MOLECULAR AND CELLULAR BIOLOGY, Nov. 1991, p. 5727–5734
0270-7306/91/115727-08$02.00/0

Vol. 11, No. 11

Structural and Functional Dissection of a Membrane Glycoprotein Required for Vesicle Budding from the Endoplasmic Reticulum

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Received 17 April 1991/Accepted 27 August 1991

Sec12p is a membrane glycoprotein required for the formation of a vesicular intermediate in protein transport from the endoplasmic reticulum to the Golgi apparatus in *Saccharomyces cerevisiae*. Comparison of the N-linked glycosylation of Sec12p, a Sec12p-invertase hybrid protein, and a derivative of Sec12p lacking 71 carboxy-terminal amino acids showed that Sec12p is a type II membrane protein. Analysis of two truncated forms of Sec12p and of a temperature-sensitive mutant indicated that the C-terminal domain of Sec12p is not essential for protein transport, whereas the integrity and membrane attachment of the cytoplasmic N-terminal domain are essential. Expression of a soluble cytoplasmic domain dramatically inhibited the growth of a *sec12* temperature-sensitive strain by increasing the transport defect at a normally permissive temperature. This growth inhibition as well as the *sec12* temperature-sensitive defect were suppressed by the overproduction of Sar1p, a small GTP-binding protein that participates in protein transport. Sar1p membrane association was enhanced by elevated levels of Sec12p. These results suggest that the cytoplasmic domain of Sec12p interacts with Sar1p and that the complex may function to promote vesicle formation.

Vesicular transport from the endoplasmic reticulum (ER) to the Golgi apparatus is a complex process that requires the participation of a large set of proteins. In the yeast *Saccharomyces cerevisiae*, temperature-sensitive (ts) mutations and suppressor analysis of some of these ts mutations have allowed the identification of 19 genes whose products function in ER-to-Golgi transport (8, 26–28, 30, 31, 35).

Among these genes, *SEC12*, *SEC13*, *SEC16*, and *SEC23* are essential for the formation of a vesicular intermediate (19). Sec12p is a bitopic membrane glycoprotein that resides in ER and Golgi membranes (25). Sec23p and Sec13p are hydrophilic proteins that are found both in the cytosol and in a membrane-bound form (16, 18b). The DNA sequence of *SEC16* also predicts a hydrophilic protein (18a). One attractive model is that Sec12p may facilitate membrane attachment of the other proteins. Such a model is supported by genetic interactions (synthetic lethality) observed between *sec12*, *sec13*, *sec16*, and *sec23* (19). This could reflect the participation of their products in a multisubunit complex required for vesicle formation.

sec12 also displays genetic interactions with *SAR1*. This gene was identified as a multicopy suppressor of a *sec12* ts mutation and encodes a small GTP-binding protein required for ER-to-Golgi transport (26). Sar1p is found both in the cytosol and in a tightly membrane-bound form (29). As was proposed for Sec13p and Sec23p, membrane attachment of Sar1p may require the function of Sec12p.

The sequence of *SEC12* predicts two hydrophilic domains, one of 354 amino acids at the N terminus and one of 98 amino acids at the C terminus, flanking a 19-residue membrane-spanning domain (25). Nakano et al. (25) showed that Sec12p is a membrane glycoprotein with a large protease-resistant domain. The glycosylation pattern and the occurrence of a nonglycosylated proteolytic fragment suggested that Sec12p is a type II membrane protein with its N-terminal domain facing the cytosol. Such an orientation of Sec12p is in agreement with the results obtained from models predicting the orientation of membrane-spanning domains on the basis of the net charge distribution of flanking residues (14).

To gain a better view of how Sec12p may function and interact with other components of the ER-to-Golgi transport machinery, we further evaluated the topology and functional organization of Sec12p. Here, we show that Sec12p is a type II membrane protein, with a lumenally oriented C-terminal domain that is not required for efficient protein transport. An intact and membrane-anchored cytoplasmic N-terminal domain is essential for Sec12p function.

MATERIALS AND METHODS

Strains, materials, plasmids, and general methods. Yeast strains used in this study are listed in Table 1. Yeast cells were grown in YP (2% Bacto Peptone and 1% yeast extract; both from Difco Laboratories, Inc., Detroit, Mich.) or MV (0.67% yeast nitrogen base without amino acids; Difco Laboratories) supplemented with the appropriate amino acids. In most experiments, 2% glucose was included in these media. For the induction of *GAL1*-regulated constructs, cells were first grown in MV containing 2% lactate (pH 5.5) and 0.1% glucose and then induced by transfer into either YP containing 2% galactose or MV containing 2% galactose and 2% lactate, pH 5.5. TG1, an *Escherichia coli* K-12 strain [Δ(*lac-pro*) *supE thi hsdD5* F′(*lacI*^{q1} *lacZ*Δ*M15* pro^{+})] was used for most cloning purposes and grown in Luria broth containing 200 μg of ampicillin per ml when appropriate.

The following reagents were obtained from the indicated sources: phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), concanavalin A (ConA)-Sepharose, and tuni-

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Reference or source[a]
RSY310	*MAT***a** *sec12-1 ura3-52*	19
RSY263	*MAT*α *sec12-4 ura3-52 leu2-3,112*	19
RSY653	*MAT*α *sec12-1 ura3-52 leu2-3,112*	
YT455	*MAT*α *ura3-52 ade2-101 suc2-Δ9*	C. Kaiser
RSY654	*MAT*α *sec12-1 ura3-52 suc2-Δ9*	
RSY655	*MAT*α *sec12-4 ura3-52 suc2-Δ9*	
YPH500	*MAT*α *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	38
W303D	*MAT*α/*MAT***a** *ura3-1/ura3-1 trp1-1/ trp1-1 ade2-1/ade2-1 leu2-3,112/ leu2-3,112 his3-11,15/his3-11/15 can1-100/can1-100*	5
RSY656	*MAT*α/*MAT***a** *SEC12/sec12Δ::LEU2 ura3-1/ura3-1 trp1-1/trp1-1 ade2-1/ ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11/15 can1-100/can1-100*	
RSY657	*MAT*α *suc2-Δ9 ura3-52 ade2-101 trp1-Δ63*	

