A Membrane Glycoprotein, Sec12p, Required for Protein Transport from the Endoplasmic Reticulum to the Golgi Apparatus in Yeast

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Abstract. *SEC12*, a gene that is required for secretory, membrane, and vacuolar proteins to be transported from the endoplasmic reticulum to the Golgi apparatus, has been cloned from a genomic library by complementation of a *sec12* ts mutation. Genetic analysis has shown that the cloned gene integrates at the *SEC12* locus and that a null mutation at the locus is lethal. The DNA sequence predicts a protein of 471 amino acids containing a hydrophobic stretch of 19 amino acids near the COOH terminus. To characterize the gene product (Sec12p) in detail, a *lacZ-SEC12* gene fusion has been constructed and a polyclonal antibody raised against the hybrid protein. The antibody recognizes Sec12p as a ~70-kD protein that sediments in a mixed membrane fraction that includes endoplasmic reticulum. Sec12p is not removed from the membrane fraction by treatment at high pH and high salt and is not degraded by exogenous protease unless detergent is present. Glycosylation of Sec12p during biogenesis is indicated by an electrophoretic mobility shift of the protein that is influenced by tunicamycin and by imposition of an independent secretory pathway block. We suggest that Sec12p is an integral membrane glycoprotein with a prominent domain that faces the cytoplasm where it functions to promote protein transport to the Golgi apparatus. In the process of transport, Sec12p itself may migrate to the Golgi apparatus and function in subsequent transport events.

A series of temperature-sensitive secretory mutants has been isolated from *Saccharomyces cerevisiae* and complementation analyses have shown that at least 27 genes are involved in the yeast secretory pathway (9, 14, 28, 31). Biochemical and morphological analyses of these *sec* mutants have revealed that the process of protein export in yeast can be dissected into several steps: protein translocation through the membrane of the endoplasmic reticulum (ER),[1] folding and posttranslational modification in the ER, transport from the ER to the Golgi apparatus, transit through the Golgi apparatus to form secretory vesicles, and discharge of the contents of secretory vesicles at the plasma membrane (36). Among these steps, the movement of proteins from the ER to the Golgi apparatus constitutes the first step of transport from a membrane-bound organelle. Protein sorting and packaging events that occur at this stage may use mechanisms that are repeated in later steps of membrane-to-membrane protein traffic.

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1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; DPAP, dipeptidyl amino-peptidase; endo H, endoglycosidase H; ER, endoplasmic reticulum; HSP, high-speed pellet; HSS, high-speed supernatant; LSP, low-speed pellet; LSS, low-speed supernatant.

The ER-to-Golgi transfer process requires ≥10 genes (31). *SEC12* is one of these 10; a temperature-sensitive mutant, *sec12*, fails to transport secretory, membrane, and vacuolar proteins from the ER to the Golgi apparatus at a restrictive temperature (29, 31, 40). To understand the function of the *SEC12* gene, we have characterized its product, Sec12p, by the application of molecular cloning techniques. We report that Sec12p is an integral membrane glycoprotein that resides in the ER and Golgi membranes.

Materials and Methods

Strains, Plasmids, and Materials

The bacterial and yeast strains used in this study are listed in Table I.

Escherichia coli plasmids pUC18 (42) and pUR290 (32); *E. coli*–yeast shuttle plasmids YEp13 (5), pSEY8, and pSEYC58 (11); and yeast integration vector YIp5 (4) have been described previously. The yeast genomic DNA library originally constructed by Nasmyth and Tatchell (27) contains DNA fragments of a Sau 3A partial digest inserted into the Bam HI site of YEp13.

YPD medium contained 1% Bacto-Yeast Extract, 2% Bacto-Peptone (both from Difco Laboratories, Inc., Detroit, MI), and 5% glucose. Minimal medium (41) was also used with 5% glucose.

All reagents used were of analytical grade. [α-^{32}P]dCTP, $H_2{}^{35}SO_4$, [α-^{35}S]dCTP, and Na^{125}I were obtained from Amersham Corp. (Arlington Heights, IL). Nitrocellulose filters were from Schleicher & Schuell, Inc. (Keene, NH), and nylon membranes were from Sartorius Filters Inc. (Hay-

Table I. Strains

Strain	Genotype	Source or reference
S. cerevisiae		
X2180-1B	*Matα gal2*	YGSC*
MBY10-7A	*Mata sec12-4 ura3-52 leu2-3,112 trpl-289 his suc*	M. Bernstein‡
MBY10-7C	*Matα sec12-4 ura3-52 leu2-3,112 trpl-289 his suc*	M. Bernstein‡
MBY12-6D	*Matα sec18-1 ura3-52 leu2-3,112 trpl-289 his*	M. Bernstein‡
SF821-8A	*Mata sec7-1 ura3-52 leu2-3,112 trpl-289 his4-580*[a]	R. Schekman and C. Field‡
ANY1-7D	*Mata ura3-52 leu2-3,112*	This study
TAY69	*Mata/Matα ura3-52/ura3-52 leu2-3,112/leu2-3,112 suc2-Δ9/suc2-Δ9 HIS4/his4-519 ADE2/ade2-1*	T. Achstetter‡
E. coli		
MC1061	*F⁻ araD139 Δ(araABOIC-leu)7679 Δlac$_x$74 galU galK rpsL hsdR*	Reference 8
BMH71-18	*Δ(lac pro) F' lacI^{q}Z ΔM15 pro$^+$*	Reference 26

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ward, CA). Lyticase was prepared as described before (37). Protein A was labeled with ^{125}I by the chloramine T method (17). Endoglycosidase H (endo H) was a gift from P. Robbins (Biology Department, Massachusetts Institute of Technology, Cambridge, MA) and used as described before (40).

Cloning and Sequence Analysis of SEC12

DNA clones that complement a *sec12* ts mutation were isolated as described previously (2). Briefly, a yeast *sec12-4* strain, MBY10-7C, was transformed with a genomic DNA library in YEp13 which contains the *LEU2* gene as a selectable marker (27). Transformants were selected on leucine-free minimal medium at 24°C for 24 h, followed by incubation at 37°C (*sec12* restrictive growth temperature). Growing colonies were chosen and plasmid DNA was isolated. Yeast plasmid DNA was propagated in *E. coli* strain MC1061 and purified. Each plasmid was confirmed for *sec12* complementation and leucine auxotrophy in the original yeast host. Two distinct clones were obtained and named pSEC1210 and pSEC1230; the latter proved to contain the authentic *SEC12* gene (Fig. 1; also see Results).

DNA manipulations including restriction endonuclease digestions, ligations, plasmid isolation, and *E. coli* transformation were carried out by the standard methods (suppliers' protocols; see also reference 25). Yeast transformation was achieved either by the spheroplast (38) or lithium acetate method (18). DNA nucleotide sequences were determined by the dideoxy method (34) in combination with deletion techniques using exonuclease III (Bethesda Research Laboratories, Gaithersburg, MD) or nuclease BAL 31 (International Biotechnologies, Inc., New Haven, CT).

Northern Blotting Analysis

The 0.7-kb Bam HI–Sal I fragment from pSEC1230 (Fig. 1, fragment *D*) was subcloned into pUC18 and the 4.0-kb Xho I–Hind III fragment (Fig. 1, fragment *B*) into YIp5. These recombinant plasmids were nick translated in the presence of [α-^{32}P]dCTP (25) and used as hybridization probes. Poly(A)$^+$RNA purified from yeast strain X2180-1B was electrophoresed in an agarose gel containing 2.2 M formaldehyde. Transfer of RNA to nitrocellulose and DNA-RNA hybridization was performed as described (25).

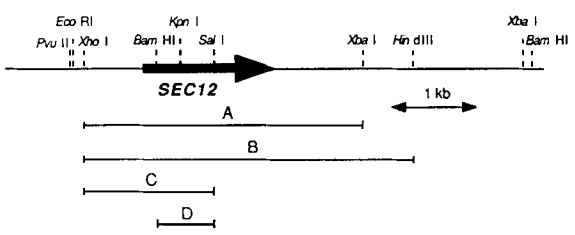

Figure 1. Restriction map of the 6.4-kb insert in plasmid pSEC1230, which contains the authentic *SEC12* gene. The heavy arrow indicates the coding region of *SEC12* and its direction of transcription. Fragments *A–D* were subcloned into different vectors as described in the text.

Sec12p Antiserum

The Bam HI–Hind III fragment from pSEC1230 was inserted into pUR290 to make an in-frame gene fusion of *lacZ* and *SEC12*. The resultant plasmid, pANF1 (see Fig. 4 *a*), was introduced into *E. coli* strain BMH71-18 and the transformant was induced to express a *lacZ-SEC12* fusion gene product (~160 kD). This hybrid protein was purified by a modification of the method of Bernstein et al. (2). BMH71-18/pANF1 was grown in 1 liter of Luria broth/amp medium at 37°C to an OD_{550} of 0.7 and isopropylthio-β-D-galactoside was added to 1 mM. After a 1-h incubation at 37°C, cells were harvested and treated with 0.2 mg/ml lysozyme. Spheroplasts were broken by freeze-thawing and subjected to a brief sonication. Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM. The lysate (10 ml) was centrifuged for 1 h at 100,000 *g* in a rotor (type 40; Beckman Instruments, Inc., Fullerton, CA) at 4°C. The pellet was resuspended in 5 ml of 0.02% SDS and again centrifuged. About 70% of total β-galactosidase activity was recovered in the final pellet fraction. This final pellet was solubilized with 3 ml of SDS gel sampling buffer in a boiling water bath and subjected to preparative SDS-PAGE. The hybrid protein band was cut out and the protein eluted from the gel electrophoretically. Eluted protein was extensively dialyzed against distilled water and lyophilized.

