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Structural and Functional Dissection of Sec62p, a Membrane-Bound Component of the Yeast Endoplasmic Reticulum Protein Import Machinery

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***SEC62* is required for the import of secretory protein precursors into the endoplasmic reticulum (ER) of *Saccharomyces cerevisiae*. The DNA sequence of *SEC62* predicts a 32-kDa polypeptide with two potential membrane-spanning segments. Two antisera directed against different portions of the *SEC62* coding region specifically detected a 30-kDa polypeptide in cell extracts. A combination of subcellular fractionation, detergent and alkali extraction, and indirect immunofluorescence studies indicated that Sec62p is intimately associated with the ER membrane. Protease digestion of intact microsomes and analysis of the oligosaccharide content of a set of Sec62p-invertase hybrid proteins suggested that Sec62p spans the ER membrane twice, displaying hydrophilic amino- and carboxy-terminal domains towards the cytosol. Sec62p-invertase hybrid proteins that lack the Sec62p C terminus failed to complement the *sec62-1* mutation and dramatically inhibited the growth of *sec62-1* cells at a normally permissive temperature. The inhibitory action of toxic Sec62p-invertase hybrids was partially counteracted by the overexpression of Sec63p. Taken together, these data suggest that the C-terminal domain of Sec62p performs an essential function and that the N-terminal domain associates with other components of the translocation machinery, including Sec63p.**

We previously isolated a set of temperature-sensitive lethal mutants of *Saccharomyces cerevisiae* that fail to localize a signal peptide-bearing cytoplasmic enzyme chimera to the lumen of the endoplasmic reticulum (ER) (14). Genetic and biochemical characterization of these mutants indicated that mutations in four genes, *SEC61* (14), *SEC62* (15, 44), *SEC63* (44), and *SEC65* (C. Stirling and R. Schekman, unpublished data), disrupt the translocation of several secretory precursor proteins across the ER membrane. Additional alleles of *SEC63* that block either protein translocation into the ER (*ptl1* [53]) or nuclear protein accumulation (*npl1* [46]) have been independently isolated in other laboratories. The specific translocation defects resulting from the *sec62* (15) and *sec63* (44) mutations have been reproduced in vitro with microsomal membranes isolated from mutant cells, suggesting that these mutations interfere with the function of a component of the yeast ER membrane translocation apparatus. Consistent with the in vitro defect exhibited by mutant microsomal membranes, DNA sequence analysis of *SEC62* (15) predicted a 32-kDa polypeptide with two potential transmembrane domains, and *SEC63* was predicted to encode a 76-kDa protein with three potential membrane-spanning segments (46).

Besides the *SEC* genes discussed above, several other genes required for proper insertion of secretory proteins into the ER have been identified. Hsp70 homologs appear to be required on both sides of the yeast ER membrane for efficient translocation of certain precursor proteins. Cytosolic Hsp70 homologs encoded by the *SSA* genes are required for efficient translocation of pre-pro-α-factor into the ER both in vivo (13) and in vitro (11, 13). Recently, two laboratories (36, 43) reported the identification of a yeast gene (*KAR2*) that encodes an ER-localized homolog of the Hsp70-like heavy-chain-binding protein (BiP). Depletion or conditional inactivation of Kar2 protein results in the accumulation of unprocessed secretory precursor proteins in the cytoplasmic compartment (55). Genes encoding homologs of mammalian translocation-promoting factors have also been identified in yeasts. Targeting of secretory proteins to dog pancreas microsomal vesicles in vitro requires the multisubunit signal recognition particle, which in part acts as a cytosolic signal peptide receptor (6). A search for homologs of the signal peptide-binding subunit of the signal recognition particle (SRP54) yielded an *S. cerevisiae* gene (21) bearing features characteristic of the mammalian protein (5, 42), although no genetic or biochemical evidence for its role in protein translocation has yet been presented.

To gain a better view of the structural and functional organization of the ER membrane translocation machinery, we initiated a detailed analysis of the *SEC62* gene product (Sec62p). Whereas the topology of the yeast ER membrane protein 3-hydroxyl-3-methylglutaryl coenzyme A reductase has been deduced by an analysis of *HIS4C* fusions (50), little is known about the ER membrane topology of the Sec proteins involved in translocation (15,46) and posttranslational processing of secretory precursors (8, 9, 12, 38). Here we show that *SEC62* encodes a 30-kDa integral protein of the ER membrane. By analogy to the approach of Beckwith and colleagues, who used alkaline phosphatase fusions to map the topology of *Escherichia coli* inner membrane proteins (31, 47), we constructed a set of *SEC62-SUC2* fusions to assess the topology of Sec62p in the ER membrane. Besides providing evidence concerning the topology of Sec62p, genetic analysis of the *SEC62-SUC2* hybrids suggested that Sec62p may assemble into complexes containing other translocator proteins, including Sec63p.

MATERIALS AND METHODS

Strains, materials, plasmids, and general methods. The following yeast strains were used in this study: RDM50-94C

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(*leu2-3 leu2-112 his4 ura3-52 sec62-1* α), *RSY151* (*leu2-3 leu2-112 ura3-52 pep4-3 sec63-1* α), RSY271 (*sec18-1 ura3-52 his4-619* α), W303-1B (*leu2-3 leu2-112 his3-11 -15 trp1-1 ura3-1 ade1-1 can1-100* α), and BF-1, which is the same as W303-1B except that the *PEP4* gene was deleted and replaced with *TRP1* (L. Silveira, personal communication). Yeast cells were grown in rich or minimal medium (14).