[a] Strains were constructed during this study unless otherwise stated.

camycin (Sigma Chemical Co., St. Louis, Mo.); protein A-Sepharose CL-4B (Pharmacia-LKB, Piscataway, N.J.); Tran^{35}S-label (ICN, Cleveland, Ohio); alkaline phosphatase immunoblotting reagents (Bio-Rad, Richmond, Calif.); ECL immunoblotting reagents and ^{35}S-dATP (Amersham Co., Arlington Heights, Ill.); and Geneclean (Bio 101, La Jolla, Calif.). All restriction enzymes and molecular biology reagents were purchased from Boehringer Mannheim (Indianapolis, Ind.). Anti-invertase, anti-carboxypeptidase Y, anti-Sec12p, anti-Sec23p, anti-α,1-6-mannose, and anti-Sar1p antibodies have been described previously (3, 16, 25, 29, 33, 39). Anti-Ypt1p and anti-phosphoglycerokinase were kindly provided by M. Rexach and J. Thorner (University of California, Berkeley), respectively.

Fusion and deletion constructs of *SEC12* starting with pANY1-4 (*CEN4-ARS1 URA3 SEC12*) (25) were performed. We first subcloned an *Hae*III fragment into the *Sma*I site of pUC119 (43). The resulting plasmid, pCE4, was cut by *Eco*RI and *Sna*BI to generate a fragment carrying the *SEC12* promoter and the first 1,200 nucleotides of the coding sequence. This fragment was subcloned into the *Eco*RI and *Sma*I sites of the *SUC2* fusion vectors pSEY304 (2μm *URA3*) (4) and pSEYC306 (*CEN4-ARS1 URA3*) (17). This cloning generates an in-frame fusion between *SEC12* and *SUC2*. The resulting plasmids are designated pCEY2 (2μm) and pCEY3 (*CEN4-ARS1*). *SEC12Δ1* was obtained by treating the *Hin*dIII site of pCEY2 and pCEY3 with the Klenow fragment of *E. coli* DNA polymerase I in the presence of deoxyribonucleoside triphosphates. This treatment introduces a frameshift between *SEC12* and *SUC2* resulting in an early translation arrest in *SUC2* (16 codons downstream of amino acid 400 of Sec12p). The resulting plasmids are pCEY8 (2μm) and pCEY9 (*CEN4-ARS1*). *SEC12Δ2* was obtained by site-directed deletion mutagenesis, as described by Boyd et al. (6). Briefly, the *Sal*I-*Eco*RI fragment of pANY1-4 was subcloned into pUC119. Single-strand DNA was produced by using the helper phage M13KO7, and deletion of the DNA encoding the Sec12p transmembrane domain and the C-terminal domain was induced by using an oligonucleotide which hybridizes both upstream of the first codon of the transmembrane domain and downstream of the last codon of the coding sequence (5′-CTCATAACTTTC TCTCAAGCTCA/TTTAGAGATTTTTTGTTTCATTG-3′) during the synthesis of the complementary strand. Plasmids carrying the deletion (351 bp) were screened for increased electrophoretic mobility on agarose gels. To ensure that no additional mutation was present, the deletion was then sequenced. Plasmids pCEY16Δ2 (*CEN4-ARS1*) and CEY17Δ2 (2μm) were obtained by subcloning the mutant *Sal*I-*Eco*RI fragment into pANY1-4 and pANY1-9 (2μm *URA3 SEC12*) (25), respectively, from which the wild-type *Sal*I-*Eco*RI fragment had been deleted. Expression of *SEC12* and its derivatives under *GAL1* control was achieved by first introducing a *Bsp*HI site at the start codon of *SEC12* or *SEC12Δ2* by site-directed mutagenesis (replacement of 5′-CTATGA-3′ by 5′-TCATGA-3′ with the following oligonucleotide: 5′-CACGAACTTCATGATGGCTCGATTG-3′). Site-directed mutants were screened for the appearance of a *Bsp*HI site, and the DNA region encompassing the mutation was sequenced. A *Bsp*HI fragment carrying *SEC12* or *SEC12Δ2* was then subcloned into the *Bam*HI site of pCGS109 (2μm circle *URA3 GAL1-GAL10*; a gift of D. Moir, Collaborative Research, Waltham, Mass.) to give plasmids pCEY5 (*GAL1-SEC12*) and pCEY4 (*GAL1-SEC12Δ2*). The *Eco*RI-*Sna*BI fragment of pCEY5 was then subcloned into the *Eco*RI and *Sma*I sites of pSEY304 to give pCEY6 (*GAL1-SEC12Δ1-SUC2*). pCEY6 was then altered at its *Hin*dIII site by treating it with the Klenow fragment of *E. coli* DNA polymerase I in the presence of deoxyribonucleoside triphosphates to give pCEY7 (*GAL1-SEC12Δ1*).

Other *SEC* plasmids were pANY2-7 (2μm *URA3 SAR1*) (26) and pSEC1610 (2μm *URA3 SEC16*) and pSEC1310 (2μm *URA3 SEC13*) (isolated by M. Bernstein in this laboratory).