The purified hybrid protein was suspended in PBS (20 mM sodium phosphate, pH 7.4, and 150 mM NaCl) and used for immunizing rabbits. Primary injections contained ~100 μg of the protein in complete Freund's adjuvant. Boosts of ~50 μg in incomplete Freund's adjuvant were given at 7–10-d intervals. The maximal titer was obtained after eight boosts.

Subcellular Fractionation

Wild-type yeast strain ANY1-7D harboring the *SEC12* multicopy plasmid (pANY1-9; pSEY8 containing Xho I–Xba I fragment from pSEC1230) was grown to 1 OD_{600} in 500 ml minimal medium and centrifuged. Cells were treated with 10 ml of 0.1 M Tris-HCl, pH 9.4, and 10 mM dithiothreitol at 30°C for 20 min, sedimented, and resuspended in 10 ml YPD medium containing 0.5% glucose and 0.7 M sorbitol. Lyticase (6,000 U) was added and incubation continued for 45 min. Spheroplasts were collected by centrifugation through a 20-ml cushion of 1.2 M sorbitol, resuspended in 10 ml lysis buffer (0.3 M mannitol, 0.1 M KCl, 50 mM Tris-HCl, pH 7.4, and 1 mM EGTA), and immediately homogenized with a motor-driven Teflon pestle five times for 1 min with 1-min intervals on ice. The extent of lysis was monitored visually in a phase-contrast microscope. As an indicator of membrane aggregation, the latency of NADPH cytochrome *c* reductase to its substrate, cytochrome *c*, was examined routinely by assaying reductase activity in the presence and absence of 0.1% Triton X-100 (13). Less than 3% of the total activity was latent in each experiment, indicating that the cytoplasmic surface of the ER membrane was almost completely accessible to exogenous cytochrome *c*. Membrane fractions were separated by differential centrifugation. First, the homogenate was centrifuged at 1,000 *g* for 10 min in a rotor (model SS-34; Sorvall Instruments Div., Newton, CT) to yield low-speed pellet (LSP) and low-speed supernatant (LSS) fractions. The LSS fraction was further centrifuged at 100,000 *g* for 1 h in a type 40 rotor (Beckman Instruments, Inc.) to give a high-speed pellet (HSP) and a high-speed supernatant (HSS).

Protein was determined by the method of Schaffner and Weissman (35). NADPH cytochrome *c* reductase was assayed as an ER marker enzyme as described by Kubota et al. (21). Heat-stable and heat-labile dipeptidyl amino-peptidases (DPAPs) were assayed as Golgi and vacuole markers, respectively (3, 20). The cytoplasmic form of invertase and carboxypeptidase Y (CPY; mature form, vacuolar) were quantified by immunoblotting.

Immunoblotting and Immunoprecipitation

Crude cell extracts or subcellular fractions subjected to SDS gel electrophoresis were transferred to nitrocellulose filters (6), which were briefly stained with Ponceau S and incubated in Tris-buffered saline (50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) containing 0.1% NP-40 and 1% nonfat dry milk for 1 h. Antibody incubations were performed in the same buffer for 3 h at room temperature. Filters were washed three times with the buffer for 15 min each, treated with ^{125}I-protein A (5×10^4 cpm/ml, 1×10^7 cpm/μg protein) for 1 h at 22°C, washed again three times, and autoradiographed.

For immunoprecipitation of Sec12p, cells were grown to an OD_{600} of 0.5–1.5 in minimal medium containing 100 μM $(NH_4)_2SO_4$. Aliquots (2 OD_{600}) were harvested and resuspended in 1 ml of minimal medium containing 5 μM $(NH_4)_2SO_4$. $H_2{}^{35}SO_4$ (0.5 mCi) was added and incubation continued for 1 h at 30°C. To inhibit N-linked glycosylation, 10 μg/ml tunicamycin (Sigma Chemical Co., St. Louis, MO) was added 15 min before the start of labeling. Labeled cells were chilled on ice, washed with 10 mM NaN_3 in PBS, and resuspended in 200 μl of PBS containing 1% SDS and 1 mM phenylmethylsulfonyl fluoride. Glass beads (0.2 g) were added and suspensions were vortexed for 2 min followed by heating in boiling water for 5 min. Extracts were adjusted to 1 ml by addition of 2% Triton X-100 in PBS and clarified by centrifugation for 2 min in a microcentrifuge (Fisher Scientific Co., Pittsburgh, PA). Aliquots (0.5 ml) were incubated with 10 μl anti-Sec12p antiserum on ice overnight and then with 5 μl (packed swollen volume) of Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) with gentle agitation at room temperature for 2 h. Sepharose bead-immune complexes were washed twice with PBS containing 1% Triton X-100 and 0.1% SDS and once with PBS alone. Immunoprecipitates suspended in SDS gel sample buffer were eluted from the beads by heating in boiling water and applied to SDS gels containing 7.5% polyacrylamide. After electrophoresis, gels were treated with Amplify (Amersham Corp.) and fluorographed.

In pulse-chase experiments, cells (2 OD_{600}) were preincubated for 15 min in 1 ml of minimal medium containing no sulfate, then labeled with 2 mCi $H_2{}^{35}SO_4$ for 5 min. Chase was performed by adding 100 mM $(NH_4)_2SO_4$, 25 mM cysteine, and 27 mM methionine. Aliquots (180 μl) were taken at appropriate time points. Extracts were prepared as described above.

Electron Microscopy

Preparation of thin sections of yeast cells and the details of the protein A-gold method used for yeast will be described elsewhere (Brada and Schekman, 1988). Briefly, a *sec7* strain harboring the *SEC12* multicopy plasmid (SF821-8A/pANY1-9) was fixed twice with a solution containing 2% formaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate-HCl, pH 6.8, 1.5 mM $CaCl_2$, and 2.5 mM $MgCl_2$ at 20°C for 30 min each. Fixed cells were embedded in 2% low-melting-point agarose and dehydrated in ethanol. Dehydrated agarose gel blocks were embedded in Lowicryl K4M resin, which was then polymerized by UV irradiation at −35°C. Ultrathin sections were prepared and subjected to immune labeling. Sections on a grid were preincubated with 1% BSA in PBS for 5 min. The grid was then incubated with 10-fold concentrated anti-Sec12p antibody for 2 h, washed twice with PBS, incubated with protein A-gold suspension (8-nm particles) in 1% Triton X-100 in PBS at an OD_{525} of 0.13 for 45 min, and washed again with PBS, 1% Triton X-100 in PBS, PBS, and water. Sections were stained with uranyl acetate and lead citrate and examined under an electron microscope. In a competition experiment, 0.1 mg/ml purified *lacZ-SEC12* hybrid protein was included in the incubation with anti-Sec12p antibody.

Results

Cloning and Genetic Analysis of the Sec12 Gene

Yeast genomic DNA clones that complement the ts mutation *sec12-4* were isolated from a DNA library constructed on the multicopy vector YEp13. Among 15,0000 Leu$^+$ transformants, 20 showed a Ts$^+$ phenotype. Analysis of plasmids purified from these Ts$^+$ transformants revealed that two distinct fragments of DNA could complement *sec12-4*. Representative clones containing each type of insert were named pSEC1210 and pSEC1230. Southern hybridization probing of one clone to the other showed no homology between the two inserts (data not shown). Smaller fragments from the inserts were subcloned into a multicopy plasmid, pSEY8, or a single-copy centromere plasmid, pSEYC58, and the complementation of *sec12-4* was examined. Every fragment from pSEC1230 that complemented *sec12-4* on a multicopy plasmid did so on a single-copy plasmid as well. On the other hand, the smallest fragment from pSEC1210 that conferred a Ts$^+$ phenotype to *sec12-4* on a multicopy plasmid failed to give Ts$^+$ transformants on a single-copy plasmid. Therefore, we tentatively assumed that pSEC1230 contained the *SEC12* gene itself, whereas pSEC1210 contained a gene that could suppress the *sec12-4* mutation when its gene dosage was elevated.

A restriction map of the insert of pSEC1230 is shown in Fig. 1. The Xho I–Xba I fragment (Fig. 1, fragment *A*) was the smallest piece of DNA that complemented *sec12-4*, either on the single-copy plasmid pSEYC58 or on the multicopy plasmid pSEY8. Any further deletion of this fragment to the Bam HI, Kpn I, or Sal I sites resulted in a failure to complement, indicating that these sites were internal to the functional region of complementing activity. To show that this activity was due to the *SEC12* gene, chromosomal mapping of the cloned fragment was done. The Xho I–Hind III fragment (Fig. 1, fragment *B*) was subcloned into the integration vector YIp5, which harbors the *URA3* gene as a marker. The recombinant plasmid was linearized by Bam HI digestion to facilitate homologous recombination and introduced into a *sec12-4* strain, MBY10-7A. A representative Ura$^+$/Ts$^+$ transformant was mated with a *SEC12–URA3* strain to form a diploid, which was then sporulated and subjected to tetrad analysis. Among 37 asci analyzed, 35 showed 4:0 segregation of the Ts$^+$/Ts$^-$ phenotypes and two showed a 3:1 pattern. The Ura$^+$/Ura$^-$ phenotype segregated ~3:1. This experiment demonstrated that the cloned DNA was integrated at or very close to the *sec12* locus, supporting the assumption that pSEC1230 contained the *SEC12* gene.

