,L.B. (1988) *Cell*, **54**, 37−46.

Nakano,A., Brada,D. and Schekman,R. (1988) *J. Cell Biol.*, **107**, 851−863.

Newman,A.P. and Ferro-Novick,S. (1987) *J. Cell Biol.*, **105**, 1587−1594.

Novick,P., Field,C. and Schekman,R. (1980) *Cell*, **21**, 205−215.

Pfeffer,S. and Rothman,J. (1987) *Annu. Rev. Biochem.*, **56**, 829−852.

Rose,M.D., Novick,P., Thomas,J.H., Botstein,D. and Fink,G.R. (1987) *Gene*, **60**, 237−243.

Ruohola,H., Kabcenell,A.K. and Ferro-Novick,S. (1988) *J. Cell Biol.*, **107**, 1465−1476.

Ruther,U. and Muller-Hill,B. (1983) *EMBO J.*, **2**, 1791−1794.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463−5467.

Schauer,I., Emr,S., Gross,C. and Schekman,R. (1985) *J. Cell Biol.*, **100**, 1664−1675.

Scott,J. and Schekman,R. (1980) *J. Bacteriol.*, **142**, 414−423.

Received on December 2, 1988; revised on March 7, 1989