The biological activity of Sec12Δ1-Suc2p and Sec12Δ1p was tested by the complementation of a *sec12* null strain. First, we constructed a *SEC12* heterozygous diploid by replacing one chromosomal copy of *SEC12* with the *LEU2* gene in a diploid strain (W303D). This was achieved by transforming W303D with a DNA fragment that contained part of the 5′ noncoding region of *SEC12* (upstream of nucleotide −140; *Hin*dIII-*Bam*HI fragment of pCEY16D12 [see next section]), the *LEU2* gene (*Bgl*II fragment of YEp13 [7]), and part of the 3′ noncoding region of *SEC12* (downstream of nucleotide +277; *Sna*BI-*Eco*RI fragment of pCE4). Transformants were selected for growth on minimal medium lacking leucine and induced to sporulate. In all cases tested, the resulting asci gave rise to tetrads with only one or two viable spores. *SEC12* is an essential gene (25), and, as expected, no Leu$^+$ spores were observed. The disrupted locus is designated *sec12Δ::LEU2*, and one of the transformants (RSY656) was used in further experiments.

Common recombinant DNA techniques were performed essentially as described by Maniatis et al. (21). DNA sequencing was performed by the dideoxy chain termination method (32) with ^{35}S-dATP and T7 DNA polymerase (U.S. Biochemicals, Cleveland, Ohio). The lithium acetate method was used for yeast transformation (2). Transfer of proteins from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels to nitrocellulose filters (Schleicher and Schuell, Inc., Keene, N.H.) was performed as described previously (41). Filters were blocked and all antibody incubations were conducted with 2% nonfat dry milk in 20 mM Tris · HCl (pH 7.4)–150 mM NaCl–0.1% Nonidet P-40. Detection of filter-bound antibodies with alkaline phosphatase- or horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G was performed according to the supplier's instructions.

Mapping and sequencing the *sec12* mutations. pANY1-4

was used for fine-structure mapping of *sec12-1* (RSY310) and *sec12-4* (RSY263). This plasmid contains unique *Bam*HI and *Sal*I sites as well as two *Sna*BI sites. Combined use of these three enzymes generated the mapping plasmids illustrated in Fig. 3. To obtain a gapped plasmid which allowed the mapping of mutations localized upstream of the *Bam*HI site, we constructed deletions of this region by use of the *Bal* 31 exonuclease. Briefly, pANY1-4 was cut with *Bam*HI and subjected to *Bal* 31 digestion. After the addition of EDTA (pH 8.0) to a final concentration of 100 mM, the exonuclease was heat inactivated. Digested plasmids were then cut with *Hin*dIII, and deleted fragments were gel purified prior to subcloning into the *Hin*dIII and *Sma*I sites of pUC119. The resulting plasmids were screened according to size, and after deletion end points were sequenced, the appropriate *Hin*dIII-*Bam*HI fragments were recloned into pANY1-4. For the present study, we used a plasmid (pCEY16D16) which lacks all the coding sequence of *SEC12* upstream of the *Bam*HI site as well as 59 bp of the 5′ noncoding sequence. This plasmid was linearized with *Bam*HI for mapping purposes. The mapping method has been described previously (37, 40). Plasmids carrying the mutant copy of the gene were recovered as described by Ausubel et al. (2), and the DNA region encompassing the *Bam*HI site was sequenced.

Protein radiolabeling and immunoprecipitation. Cells were grown to an optical density at 600 nm (OD_{600}) of 0.2 in minimal medium containing 100 μM $(NH_4)_2SO_4$ and were then transferred to minimal medium without $(NH_4)_2SO_4$ to a final OD_{600} of 5. Cells (1 ml) were then treated with or without 10 μg of tunicamycin per ml for 15 min. Radiolabeling was performed with Tran^{35}S-label (30 μCi per OD_{600} unit cells) for various times. Chase was achieved by the addition of cold methionine and cysteine (final concentration, 1 mg/ml). Cell lysis, immunoprecipitations, and ConA precipitations were performed essentially as described previously (25). For double immunoprecipitations, glass bead extracts (1 ml) were first immunoprecipitated with anti-Sec12p antiserum (cell extract [OD_{600} cell equivalent = 1.5] precipitated with 15 μl of anti-Sec12p antiserum and 15 μl of swollen protein A-Sepharose) overnight at 4°C. Bound material was eluted from the beads by heating at 95°C in 1% SDS for 5 min. A third of this eluted material was reprecipitated with either ConA-Sepharose (10 μl of swollen beads) or anti-α,1-6-mannose serum (5 μl of serum plus 5 μl of protein A-Sepharose) for 2 h at room temperature. Washed precipitates were resuspended in 40 μl of SDS-PAGE sample buffer and heated to 95°C for 5 min, and entire samples were loaded onto 10% polyacrylamide gels.

Subcellular fractionation. Cells were grown to mid-logarithmic phase in YP containing galactose (2%) or in MV containing lactate (2%) and galactose (2%), harvested by centrifugation, and treated for 10 min with 100 mM Tris · HCl (pH 9.5) and 10 mM DTT. After centrifugation, the cell pellet was resuspended in 1 ml of 75% YP containing 0.7 M sorbitol, 0.5% galactose, and 10 mM Tris · HCl (pH 7.4) (100 OD_{600} cells per ml), and lyticase (Fr. II [34]) was added (20 U per OD_{600} cell). Cells were converted to spheroplasts during a 60-min incubation with gentle agitation at 30°C. Spheroplasts were then centrifuged through a cushion of 0.8 M sucrose, 1.5% Ficoll 400, and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4) (10,000 × *g*, 10 min, 4°C). The pellet fraction was resuspended in lysis buffer (100 mM sorbitol, 20 mM HEPES [pH 7.4], 50 mM potassium acetate, 2 mM EDTA, 1 mM DTT, 1 mM PMSF; 200 OD_{600} cells per ml) at 4°C and homogenized by gentle agitation with glass beads (0.8 g/ml). The homogenate (Fig. 5, T) was then fractionated by differential centrifugation (500 × *g*, 10 min, 4°C), which allowed the separation of mostly unbroken cells (Fig. 5, L) from membranes and cytosol (low-speed supernatant). The low-speed supernatant fraction was further fractionated into a high-speed pellet (Fig. 5, H) and high-speed supernatant fractions (Fig. 5, S) by a 100,000 × *g* centrifugation (30 min, 4°C). Each pellet was resuspended in lysis buffer at a concentration of 200 OD_{600} cell equivalents per ml.