The Xho I–Sal I fragment (Fig. 1, fragment *C*) on a centromere plasmid, which itself did not complement *sec12-4*, was capable of converting the ts allele at a much higher frequency than by spontaneous reversion. Plasmid pSEYC58, with or without fragment *C* as an insert, was introduced into MBY10-7A. Several transformants were picked and grown in liquid culture to promote recombination between the plasmid and chromosomes. Cells were plated and the appearance of Ts$^+$ colonies was examined. The plasmid with fragment *C* conferred a Ts$^+$ phenotype at a frequency of 1.5×10^{-6}, whereas the vector alone gave no Ts$^+$ transformants out of 4×10^7 cells. This observation suggested that fragment *C* contained the site of the *sec12-4* mutation. Thus, from two lines of evidence, we confirmed that clone pSEC1230 contained the authentic *SEC12* gene.

The plasmid pSEC1210 allowed growth of *sec12-4* at the restrictive temperature by a different mechanism. This apparent complementation may result from overproduction of another protein that suppresses the effect of the *sec12-4* muta-

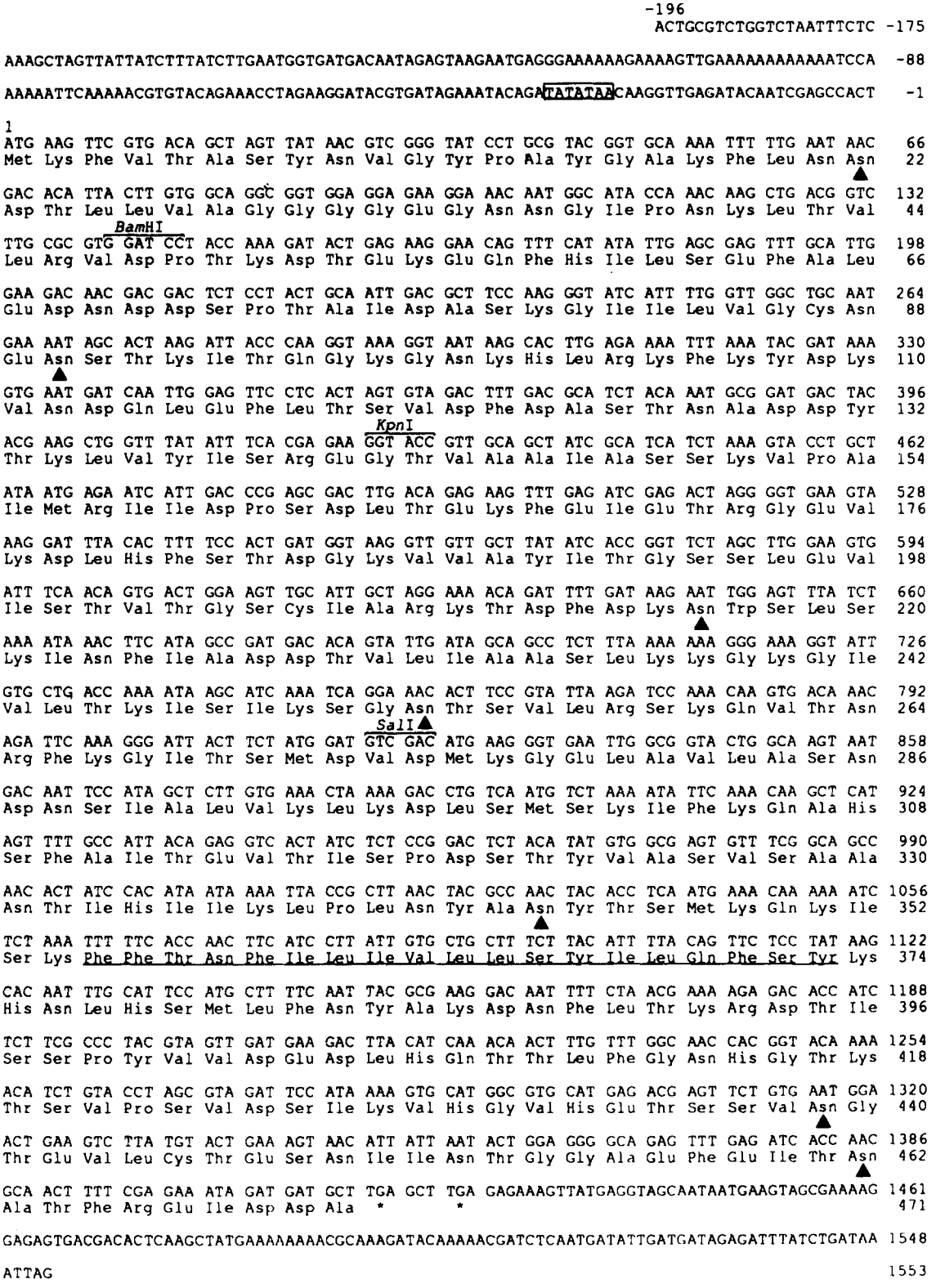

Figure 2. Nucleotide sequence of *SEC12* gene and its predicted amino acid sequence. Solid triangles indicate possible sites of N-glycosylation. Underline indicates a hydrophobic stretch of 19 amino acid residues.

tion. pSEC1210 suppressed three separate ts isolates of *sec12 (sec12-1, -3,* and *-4*), but did not suppress the effect of a disrupted *SEC12* gene (see below). Hence, the suppressor function on this DNA clone must work in concert with a partly defective Sec12 protein, and not in the absence of the protein. The nature of the suppressor gene is currently being investigated.

As with all other yeast *sec* mutants, *sec12* was isolated as

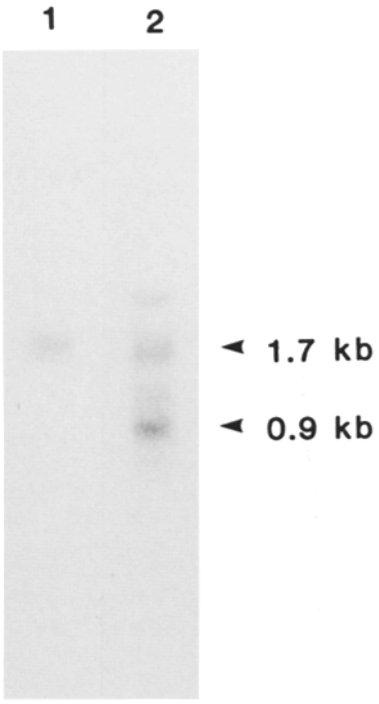

Figure 3. Northern hybridization analysis of *SEC12* transcript. Poly(A)$^+$ RNA from wild-type yeast was resolved on an agarose gel and blotted to nitrocellulose. Nick-translated DNA probes containing the internal fragment of *SEC12* (lane *1*) and whole *SEC12* and *URA3* genes (lane *2*) were used for hybridization. *1.7 kb*, *SEC12* message; *0.9 kb*, *URA3* message.

a conditional ts lethal, supporting the assumption that protein secretion is coupled to cell surface expansion and therefore to cell growth (28). To test the possibility that *sec12* isolates were null mutations that exposed an independent thermosensitive process, the chromosomal copy of *SEC12* was disrupted and the phenotypic consequences monitored. The internal Bam HI–Sal I fragment of the *SEC12* gene (Fig. 1, fragment *D*) was subcloned into YIp5, linearized by cleavage with Kpn I, and introduced into a *SEC12/SEC12*, *ura3/ura3* diploid strain, TAY69. Stable transformants were induced to sporulate and the resulting tetrads dissected. Among 33 asci analyzed, 24 gave rise to two viable spores and 9 tetrads produced only one progeny. None of the viable spores were Ura$^+$. A Southern hybridization experiment confirmed that the diploid transformants contained both the wild-type and disrupted copies of *SEC12*, whereas all viable progeny contained only the wild-type copy (data not shown). These observations indicated that spores with the disrupted *SEC12* gene could not initiate cell division. Hence *SEC12* is directly essential for cell growth.

DNA Sequence and Transcription of SEC12

The nucleotide sequence of a 1.7-kb DNA fragment containing the *SEC12* gene was determined. As shown in Fig. 2, *SEC12* contained a single long open reading frame uninterrupted with introns, preceded by several stretches of A's and a TATA sequence (Fig. 2, *box*), and terminated by two TGA codons. A polypeptide of 471 amino acids is predicted with an amino acid composition of high hydrophilicity (25% charged). Hydropathic analysis by the method of Kyte and Doolittle (22) detected a hydrophobic stretch of 19 amino acids (Fig. 2, *underline*) flanked by three positively charged amino acids on both sides. This is a feature typical of the membrane-spanning domains of integral membrane proteins. Other hydrophobic sequences predicted in *SEC12* were not of sufficient length to span a membrane lipid bilayer. Seven triangles in Fig. 2 depict possible N-glycosylation sites. The protein is rich in serine and threonine residues which are possible acceptors of O-linked oligosaccharides (see below). No significant homology in amino acid sequence was found to proteins catalogued in the protein data bank from the National Biomedical Research Foundation (23).