RESULTS

Topology and glycosylation of Sec12p. The topology of ER membrane proteins has been studied by the application of gene fusions involving the secretory protein invertase, encoded by the *SUC2* gene (10, 36). Fusion of Suc2p to normally cytoplasmic domains results in hybrid proteins whose N-linked glycosylation is unchanged, whereas fusion to luminal domains results in hybrid proteins which are extensively core glycosylated. To study the topology of Sec12p, we constructed a fusion between *SEC12* and *SUC2*, *SEC12Δ1-SUC2*, which encodes the first 400 amino acids of Sec12p and the mature part of invertase (Fig. 1A). Invertase contains 12 to 13 potential glycosylation sites, of which, on average, 10 are modified per polypeptide, resulting in an increased M_r of ~27,000 (42). If Sec12p is a type I membrane protein, Sec12Δ1-Suc2p should bear the same glycosylation characteristics as Sec12p, whereas if Sec12p is a type II membrane protein, the fusion protein should be extensively glycosylated (Fig. 1A). Cells expressing Sec12Δ1-Suc2p [RSY657(pCEY6)] were pulse labeled in the absence or presence of tunicamycin, an inhibitor of N glycosylation, and immunoprecipitated with anti-Sec12p antibodies. Results presented in Fig. 1B (lanes 5 to 6) show that Sec12Δ1-Suc2p migrated as a polypeptide of 135 kDa with ca. 30 kDa of N glycosylation, suggesting that the Suc2 moiety of the fusion protein projected into the ER lumen. Similar results were obtained when Sec12-Suc2p was immunoprecipitated with anti-invertase antibodies (data not shown). This topology was confirmed by studying another derivative of Sec12p, Sec12Δ1p, which lacked the C-terminal 71 amino acids and consequently the two potential N-glycosylation sites (Fig. 1A). As shown in Fig. 1B (lanes 7 and 8), Sec12Δ1p was not glycosylated. Sec12p is thus a type II membrane protein with an N-terminal domain facing the cytosol and an N-glycosylated C terminus oriented in the ER lumen.

Nakano et al. (25) suggested that Sec12p is subject to slow additional glycosylation, possibly by O-linked mannose, which occurs both in the ER and in the Golgi apparatus. To test whether Sec12Δ1p and Sec12Δ1-Suc2p were subject to the same kind of glycosylation, we compared their biosynthesis with that of Sec12p in a pulse-chase experiment. Results presented in Fig. 2 showed that when N glycosylation was prevented by the addition of tunicamycin, Sec12p was precipitated by ConA, consistent with a non-N-linked carbohydrate modification (Fig. 2, lanes 2 and 5). After a lengthy chase of a pulse-labeled sample, approximately 20% of Sec12p was precipitated with α,1-6-mannose-specific antibodies (lanes 10 to 12), indicating that at least a fraction of the N-linked oligosaccharides were extended by α,1-6 mannose residues, a modification characteristic of an early Golgi event (12). This modification was not detected on Sec12p after the 10-min pulse-label or in cells treated with tunicamycin (Fig. 2, lanes 3, 6, and 9). In contrast, Sec12Δ1p was not precipitated by ConA when labeling was conducted in the presence or absence of tunicamycin (lanes 2, 5, 8, and 11).

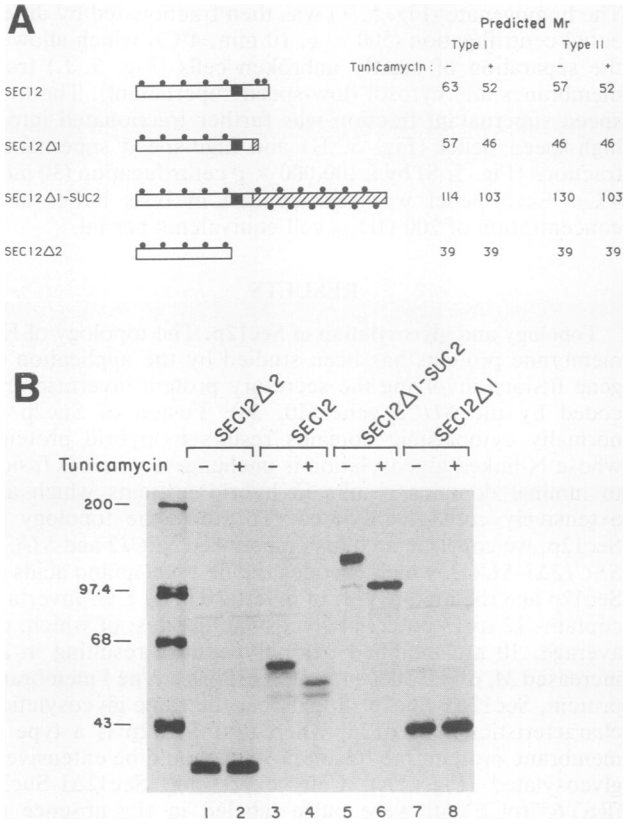

FIG. 1. N glycosylation of Sec12p derivatives. (A) Schematic representation of the four Sec12p derivatives used in this study. Open boxes indicate the N-terminal and C-terminal domains of Sec12p. A black box indicates the Sec12p transmembrane domain. Sec12p contains 471 amino acids, Sec12Δ1p contains 400 amino acids, and Sec12Δ2p contains 354 amino acids. Invertase, the product of the *SUC2* gene, is indicated by a hatched box. Potential N-glycosylation sites are indicated by black dots. The two potential orientations of Sec12p (type I membrane protein, lumenal N-terminal domain versus type II membrane protein, cytoplasmic N-terminal domain) were envisioned for predictions of the molecular weights of the four proteins as well as for the effect of inhibition of N glycosylation by tunicamycin. Molecular weights are expressed in thousands and are predicted on the basis of previously obtained results for Sec12p (25) and invertase (42). Each N-linked oligosaccharide is predicted to add 2,500 units to the M_r of a glycoprotein (42). (B) *suc2* cells (RSY654) transformed with pCEY4 (*SEC12Δ2*, lanes 1 and 2), pCEY5 (*SEC12*, lanes 3 and 4), pCEY6 (*SEC12Δ1-SUC2*, lanes 5 and 6) and pCEY7 (*SEC12Δ1*, lanes 7 and 8) were grown in MV containing lactate (2%) and galactose (2%) and pulse-labeled for 10 min with Tran^{35}S-label in the presence (+) or absence (−) of 10 μg of tunicamycin per ml, as indicated. Glass bead extracts were treated with anti-Sec12p serum, and the immunoprecipitates were subjected to SDS-PAGE and autoradiography.

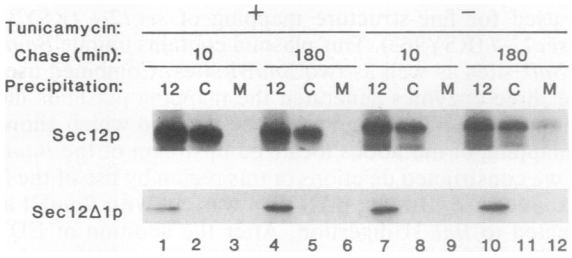

FIG. 2. Glycosylation of Sec12p and Sec12Δ1p. Cells (RSY657) transformed with pCEY5 (Sec12p) or pCEY7 (Sec12Δ1p) were grown in MV containing lactate (2%) and galactose (2%), pulse-labeled for 10 min with Tran^{35}S-label in the presence (+) or absence (−) of 10 μg of tunicamycin per ml, as indicated, and then incubation was continued in the presence of cold methionine and cysteine (1 mg/ml) for the indicated times. Glass bead extracts were immunoprecipitated with anti-Sec12p serum. Bound material was released by heating to 95°C in 1% SDS and reprecipitated with ConA-Sepharose (C) or anti-α,1-6-mannose serum (M). Gel lanes designated 12 contain primary immunoprecipitates with anti-Sec12p serum. The precipitates were subjected to SDS-PAGE and autoradiography.

Although Sec12Δ1-Suc2p was N glycosylated (Fig. 1, lanes 5 and 6), no N-glycosylation-independent carbohydrate was detected (data not shown). From these results, we propose that both the N-linked and the non-N-linked glycosylation of Sec12p requires the integrity of its C terminus, and both may be covalently linked to this domain.

Essential aspects of Sec12p domains. The *SEC12Δ1* and *SEC12Δ1-SUC2* constructs were used to evaluate the role of the C-terminal domain of Sec12p in ER-to-Golgi transport. Single- or multicopy plasmids carrying these constructs were introduced into two *sec12* ts strains (RSY654 [*sec12-1*] and RSY655 [*sec12-4*]) whose nonpermissive temperature is 30°C. In both cases, growth was restored at 30°C by a single-copy plasmid and at 37°C by a multicopy plasmid. These results were consistent with full replacement of the ts form of Sec12p or with interallelic complementation. These possibilities were distinguished by introducing plasmids encoding the truncated or hybrid Sec12 protein vectors into a diploid strain containing wild-type and null alleles of *SEC12* (RSY656). Results presented in Table 2 show that viable spores carrying a *sec12* deletion (*sec12Δ::LEU2*, Leu$^+$ phenotype) were obtained from RSY656 transformed by a multicopy plasmid carrying either *SEC12* (pANY1-9), *SEC12Δ1-SUC2* (pCEY2), or *SEC12Δ1* (pCEY8). Among single-copy plasmid transformants of RSY656, only intact *SEC12* yielded viable Leu$^+$ spores (data not shown). We conclude that the lumenal domain of Sec12p is dispensable for viability. Transformants harboring *SEC12Δ1* in a *sec12* null strain showed no accumulation of carboxypeptidase Y precursors; thus, the lumenal domain of Sec12p was also

TABLE 2. Dispensability for transport of the lumenal domain of Sec12p

Plasmid	Genotype	Spore viability segregation					Phenotype[a]			
		4:0	3:1	2:2	1:3	0:4	L^+U^+	L^+U^-	L^-U^+	L^-U^-
pSEY8	*URA3*	0	0	8	4	1	0	0	14	6
pANY1-9	*URA3 SEC12*	5	1	2	1	3	12	0	8	8
pCEY2	*URA3 SEC12Δ1-SUC2*	8	4	3	3	2	21	0	28	4
pCEY8	*URA3 SEC12Δ1*	6	8	13	1	0	23	0	34	18

[a] Indicated phenotypes are growth (L^+) or no growth (L^-) on MV lacking leucine and growth (U^+) or no growth (U^-) on MV lacking uracil.