Transcription of *SEC12* was examined by Northern hybridization. The DNA fragment internal to *SEC12* (Fig. 1, fragment *D*) was used as a probe to identify *SEC12* mRNA. As shown in Fig. 3, lane *1*, a single species of ~1.7 kb was seen, which corresponded to the size of *SEC12* message predicted from the DNA sequence. To estimate the abundance of this mRNA, the hybridization intensity was compared to *URA3* mRNA (0.9 kb) using a nick-translated probe containing both the *SEC12* and *URA3* genes (YIp5 containing fragment *B* of Fig. 1). Lane *2* in Fig. 3 shows that the probe hybridized *SEC12* mRNA about half as strongly as *URA3* mRNA. Assuming that the *URA3* message represented ~0.1% of total mRNA (1), a simple calculation leads to an estimate that the *SEC12* message was ~0.02–0.03% of total mRNA or two to four copies per cell. A similar Northern blot experiment using single-stranded DNA probes confirmed the direction of transcription as shown in Fig. 1 (data not shown).

Identification of the SEC12 Gene Product, Sec12p

To identify and characterize the *SEC12* gene product, Sec12p, we prepared an antibody against a *lacZ–SEC12* fusion gene product. The fusion (Fig. 4 *a*) contained the promoter region and almost the whole coding sequence of the *E. coli lacZ* gene, contained on the fusion vector pUR290 (32), joined to 90% of the *SEC12* coding sequence at the Bam HI site. *E. coli* strain BMH71-18 transformed with this gene fusion (pANF1) produced a novel 160-kD protein upon induction with isopropylthio-β-D-galactoside. This hybrid protein was purified and used to immunize rabbits. The resulting polyclonal antiserum showed titer against both *E. coli* β-galactosidase and yeast Sec12p epitopes, and was used without further purification.

Sec12p was detected in yeast extracts by two methods. First, wild-type or *sec12* yeast cells that contained a multicopy plasmid with or without the *SEC12* gene insert were lysed and analyzed by SDS-PAGE and Western immunoblotting (Fig. 4 *b*). The antibody recognized a single band with an apparent molecular mass of 70 kD when the *SEC12* plasmid was present in the cells (Fig. 4 *b*, lanes *2* and *4*). This band was not readily detected in the absence of the multicopy *SEC12* gene (Fig. 4 *b*, lanes *1* and *3*), indicating that Sec12p was not abundant. The mutant *sec12* strain harboring the *SEC12* multicopy plasmid (Fig. 4 *b*, lane *4*) had a higher level of Sec12p expression than the wild-type strain containing the same plasmid (Fig. 4 *b*, lane *2*), though both transformants grew similarly at 37°C.

Sec12p was also detected by immune precipitation from extracts of radiolabeled cells. The wild-type strain over-

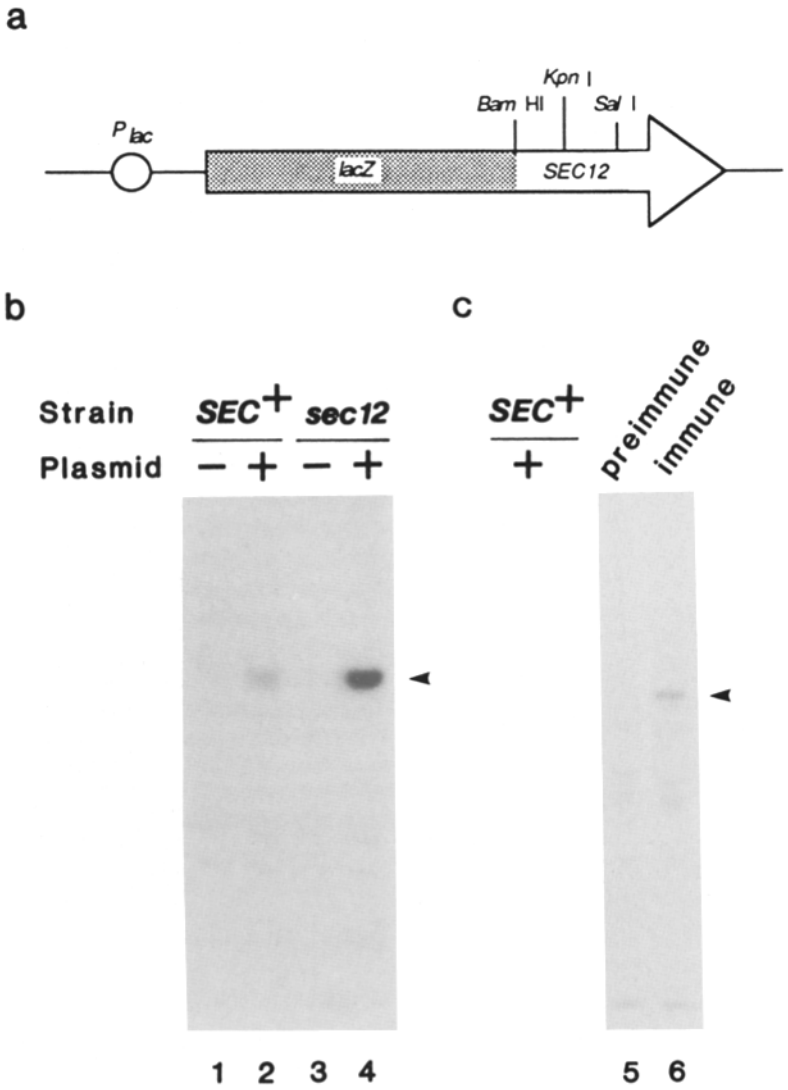

Figure 4. Identification of *SEC12* gene product, Sec12p. (*a*) A construct of *lacZ–SEC12* gene fusion used for raising anti–Sec12p antibody. The hybrid protein was produced in *E. coli*, purified, and injected into rabbits to yield a polyclonal antiserum. (*b*) Western blot analysis of yeast extracts using the obtained anti–Sec12p antibody. The wild-type (*SEC*$^+$, ANY1-7D) and mutant (*sec12*, MBY10-7A) strains with a multicopy plasmid containing the *SEC12* gene (+, pANY1-9) or vector alone (−, pSEY8) were grown in a minimal medium lacking uracil to maintain the plasmid. Extracts were prepared by agitation with glass beads in the SDS gel sampling buffer. Proteins were resolved on a polyacrylamide gel and blotted to a nitrocellulose filter. The filter was decorated with anti–Sec12p antibody and [^{125}I]protein A, and exposed to x-ray film. *Arrowhead*, Sec12p (70 kD). (*c*) Immunoprecipitation of Sec12p. A wild-type strain harboring the *SEC12* multicopy plasmid (ANY1-7D/pANY1-9) was labeled with [^{35}S]sulfate at 30°C for 1 h and an extract prepared by agitation with glass beads in the presence of 1% SDS. The extract was adjusted to 1.6% Triton X-100, 0.2% SDS, and subjected to immunoprecipitation using a preimmune serum or the immune anti–Sec12p antiserum in combination with protein A–Sepharose. The immunoprecipitates were resolved on an SDS gel and fluorographed. *Arrowhead*, Sec12p.

producing Sec12p was grown in minimal medium and labeled with [^{35}S]SO_4^{2-} at 30°C for 1 h. Extracts treated with anti–Sec12p antibody and Protein A–Sepharose precipitated a single species (Fig. 4 *c*). This protein was not precipitated by preimmune serum (Fig. 4 *c*, lane *5*) or when the immune serum incubation was performed in the presence of excess purified *lacZ–SEC12* hybrid protein (data not shown).

Sec12p, a Membrane Protein That Fractionates with Rapidly Sedimenting Membranes

Sec12p was examined in subcellular fractions obtained from a wild-type strain harboring the *SEC12* multicopy plasmid. Cells were converted to spheroplasts, homogenized, and subjected to differential centrifugation. All fractions were analyzed by Western immunoblotting. The data in Fig. 5 *a* show that Sec12p was almost exclusively found in a LSP fraction. Only a small amount was detected in LSS or HSP fractions; nothing remained soluble after a high speed centrifugation. Table II shows the distribution of various marker proteins in these fractions. A cytosolic protein (cytoplasmic invertase) was recovered in the HSS. Vacuolar proteins were found predominantly in the LSP. However, 40% of a soluble protein (mature CPY) was recovered in the HSS and a significant amount of a vacuolar membrane protein (heat-labile DPAP) was detected in the HSP. A Golgi enzyme, heat-stable DPAP, fractionated equally in the LSP and HSP. The bulk of the ER marker enzyme, NADPH cytochrome *c* reductase, was recovered in the LSP. Hence, the Sec12p was contained in a mixed membrane fraction that included most of the ER.

Two experiments were conducted to test whether Sec12p was soluble in a membrane-enclosed compartment or was an integral membrane protein. First, the LSP fraction was treated with 0.1 M Na_2CO_3, pH 11.5, and centrifuged. This treatment generated membrane sheets, removing soluble and peripherally associated membrane proteins (15). Fig. 5 *b* shows that Sec12p remained sedimentable after this treatment, suggesting a firm association with the lipid bilayer. Second, a "detergent blotting" method developed by Ito and Akiyama (19) was used to assess membrane association of Sec12p. In a modification of the normal immunoblot procedure, this technique uses a polyacrylamide gel containing the detergent NP-40 inserted between the original SDS gel and a nylon membrane. During electrophoretic transfer from the SDS gel to nylon, integral membrane proteins form large

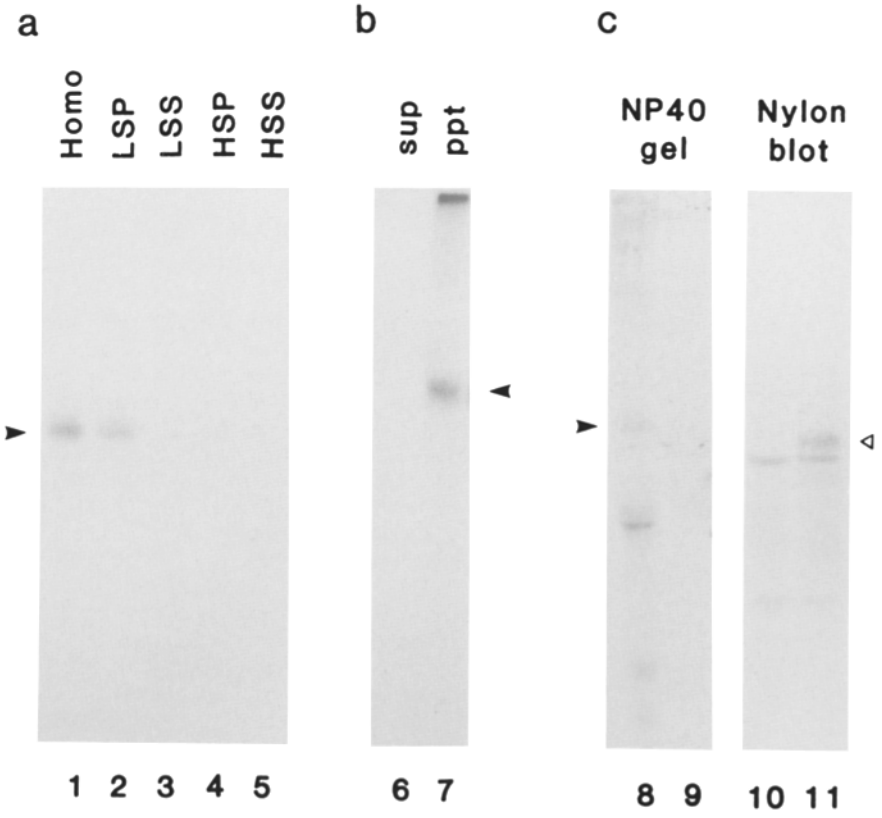

Figure 5. Determination of Sec12p localization by biochemical analyses. (*a*) Subcellular fractionation of membranes. Wild-type cells containing the *SEC12* multicopy plasmid (ANY1-7D/pANY1-9) were converted to spheroplasts and homogenized. The homogenate (2.9 mg protein/ml) in 0.3 M mannitol, 0.1 M KCl, 50 mM Tris-HCl, pH 7.4, 1 mM EGTA was centrifuged at 1,000 *g* for 10 min to separate LSP from LSS fractions. LSS was further fractionated by centrifugation at 100,000 *g* for 1 h into HSP and HSS fractions. Each fraction equivalent to 1 OD_{600} of cells was resolved on an SDS gel and subjected to immunoblot analysis using anti-Sec12p antibody. *Arrowhead*, Sec12p. (*b*) Sodium carbonate treatment of LSP. The LSP fraction at a final protein concentration of 10 mg/ml was treated with 0.1 M Na_2CO_3, pH 11.5, on ice for 30 min and centrifuged at 100,000 *g* for 1 h. Supernatant (*sup*) and pellet (*ppt*) fractions derived from the same amount of LSP were analyzed by immunoblot to determine the distribution of Sec12p. *Arrowhead*, Sec12p. (*c*) "Detergent blot" analysis of Sec12p. The same wild-type cells (ANY1-7D/pANY1-9) were labeled with $[^{35}S]SO_4^{2-}$ at 30°C for 1 h, lysed by agitation with glass beads in the presence of 1% SDS, and subjected to immunoprecipitation with anti-Sec12p (lanes *8* and *10*) or anti-CPY (lanes *9* and *11*) antibodies. Immunoprecipitates were resolved on an SDS-polyacrylamide gel and electrophoretically transferred to a nylon membrane. In this transfer, another polyacrylamide gel containing 1% NP-40 was placed between the original SDS gel and nylon (19). After transfer, the NP-40 gel and nylon were dried and autoradiographed. *Arrowhead*, Sec12p; *open triangle*, CPY. Other prominent bands are contaminants in the immunoprecipitates.

micelles with NP-40 molecules and are markedly retarded in migration. Sec12p, as well as the soluble vacuolar protein CPY as a control, were immunoprecipitated from wild-type cells labeled with $[^{35}S]SO_4^{2-}$ and subjected to detergent blotting. Fig. 5 *c* shows that Sec12p was trapped in the NP-40 gel (Fig. 5 *c*, lane *8*), whereas CPY passed through and was blotted to nylon (Fig. 5 *c*, lane *11*). These results substantiated the prediction that Sec12p is an integral membrane protein.

The orientation of Sec12p with respect to the bilayer was probed by treatment of homogenates with trypsin in the presence or absence of Triton X-100. Without detergent, Sec12p was not degraded during 30 min at 0°C (Fig. 6 *a*, lanes *1–4*). When the detergent was present, Sec12p was degraded rapidly to produce a 43-kD fragment (Fig. 6 *a*, lanes *5–8*). No shift in the mobility of Sec12p was produced in incubations that contained detergent but no protease (Fig. 6 *a*, lanes *9* and *10*). Resistance to trypsin in the absence of the detergent was not seen for cytosolic proteins (data not shown). These observations indicate that some site(s) in Sec12p was accessible to trypsin only when the membrane was permeabilized. Similar experiments using proteinase K instead of trypsin and saponin instead of Triton X-100 gave the same results (data not shown).

Table II. Subcellular Fractionation of Wild-Type Yeast

Protein	Localization	% Total LSP	HSP	HSS
Total protein		26	23	51
Invertase*	Cytosol	3	3	94
Mature CPY*	Vacuole content	50	10	40
Heat-labile DPAP	Vacuole membrane	65	24	11
Heat-stable DPAP	Golgi membrane	45	46	9
Cytochrome *c* reductase	ER membrane	76	14	10
Sec12p*		90	9	<1

The same fractions as in Fig. 5 *a* were analyzed. Proteins were quantified by Western blotting (*) or their activities were determined.

The presence or absence of N-linked carbohydrate on Sec12p and on the 43-kD fragment was used to probe further the orientation of Sec12p with respect to the membrane. Five potential N-glycosylation sites are found in the NH_2-terminal hydrophilic domain that precedes the putative membrane anchor peptide (Fig. 2); two such sites are represented on the COOH-terminal domain. If the NH_2-terminal domain faces the lumen of the organelle in which Sec12p resides, and proteolysis in the presence of detergent removes this domain from the whole protein, the 43-kD fragment should be glycosylated. If the 43-kD fragment instead represents a cytoplasmic, protease-resistant domain attached to the membrane anchor, with a trypsin cleavage site in the lumenal COOH-terminal domain, then the 43-kD fragment