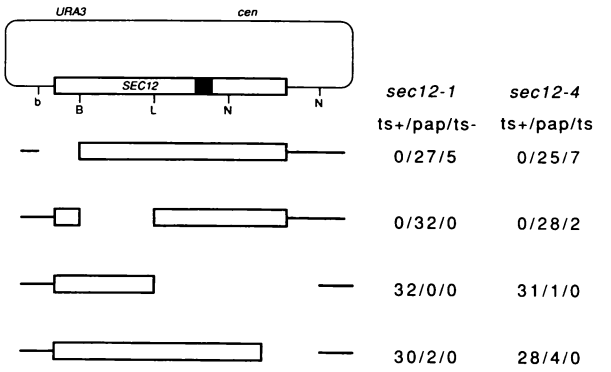

FIG. 3. Deletion mapping of *sec12* mutations by transformation with gapped linear plasmids. Each mapping plasmid used in this study is shown; each was generated by cutting pANY1-4 (*CEN4-ARS1 URA3 SEC12*) at two different restriction sites (B, *Bam*HI; L, *Sal*I; N, *Sna*BI; b, *Bam*HI site obtained after *Bal* 31 digestion) and was used to transform each of the *sec12* ts strains (*sec12-1* [RSY310] and *sec12-4* [RSY263]) at the permissive temperature (24°C). The absence of wild-type recombinants (ts+) between a chromosomal mutation and a specific plasmid deletion localizes the mutation within or close to that deletion interval. In each case, many transformants were recovered; only those tested for the marker recovery of the temperature-sensitive phenotype are presented. Three different phenotypes were observed at 37°C: growth (ts+), death (ts−), and formation of papillae (pap). The papillation phenotype probably is due to an abnormal increase in the copy number of the single-copy plasmid, resulting in stable ts^+ transformants which carry several copies of the *sec12* mutant gene. The transmembrane domain of Sec12p is shown as a black box, and *SEC12* is transcribed from left to right.

dispensable for protein transport. Full complementation of the Sec12p defect due to either a thermosensitive mutation or a null mutation required the expression of Sec12Δ1p or of Sec12Δ1-Suc2p from a multicopy plasmid. This may not reflect the need for elevated expression of the truncated or hybrid protein, since they were less abundant than Sec12p when lysates of multicopy transformants were evaluated by immunoblotting (data not shown).

The exact nature of the ts mutations in *sec12-1* and *sec12-4* was evaluated by marker recovery and DNA sequence analyses. Nakano et al. (25) suggested that the site of one of the *sec12* ts mutations, *sec12-4*, was contained in the region of the gene encoding the cytosolic domain. To confirm this result, the *sec12-4* and *sec12-1* mutations, which had been obtained in two separate mutageneses (31), were mapped in vivo by a marker recovery method depicted in Fig. 3. Most efficient marker recovery of both mutations was obtained with a DNA fragment encoding the first 274 amino acids of Sec12p. Mutant *sec12* alleles were recovered from the yeast chromosome onto recombinant plasmids, and the region determined by mapping to contain the mutation was sequenced. The same change was found in both mutants, a transition from C to T at position 218 in the coding sequence resulting in the replacement of proline 73 by leucine. Equivalent levels of overproduced wild-type and Sec12 ts protein were detected by immunoblotting lysates of multicopy transformants or by immunoprecipitating them from radiolabeled cells (data not shown). We conclude that alteration of the structure of the cytosolic domain of Sec12p, rather than protein stability, accounts for the effect of the *sec12-1/4* mutation.

Overproduction of Sec12 ts protein by the introduction of the mutant gene on a multicopy plasmid allowed growth of a *sec12* ts strain at 37°C. Normal levels of the Sec12 ts protein may retain some activity below a threshold required for efficient growth at 37°C.

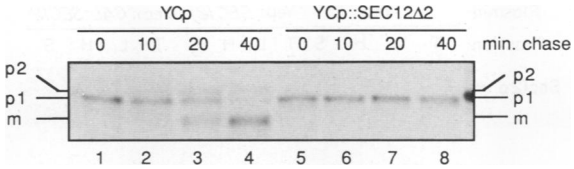

FIG. 4. Sec12Δ2p increases the transport defect of a *sec12* ts strain. *sec12-1* cells (RSY653) transformed with either the single-copy plasmid pSEYC68 (YCp, lanes 1 to 4) or pCEY16Δ2 (YCp::SEC12Δ2, lanes 5 to 8) were grown in minimal medium at 18°C and transferred at 24°C for 30 min. Cells were pulse-labeled at 24°C for 5 min, and incubation was continued in the presence of cold methionine and cysteine (1 mg/ml) for the indicated times. Glass bead extracts were immunoprecipitated with anti-CPY serum. The precipitates (see Materials and Methods) were subjected to SDS-PAGE and autoradiography. The ER (p1), Golgi (p2), and vacuolar (m) forms of CPY are indicated.

Sec12p cytosolic fragment toxic in *sec12*. Results presented above show that the cytosolic N-terminal domain is essential for Sec12p function and could therefore be the site of interactions with other cytosolic components of the transport machinery. We reasoned that the production of a cytosolic fragment that would be transport incompetent by virtue of its mislocalization might interfere with the transport machinery and thus inhibit ER-to-Golgi transport (dominant lethal phenotype [15]). A truncated form of *SEC12* which expressed only the cytosolic domain of Sec12p (354 amino acids) was constructed by fusing the region of *SEC12* encoding the N-terminal domain to the 3′ noncoding region by in vitro deletion mutagenesis (see Materials and Methods for details). The resulting construct, *SEC12Δ2*, was cloned on either a single-copy plasmid (pSEYC68) or a multicopy plasmid (pSEY8 or pCGS109) and expressed under the control of either the *SEC12* promoter, a weak promoter (25), or the *GAL1* promoter, a strong inducible promoter (18). When these plasmids were introduced in a wild-type strain (RSY657), expression of a soluble 39-kDa nonglycosylated protein was observed, as expected from the DNA sequence of *SEC12* and from the topology of Sec12p (Fig. 1B). Fractionation of lysates of cells expressing Sec12Δ2p under the control of the *GAL1* promoter resulted in the truncated protein in both the supernatant (~45%) and pellet fraction (~35%) after centrifugation at 100,000 × *g* for 1 h. Thus, around half of the Sec12Δ2p is cytosolic. Expression of Sec12Δ2p did not impair growth, but it did decrease the rate of transport of the vacuolar enzyme carboxypeptidase Y (CPY) from the ER by a factor of 2 to 3 (data not shown).