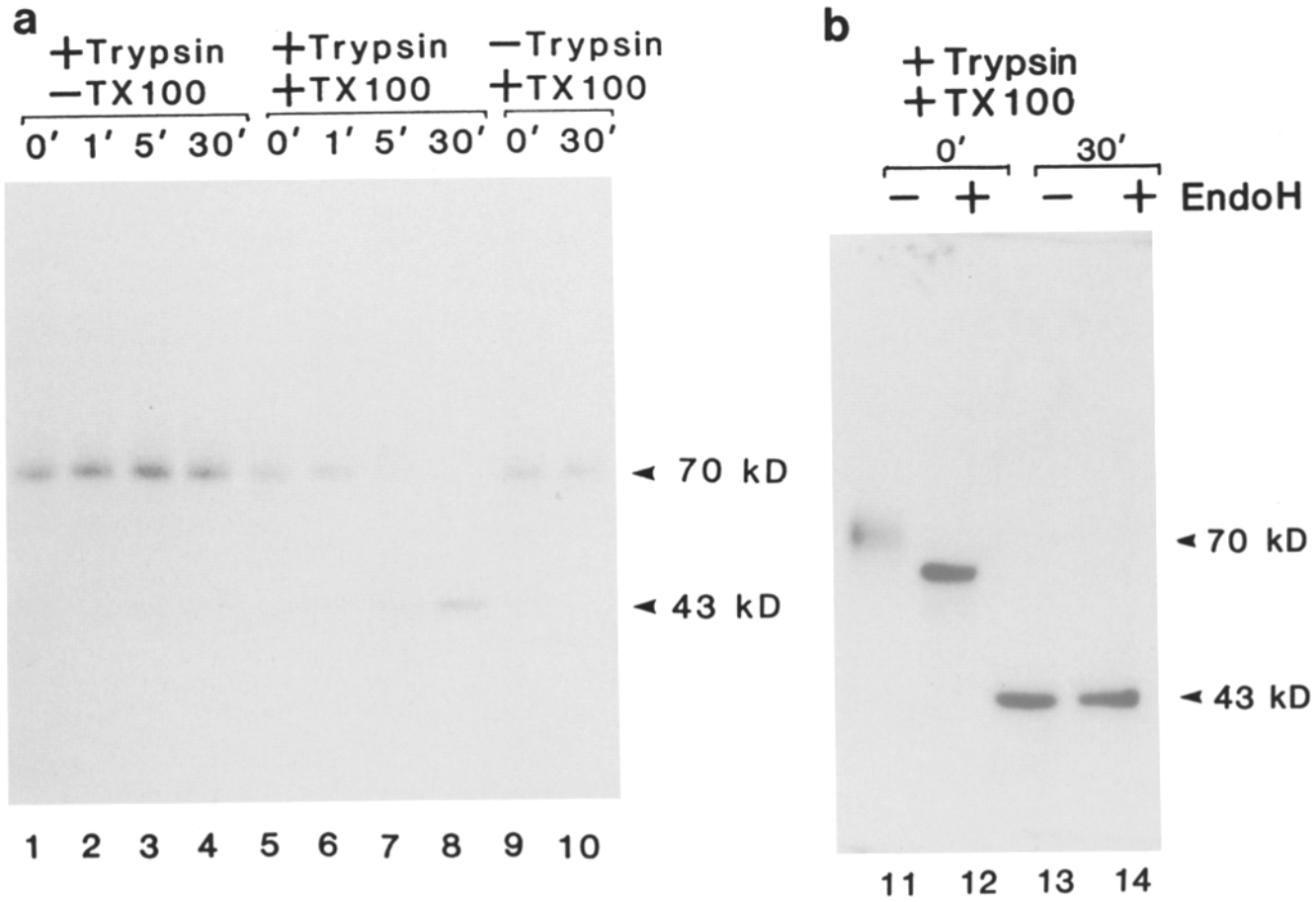

Figure 6. Susceptibility of Sec12p to tryptic digestion from the cytoplasmic side of the membranes. (*a*) The homogenate from wild-type cells (ANY1-7D/pANY1-9), identical to that shown in Fig. 5 *a*, lane *1*, was treated on ice with (lanes *1–8*) or without (lanes *9* and *10*) 0.5 mg/ml trypsin in the absence (lanes *1–4*) or presence (lanes *5–10*) of 0.1% Triton-X-100. After the indicated times, tryptic action was stopped by the addition of 1.1 mg/ml soybean trypsin inhibitor. Each sample equivalent to 1 OD_{600} of cells was resolved on an SDS gel and subjected to immunoblotting with anti-Sec12p antibody. *Arrowheads*, mature 70-kD form and 43-kD fragment of Sec12p. (*b*) Endo H sensitivity of the 70-kD protein and 43-kD fragment was examined. A homogenate was treated with 0.5 mg/ml trypsin in the presence of 0.1% Triton X-100 on ice for 0 or 30 min. Digestion was stopped by the addition of trypsin inhibitor followed by heating to 95°C in the presence of 1% SDS. Samples were divided in half, brought to final concentrations of 0.3% SDS, 0.15 M sodium citrate, pH 5.5, and 5 mM NaN_3, and treated with or without 0.05 U/ml endo H at 37°C for 18 h. Sec12p polypeptides were resolved on an SDS gel and detected by immunoblotting with anti-Sec12p antibody.

will not be glycosylated. Samples from untreated or trypsin + Triton X-100–treated fractions were exposed to endo H. The data in Fig. 6 *b* shows that heterogeneous 70-kD Sec 12p was converted to a more defined 65-kD species by removal of N-linked carbohydrate. In contrast, the 43-kD proteolytic fragment migrated sharply and was not reduced in apparent molecular mass by endo H treatment. This result suggested that the 43-kD fragment was not glycosylated and may represent a cytoplasmically oriented, protease-resistant domain of Sec12p.

Progressive Glycosylation of Sec12p

The 70-kD form of Sec12p detected by immunoblotting was larger than expected from the DNA sequence (Fig. 2; 51.6-kD polypeptide predicted). At least part of this difference was due to N-linked carbohydrate (Fig. 6 *b*). To detect additional covalent modifications of the polypeptide that would account for this discrepancy, the biosynthesis of Sec12p was examined in a pulse–chase experiment. Wild-type cells were pulse labeled with $[^{35}S]SO_4^{2-}$ for 5 min and chased for the indicated times in the presence (Fig. 7, lanes *1–5*) or absence (Fig. 7, lanes *6–10*) of tunicamycin. In the control, newly synthesized Sec12p displayed a gradual increase in apparent molecular mass up to 70 kD during the chase (Fig. 7, lanes *6–10*). In the presence of tunicamycin, Sec12p increased in size over a lower range of molecular mass (Fig. 7, lanes *1–5*). The apparent size difference was ~5 kD at each time point. Endo H treatment of Sec12p labeled in the absence of tunicamycin gave the same decrement of 5 kD in apparent molecular mass (Fig. 6 *b*). This result suggested that only part of the size anomaly of Sec12p was due to N-glycosylation with one or two oligosaccharide chains.

The progressive decrease in electrophoretic mobility of Sec12p was not due to modification of N-linked sugars because it occurred in the presence or absence of tunicamycin. To test the possibility that some other glycosyl modification took place, binding of Sec12p to concanavalin A (Con A) was examined (Fig. 8); and samples from a pulse–chase experi-

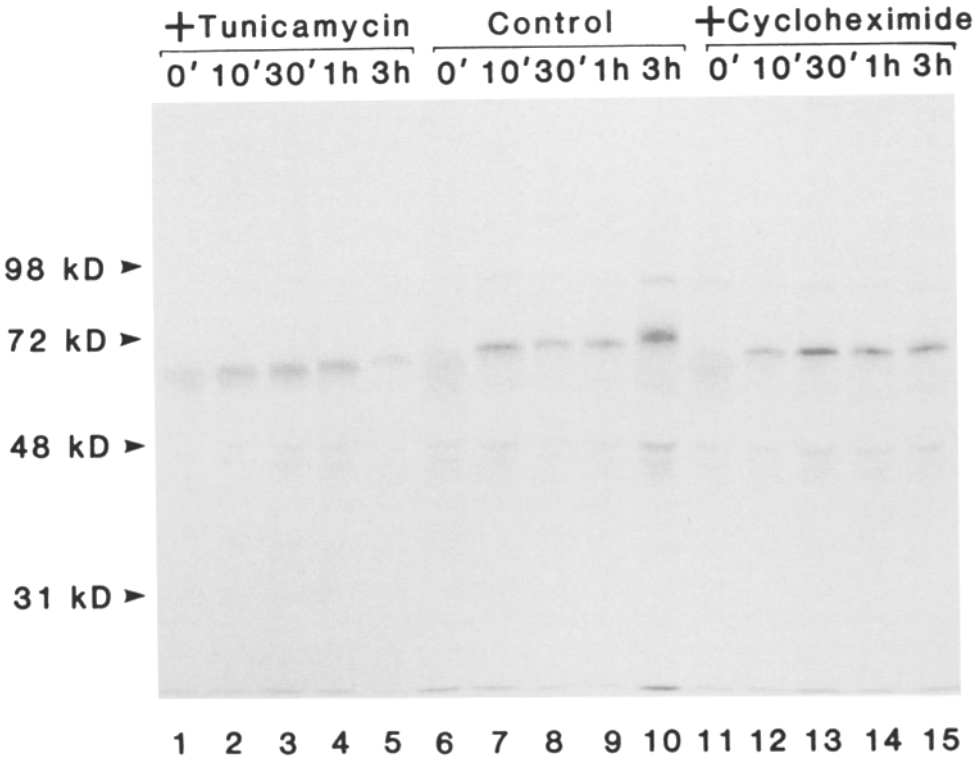

Figure 7. Biosynthesis and modification of Sec12p. Wild-type cells (ANY1-7D/pANY1-9) were grown in a low sulfate medium, washed, and resuspended in minimal medium with no sulfate. Three tubes, each containing 2 OD_{600} cells in 1 ml medium, were preincubated at 30°C for 15 min. Cells were then pulse labeled with 2 mCi $[^{35}S]SO_4^{2-}$ at 30°C for 5 min, and chased by the addition of cold sulfate, cysteine, and methionine. The first tube (lanes *1–5*) contained 10 μg/ml tunicamycin from the period of preincubation through the chase, the second tube contained no drugs (*Control*, lanes *6–10*), and the third tube received 100 μg/ml cycloheximide at the beginning of the chase period (lanes *11–15*). After chase at 30°C for the times indicated, 180-μl aliquots were withdrawn from each tube. Extracts were prepared by agitation with glass beads in the presence of 1% SDS, and Sec 12p was immunoprecipitated.

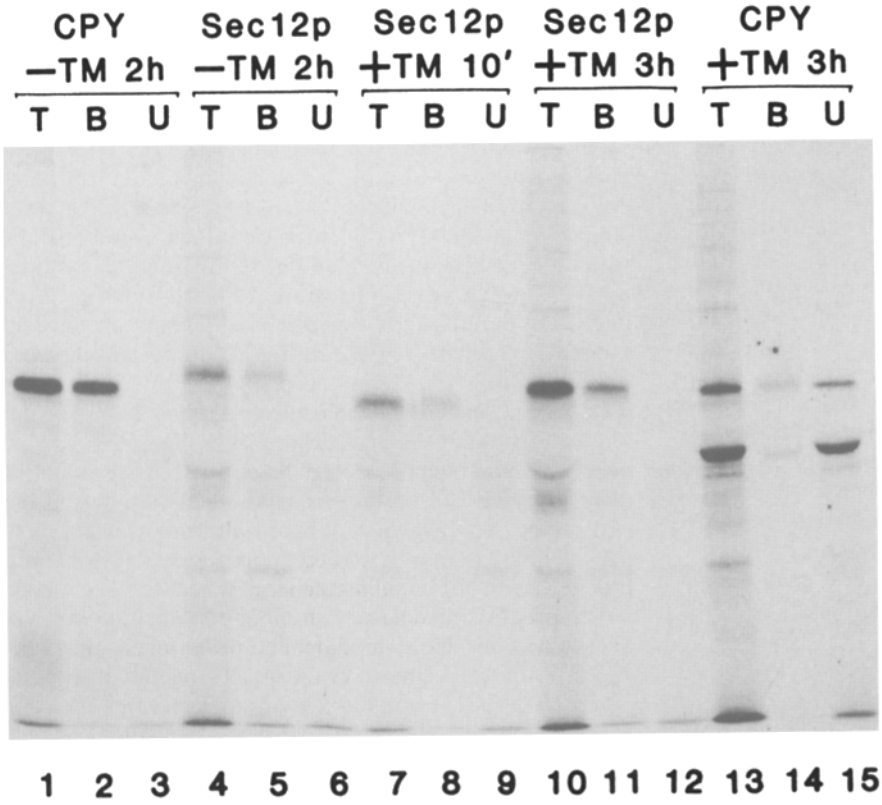

Figure 8. Con A binding of Sec12p. Wild-type cells (ANY1-7D/pANY1-9) were pulse labeled and chased in the presence or absence of tunicamycin (*TM*) as described in the legend to Fig. 7. Extracts were prepared from samples taken at the indicated chase times and incubated with Con A–Sepharose CL-4B in 1.6% Triton X-100, 0.1% SDS, 0.5 M NaCl, and 20 mM sodium phosphate, pH 7.4. To ensure quantitative binding of glycoproteins, 5 μl of swollen Con A–Sepharose beads were used for extracts equivalent to 0.1 OD_{600} of cells. After gentle agitation at room temperature for 3 h, beads were collected by a brief spin in a microfuge and the supernatant fraction containing unbound material was saved. Beads were washed three times with 0.05% SDS, 0.5 M NaCl, and 20 mM Tris HCl, pH 7.4, and treated with 1% SDS in a boiling water bath for 5 min. Eluted material was adjusted to 1.6% Triton X-100 and 0.2% SDS in PBS and subjected to immunoprecipitation with anti-Sec12p or anti-CPY antibody. Immunoprecipitation was also performed for total extracts and unbound fractions in parallel. *T*, total extract; *B*, bound fraction; *U*, unbound fraction. Two major bands in lanes *13–15* derive from precursor and mature forms of CPY (40).

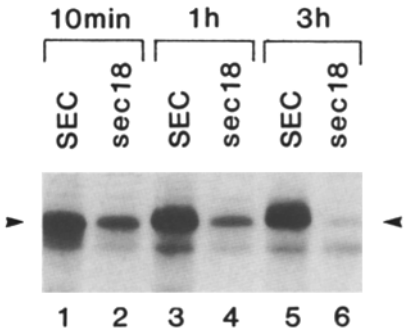

Figure 9. Modification of Sec12p in an ER-accumulating mutant, *sec18*. A *sec18* strain harboring the *SEC12* multicopy plasmid (MBY12-6D/pANY1-9) as well as the wild-type strain (*SEC*$^+$, ANY1-7D/pANY1-9) were suspended in minimal medium containing no sulfate. After preincubation for 15 min at 30°C, cells (6 OD_{600}) were labeled with 6 mCi $[^{35}S]SO_4^{2-}$ for 5 min and chased at 30°C. After 10 min, 1 h, and 3 h of chase, 2 OD_{600} cells were withdrawn and mixed with an equal volume of 20 mM NaN_3 on ice. SDS extracts were prepared and Sec12p immunoprecipitated. *Arrowhead*, Sec12p.

ment were incubated with Con A–Sepharose beads. After 3 h at room temperature, samples were centrifuged and the supernatant fraction was removed. The bound material was eluted in an SDS solution. Sec12p and CPY were immunoprecipitated from total (*T*), bound (*B*), and unbound (*U*) fractions. As shown in Fig. 8, lanes *4–12*, Sec12p, made in the absence (*−TM*) or presence (*+TM*) of tunicamycin, bound to Con A–Sepharose. On the other hand, CPY, which is known to have only N-linked oligosaccharides, bound to Con A–Sepharose when synthesized in the absence of tunicamycin (Fig. 8, lanes *1–3*), but did not when made in the presence of the drug (Fig. 8, lanes *13–15*). The binding of Sec12p to Con A was due to sugar–lectin interaction because 0.5 M α-methyl-D-mannoside completely released Sec12p from the beads (data not shown). Thus, we conclude that Sec12p undergoes two different types of glycosylation: one N-linked and the other of unknown character. Both glycosylation reactions were detected on newly synthesized Sec12p (even in the 10-min chase; see Fig. 8, lanes *7–9*), although the gradual decrease in electrophoretic mobility required as long as 3 h for completion (Fig. 7). This processing was extremely slow compared to the time required for glycosylation of a typical secretory protein such as invertase (30).

A role for ongoing protein synthesis in the posttranslational modification of Sec12p was examined with pulse-labeled cells chased in the presence of cycloheximide. The data in Fig. 7, lanes *11–15*, show that when cells were treated with cycloheximide the electrophoretic mobility of Sec12p did not decrease and become as heterogeneous as in the control. Sec12p matured in the presence of cycloheximide had the same mobility as the form produced during a 1-h chase in the control sample. Therefore, early processing of Sec12p occurred normally in the absence of protein synthesis whereas later events were blocked.

Sec12p in the Golgi Apparatus

The extensive modification of Sec12p resembled the kind of heterogeneous glycosylation that accompanies transport of secretory molecules through the Golgi apparatus (12). This possibility was tested with another *sec* mutant strain, *sec18*, in which protein transport from the ER is defective. *sec18* (31) and wild-type strains harboring the *SEC12* multicopy plasmid were labeled with $[^{35}S]SO_4^{2-}$ for 5 min at 30°C (a restrictive temperature for *sec18-1*), followed by a chase period of 10 min, 1 h, or 3 h at 30°C. In the earliest time point, Sec12p immunoprecipitated from extracts of wild-type or mutant cells migrated at the 65-kD position seen earlier (compare Fig. 7, lane *7* with Fig. 9, lanes *1* and *2*). After 1 h of continued incubation at 30°C, and more dramatically after 3 h, Sec12p in the wild-type strain was converted to a species of heterogeneous migration, while that produced in the *sec18* strain persisted at the same position of migration (Fig. 9, lanes *3–4*). In addition, Sec12p accumulated in *sec18* appeared to be labile, perhaps a result of degradation as a consequence of a failure to mature or localize the protein properly. From this we suggest that the early processing of Sec12p seen in Fig. 7, lanes *6–8*, occurred in the ER, while the later modification (Fig. 7, lanes *9* and *10*) required transport to the Golgi apparatus.

Immunoelectron microscopy using a protein A–gold method was used to localize Sec12p in thin sections. As shown in Fig. 10, *a* and *c*, Sec12p was observed in the Golgi apparatus when this organelle was accumulated in a *sec7* strain (Golgi-accumulating mutant) that was overproducing Sec12p. Gold particles were not observed beyond a background level when the labeling was competed with *lacZ–SEC12* hybrid protein (Fig. 10, *b* and *d*) or when Sec12p was not overproduced (not shown). This result supported a role for the Golgi apparatus in Sec12p biogenesis. Unfortunately, Sec12p was not reproducibly visualized in wild-type or *sec18* cells, even with cells that contained the multicopy SEC12 plasmid. Detection by this method may require a high local concentration of the antigen. Sec12p may be diluted over the large luminal surface of the ER, or the protein may be masked by close interaction with other components in this organelle.

Discussion

Molecular cloning and sequence analysis of the *SEC12* gene predicts a protein with a single potential membrane-spanning domain. Antibody generated against the *SEC12* product detects a membrane glycoprotein in yeast that resides in an intracellular compartment. This glycoprotein, Sec12p, is overproduced when yeast cells are transformed with a *SEC12* multicopy plasmid, and immune precipitation of the yeast protein is competed by excess *SEC12* product made in *E. coli*.

Routine immune detection of Sec12p has been possible only in lysates of cells that harbor the *SEC12* gene on a multicopy plasmid, therefore it appears that Sec12p is not an abundant protein in normal cells. Although our conclusions concerning Sec12p localization, or the rate of its maturation, may be influenced by the requirement for detecting an overproduced protein, the apparent size of Sec12p does not depend on *SEC12* gene dosage. Prolonged autoradiography of SDS gels containing normal and overproduced levels of Sec12p show the same ~70-kD species.