In contrast to the marginal effect of *SEC12Δ2* on wild-type cells, single- or multicopy transformants of two *sec12* ts strains (RSY310 [*sec12-1*] and RSY263 [*sec12-4*]) could only be obtained at 17°C and were unable to grow at 24°C. Results presented in Fig. 4 show that this growth defect was associated with a transport lesion in the *sec12* ts strain. The ER form of CPY (p1) accumulated at 24°C only when *sec12-1* was transformed by a plasmid carrying *SEC12Δ2* (Fig. 4; compare lanes 1 to 4 and 5 to 8). This growth defect was suppressed by the overproduction of the Sec12 ts protein, suggesting that the two mutant forms of Sec12p compete to interact with cytosolic components of the transport machinery.

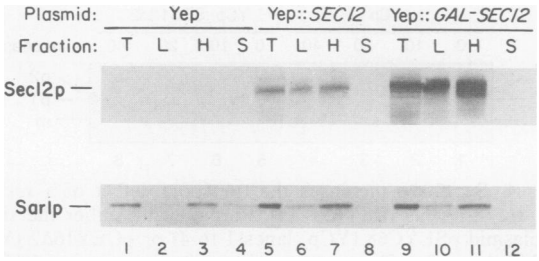

FIG. 5. Sec12p levels influence Sar1p membrane association. Wild-type cells (YPH500) transformed with either the multicopy vector pSEY8 (YEp, lanes 1 to 4), pANY1-9 (YEp::*SEC12*, lanes 5 to 8) or pCEY5 (YEp::*GAL1-SEC12*, lanes 9 to 12) were grown in YP galactose (2%) to mid-logarithmic phase, converted to spheroplasts, and lysed by homogenization. The initial lysate (T) was centrifuged at 500 × *g*, yielding low-speed pellet (L) and supernatant fractions. This supernatant was centrifuged at 100,000 × *g* to generate high-speed pellet (H) and supernatant (S) fractions. Equal portions of each fraction were resolved by SDS-PAGE and immunoblotted with either anti-Sec12p or anti-Sar1p antibodies.

TABLE 3. Influence of Sec12p levels on Sar1p membrane association[a]

Protein	% in fraction with plasmid[b]:								
	YEp			YEp::*SEC12*			YEp::*GAL1-SEC12*		
	LSP	HSP	HSS	LSP	HSP	HSS	LSP	HSP	HSS
Sec12p	ND[c]	ND	ND	40	60	ND	38	62	ND
Sar1p	16	62	22	24	72	4	25	75	ND
Ypt1p	13	61	26	15	58	28	18	58	24
Sec23p	6	94	ND	11	89	ND	3	97	ND
PGK[d]	6	25	69	8	26	66	7	24	69

[a] Proteins immunodetected in the various fractions obtained after differential centrifugation of a cell lysate (as described in the legend to Fig. 5) were quantified by densitometry. Each value is presented as the percentage of the sum of the three fractions.

[b] Plasmids: YEp, YPH500(pSEY8); YEp::*SEC12*, YPH500(pANY1-9); YEp::*GAL1-SEC12*, YPH500(pCEY5). Fractions: LSP, 500 × *g* pellet; HSP, 100,000 × *g* pellet; HSS, 100,000 × *g* supernatant.

[c] ND, not detectable.

[d] PGK, phosphoglycerokinase.

Genetic interactions suggest some interplay between Sec12p, Sec13p, Sec16p, and Sar1p (19, 26). If the thermosensitive phenotype of the *sec12-1/4* mutation is the result of a reduced affinity of Sec12p for one of these proteins, the toxic effect of the soluble cytosolic domain might be suppressed by the overproduction of the interacting protein. Multicopy plasmids carrying either *SEC13*, *SEC16*, or *SAR1* were introduced into a *sec12-1* strain (RSY653) previously transformed by a single-copy plasmid carrying *SEC12Δ2*. Only the *SAR1* plasmid restored growth of this strain at 24 and 37°C. This suggested that Sar1p overproduction suppressed both the *sec12* ts defect and the synthetic defect induced by Sec12Δ2p production.

Sar1p localization and interaction with Sec12p. DNA sequence analysis of the *SEC13* and *SAR1* genes predicts hydrophilic proteins (18a, 26). Fractionation data show that Sec13p and Sar1p are found both in the cytosol and in a membrane-bound form (18b, 29). If Sec13p or Sar1p association with membrane is dependent on an interaction with Sec12p, localization of these proteins should be influenced by the levels of Sec12p found in the membrane. To test whether this is the case, we examined three different derivatives of a wild-type strain which expressed various levels of Sec12p: YPH500(pSEY8) (11), with a low level of expression from the chromosomal *SEC12* gene (Fig. 5, YEp); YPH500 (pANY1-9), with a medium level of expression from a multicopy plasmid carrying *SEC12* under the control of its own promoter (Fig. 5, YEp::*SEC12*); and YPH500(pCEY5), with a high level of expression from a multicopy plasmid carrying *SEC12* under *GAL1* control (Fig. 5, YEp::*GAL-SEC12*). Cell extracts were fractionated by differential centrifugation, and membrane and cytosol fractions were evaluated by immunoblotting them with Sec12p and Sar1p antibodies. As before, Sec12p was detected only in lysates of cells carrying *SEC12* on a multicopy plasmid, and this material was found exclusively in the 100,000 × *g* pellet (Fig. 5; compare lanes 3, 7, and 11 [pellet] with lanes 4, 8, and 12 [supernatant]) (25). Sar1p was found both in the 100,000 × *g* pellet and in supernatant fractions (lanes 3 and 4). However, the ratio of membrane-bound Sar1p to soluble Sar1p increased with the level of expression of Sec12p (Fig. 5; compare lanes 3 and 4, 7 and 8, and 11 and 12) (see Table 3 for quantitation). The localization of two other peripheral membrane proteins involved in protein transport, Sec23p and Ypt1p, was not influenced by Sec12p levels, nor was the localization of a cytoplasmic protein, phosphoglycerokinase (Table 3). In other studies, we have found that isolated Sar1p binds better to membranes isolated from Sec12p-overproducing cells than to membranes from untransformed cells (9).

DISCUSSION

We have used a Sec12-invertase hybrid protein to demonstrate that Sec12p is a type II membrane protein whose large 354-residue N-terminal domain faces the cytosol. Genetic analysis of two truncated derivatives of *SEC12*, one of which lacks the C-terminal 71 amino acids (*SEC12Δ1*) and the other of which lacks both the single transmembrane domain and the lumenal C-terminal domain (*SEC12Δ2*), and of a thermosensitive mutant of *SEC12* (*sec12-1/4*) (31) shows that (i) the C-terminal domain of Sec12p is not required for efficient ER-to-Golgi transport, (ii) the integrity and membrane attachment of the cytoplasmic domain of Sec12p is essential, and (iii) this cytoplasmic domain may be the site of a Sec12p-Sar1p interaction, as previously suggested by Nakano and Muramatsu (26).

To study the topology of Sec12p, we have taken advantage of the N glycosylation characteristics of a Sec12-invertase hybrid protein (Sec12Δ1-Suc2p). Fusion proteins have been used successfully to assess the topology of cytoplasmic membrane proteins in *E. coli* (22) or ER membrane proteins in *S. cerevisiae* (10, 36). Although this technique may be misleading when used for multispanning membrane proteins (36), the results with this approach concur with proteolytic mapping of Sec12p obtained previously by Nakano et al. (25). Furthermore, the biological activity of both Sec12Δ1-Suc2p and Sec12Δ1p argue that the deletion and protein fusion events have not reoriented Sec12p in the ER membrane.

By studying the glycosylation of Sec12p, Nakano et al. (25) showed that in addition to its N-linked oligosaccharides, Sec12p is modified by non-N-linked carbohydrates, possibly O-linked mannoses. We have now shown that the lumenal domain of Sec12p is required for efficient non-N-linked glycosylation, and we propose that this domain carries the modification. Previous results also suggested that Sec12p may circulate between the ER and Golgi complex and that

the non-N-linked glycosylation is slowly enlarged when Sec12p is transferred into the Golgi apparatus. α,1-6-Mannose, an outer-chain linkage that yeast glycoproteins acquire within the Golgi apparatus (12, 20), is part of this modification. These conclusions may apply only in cells that are overproducing Sec12p; immunodetection of the protein in untransformed cells has not yet been possible.

A genetic study of three different *SEC12* derivatives, *SEC12Δ1*, *SEC12Δ2*, and *sec12-1/4*, shows that membrane attachment of the cytosolic domain of Sec12p is crucial. This domain may be the site of interaction with other components required for vesicle budding from the ER, such as Sec13p, Sec16p, and Sec23p (19) and Sar1p, a small GTP-binding protein required for ER-Golgi transport (26). Results presented in this study show that Sar1p binding to membranes is increased by Sec12p overproduction. Enhanced binding of isolated Sar1p to Sec12p-rich membranes in simple mixing experiments has also been observed (9). Conversely, overproduction of Sar1p leads to an increased proportion of the protein recovered in a cytosolic fraction (29). Sec12p may in this regard act as a membrane anchor for Sar1p or modify Sar1p in such a way that it binds more tightly to membranes.

Several small GTP-binding proteins are believed to be anchored to membranes by lipid modification of cysteine residues near the C terminus (13, 23, 24, 44, 45). However, Sar1p does not have such a cysteine (26); hence, membrane binding may be more directly dependent on Sec12p. A cycle of Sar1p association and dissociation from the membrane may be influenced by GTP-GDP exchange or nucleotide hydrolysis by Sar1p, processes which could be influenced by interaction with Sec12p. For example, the *ras*-like GTP-binding protein, *smg* p25A/rab3A, becomes more tightly bound to membranes in the GTP-bound form than it does in the GDP-bound form (1). Sec12p-mediated membrane binding of Sar1p could regulate the subsequent assembly of a vesicle budding machinery on the cytoplasmic side of the ER.

In view of the Sec12p-Sar1p interaction, we think it is likely that the Sec12 ts protein has a reduced affinity for Sar1p, such that the formation of the complex is reduced below a threshold required for efficient growth at 37°C. This hypothesis explains suppression of the *sec12* ts mutation by overproduction of either Sar1p or the Sec12 ts mutant protein and is consistent with the fact that a *sec12* null mutation is not suppressed by Sar1p overproduction (reference 26 and this study). Likewise, the Sec12p cytosolic fragment may be toxic to *sec12-1/4* cells because of a relatively higher affinity of Sar1p for the soluble fragment than for the membrane-bound mutant protein. Conversely, the soluble fragment may not compete effectively with the intact wild-type protein, thus accounting for the mild effect of the truncated protein in normal cells.

At least 71 amino acids of the 98-amino-acid C-terminal domain of Sec12p are not required for ER-to-Golgi transport. Although overproduction of Sec12Δ1p and Sec12Δ1-Suc2p is required for full complementation of the *sec12* thermosensitive and null mutations, our data suggest that this requirement is due to a low level of expression of these proteins. Such a low level could be due to a reduced stability of the two proteins or a reduced transcription level created by the cloning strategy. In any case, it remains possible that the C-terminal domain serves some role in transport which we have failed to detect or which is served by the ER lumenal portion that remains in the Sec12Δ1p truncated molecule.

ACKNOWLEDGMENTS

We thank R. Deshaies, C. Kaiser, C. Stirling, and D. Feldheim for many helpful discussions, as well as other members of the Schekman laboratory for constant encouragement. Useful comments were received from J. Brodsky, C. Kaiser, and N. Pryer, and P. Smith's expert assistance in preparing the manuscript was, as always, greatly appreciated. We thank S. Emr and D. Moir for providing plasmids.

This work was supported by grants from the National Institutes of Health (NIGMS) and the Howard Hughes Research Institute. C. d'Enfert was supported by the French INRA, and C. Barlowe was supported by a postdoctoral fellowship from the Damon Runyon-Walter Winchell Cancer Research Foundation.

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