Sec12p resides in a rapidly sedimentable intracellular compartment(s), possibly the ER. Proteolysis of homogenates shows that Sec12p has a site(s) susceptible to proteases within a membrane-enclosed compartment. This observation

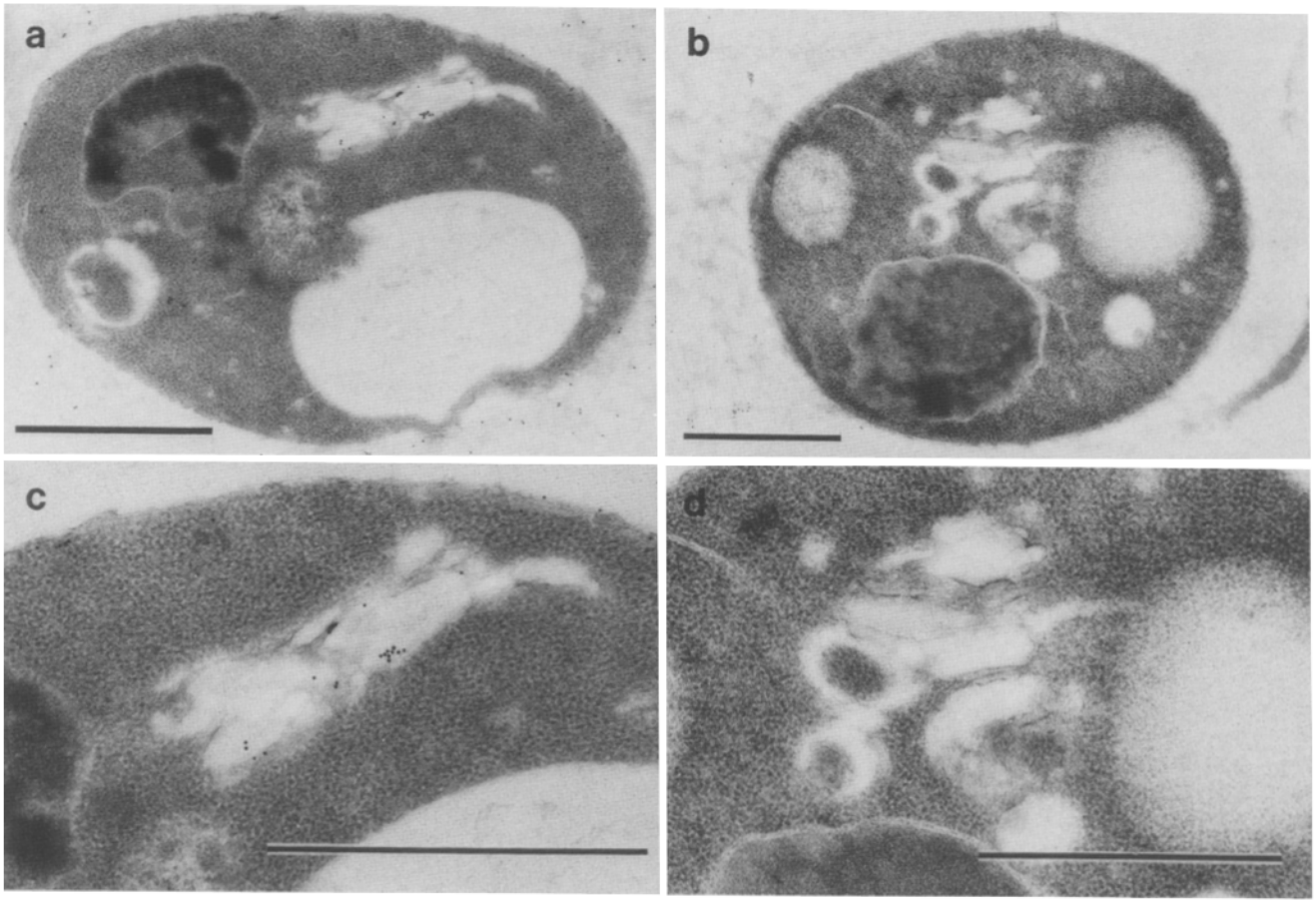

Figure 10. Electron microscopic localization of Sec12p in a Golgi-accumulating mutant, *sec7*. A *sec7* strain harboring the *SEC12* multicopy plasmid (SF821-8A/pANY1-9) was fixed and embedded in Lowicryl K4M. (*a* and *c*) Thin sections were incubated with anti-Sec12p antibody followed by protein A–gold (8-nm particles). (*b* and *d*) The same incubation as in *a* was performed in the presence of purified *lacZ-SEC12* hybrid protein. Bars, 1 μm.

diminishes the possibility that Sec12p resides in the plasma membrane which in homogenates of yeast spheroplasts remains in open sheets with both cytoplasmic and extracellular surfaces exposed (7, 39). Two possible orientations of Sec12p within an organelle membrane are shown in Fig. 11.

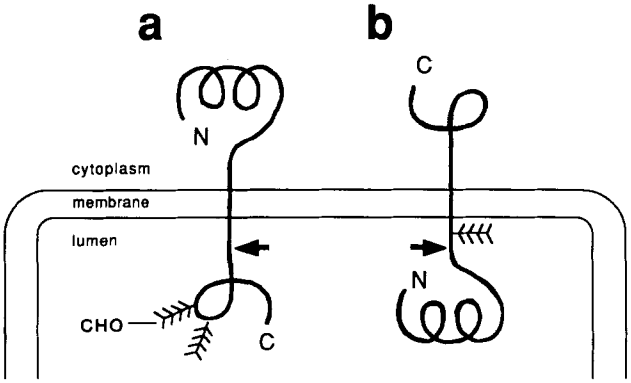

Figure 11. Models of Sec12p structure in a membrane. *N* and *C* indicate amino and carboxyl termini of Sec12p, respectively. *Arrows*, putative sites susceptible to tryptic digestion; *CHO*, N-linked carbohydrates.

Both models seek to account for the production of a nonglycosylated 43-kD fragment of Sec12p produced when membranes are treated with trypsin in the presence of detergent. Model *a* suggests the COOH-terminal domain, which from the sequence data contains two potential N-glycosylation sites, occupies a lumenal position. A potential trypsin cleavage site is located on the COOH-terminal side of the putative membrane-spanning domain. Proteolysis at this position would, in the absence of cleavage at any more NH_2-terminal site, generate a 374-amino acid-long polypeptide (~41 kD). Model *b* places the NH_2-terminal domain in the lumen with one glycosylation site separating the membrane anchor from the trypsin cleavage site. A 337-amino acid-long (~37 kD) possible proteolytic fragment is the largest NH_2-terminal segment that conforms to this model. Furthermore, for model *b* to be correct, the four other potential N-glycosylation sites in the NH_2-terminal domain of sec12p must somehow escape recognition by the oligosaccharide transfer apparatus. For these reasons we favor model *a* and suggest that the protease resistance of the exposed NH_2-terminal domain may derive from unusual folding or through interaction with other proteins. In any case, this domain may provide an anchoring site for cytoplasmic proteins involved in membrane sorting or vesicle budding.

Glycosylation of Sec12p proceeds in an unusual manner

that can only partly be accounted for by addition of N-linked oligosaccharides. Of the ~18-kD apparent molecular mass difference between the polypeptide predicted from the DNA sequence and the protein detected on immunoblots, ~5 kD is due to N-glycosylation. An additional glycosyl modification independent of N-linked oligosaccharides occurs during or shortly after synthesis of Sec12p. This species, labeled in the presence of tunicamycin, binds Con A and is eluted by α-D-methylmannoside. Among the possibilities for this modification are O-linked mannose, whose assembly on yeast glycoproteins begins in the ER (16), or a mannose-containing glycolipid anchor (24), a structure that has not yet been examined in yeast.

Yet another modification of Sec12p, accounting for ~4 kD of apparent molecular mass, occurs slowly and depends upon transit to the Golgi apparatus. This component is independent of N-linked glycosylation and may represent maturation of the other carbohydrate structure, or an altogether new modification. Although protein transport through the yeast secretory pathway ordinarily is rapid and does not require ongoing protein synthesis (30, 31), the slow modification of Sec12p is blocked by cycloheximide. One speculative interpretation of this result is that Sec12p migrates rapidly in a cycle from the ER to the Golgi apparatus and back, experiencing progressive modification in successive passages through the Golgi apparatus. Transport of Sec12p from the ER may require a cargo of newly made proteins such that inhibition of protein synthesis retards its cycling. A precedent for ligand-dependent cycling is seen in epidermal growth factor–stimulated internalization of the epidermal growth factor receptor in liver (10). Alternatively, the enzymes that slowly modify Sec12p may be turned over rapidly, resulting in a depletion of activity during prolonged inhibition of protein synthesis. Since protein transport from the ER to the Golgi apparatus is interrupted in *Sec12* cells, these results and the immune EM visualization of Sec12p in Golgi structures suggest that this protein may serve both in protein transport from the ER and within the Golgi apparatus.

One possibility for the function of Sec12p is in packaging the precursors of secretory, plasma membrane, and vacuolar proteins into the vesicular intermediates that mediate compartmental traffic within the cell. Accordingly, Sec12p should be enriched in these vesicles. Thus far, the only such vesicle that has been identified in yeast is the mature secretory vesicle involved in transport from the Golgi apparatus to the plasma membrane (28), which does not appear to contain Sec12p (Holcomb, C., and A. Nakano, unpublished results). Hence, either Sec12p plays no role in the formation of secretory vesicles derived from the Golgi apparatus, or it does so without being packaged into the membrane of this vesicle.

In the course of cloning the *SEC12* gene, a suppressor gene was obtained which, when its dosage is raised, allows *sec12* ts mutant cells to grow at 37°C. Deletion of the chromosomal locus of the suppressor gene in a wild-type cell is a lethal event, hence the suppressor product is essential for normal growth. Interestingly, the sequence of this gene predicts a *ras*-related GTP-binding protein (Nakano, A., manuscript in preparation). A similar finding has been reported for the *SEC4* gene, which encodes a distinct *ras*-homologue and which partially suppresses the ts growth phenotype of several mutants that accumulate secretory vesicles at 37°C (33). From these results, it appears that different GTP-binding proteins may regulate each stage in the secretory pathway. A further test of this hypothesis requires the generation and analysis of conditional alleles of the *SEC12* suppressor gene.

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