The following reagents were obtained from the indicated sources: 3,3′,5,5′-tetramethylbenzidine, peroxidase, phenylmethylsulfonyl fluoride (PMSF), pepstatin, leupeptin, 1,10-phenanthroline, tunicamycin, 3β-indoleacrylic acid, and Freund complete and incomplete adjuvants (Sigma Chemical Co., St. Louis, Mo.); CNBr-activated Sepharose 4B, protein A–Sepharose CL-4B, and immunoglobulin G(IgG)-Sepharose 6 (Pharmacia-LKB, Piscataway, N.J.); Tran^{35}S-label (ICN, Cleveland, Ohio); alkaline phosphatase immunoblotting reagents (Bio-Rad, Richmond, Calif.); glucose oxidase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); Citifluor (Citifluor Ltd., London, United Kingdom); and Geneclean (Bio 101, La Jolla, Calif.). Anti-Sec23p (L. Hicke, Division of Biochemistry and Molecular Biology, University of California at Berkeley), anti-phosphoglycerate kinase (J. Thorner, same location as L. Hicke), anti-dipeptidyl aminopeptidase B (DPAP-B; T. Stevens, Institute of Molecular Biology, University of Oregon), anti-Kar2p (M. Rose, Biology Department, Princeton University), and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit (K. Radding, Biochemistry Department, Stanford University) sera were kindly provided by the indicated investigators. Anti-invertase serum was described previously (48), and anti-pro-α-factor serum was prepared by B. Koch and A. Eun (our laboratory) with the *LacZ-MFα1* fusion construct (pEX-α6) described by Rothblatt and Meyer (45). Lyticase (fraction II) was prepared by A. Eun and one of us (R.S.) with minor modifications as described previously (49).

Plasmids pRIT2T (35), pEX2 (51), and pATH3 (16) were used to generate *SEC62* gene fusions for the production and purification of anti-Sec62p sera. The multicopy yeast plasmids YEp351 and YEp352 were described previously (25). pRD6 (15) contains the 4.4-kb *Hin*dIII-*Sph*I fragment of *SEC62* inserted into YEp351, and pRD34 contains the 1.7-kb *Eco*RVc-*Sph*I fragment of pRD5 (15) inserted into YEp352. Overexpression of *SEC63* was achieved by inserting the 2.7-kb *Hin*dIII fragment of pTK1 (46) into either YEp351 (pDF14) or YEp352 (pDF11) to generate the indicated plasmids (D. Feldheim, unpublished results). *SEC62-SUC2* fusions were constructed in the *SUC2* fusion vectors pSEY304 (2μm circle *URA3* [3]), pSEYC306 (*CEN4-ARS1 URA3* [26]), and pCS29. All of these vectors contain the coding sequence for 511 (of 513) amino acids of mature invertase (52) preceded by a polylinker. pCS29 was constructed by replacing the polylinker of pSEY304 with that of pUC119 (54; C. Stirling, unpublished results).

Common recombinant DNA techniques were performed essentially as described by Maniatis et al. (30) or Ausubel et al. (2). The lithium acetate method was used for yeast transformation (2). Total protein was measured as described by Markwell et al. (33). Transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to nitrocellulose filters was performed as described elsewhere (22). Filters were blocked, and all antibody incubations and washes were conducted with 2% nonfat dry milk–50 mM Tris (pH 7.4)–150 mM NaCl–0.1% Nonidet P-40–10 mM NaN_3. Detection of filter-bound antibodies with alkaline phosphatase-conjugated goat anti-rabbit IgG was performed in accordance with supplier instructions (Bio-Rad).

Sec62p antisera. Antisera were elicited against both protein A-Sec62p and β-galactosidase–Sec62p fusion proteins. pRD19 was used to express a protein A-Sec62p hybrid protein. pRD19 contained a filled-in 425-bp *Acc*I-*Eco*RI fragment encoding the C-terminal domain of Sec62p from amino acids 218 to 283 (15) inserted into the filled-in *Bam*HI site of pRIT2T. Pilot experiments indicated that pRD19 directed the heat-inducible expression of a predominantly soluble, ~36-kDa hybrid protein. A 2-liter culture of *E. coli* POP2136 cells (containing an integrated copy of the *c*I ts857 gene; J. Rothblatt, Dartmouth University) containing pRD19 was grown to an optical density at 600 nm of 2.1 at 30°C, diluted fourfold with prewarmed L broth, and shifted to 42°C for 90 min to induce the synthesis of protein A-Sec62p. Cells were harvested by centrifugation (5 min, 10,000 × *g*), washed with 100 mM NaCl–50 mM Tris hydrochloride (pH 7.5)–1 mM EDTA (STE), frozen in liquid nitrogen, and stored at −20°C. The thawed cell pellet was suspended in 20 ml of lysis buffer (100 mM sodium P_i [pH 7.0], 10% glycerol, 5 mM EDTA, 5 mM 1,10-phenanthroline, 0.5 mM PMSF, 10 μM leupeptin) and disrupted by four 30-s pulses with a Sonifier (Heat Systems-Ultrasonics, Plainview, N.Y.) equipped with a microtip. The extract was centrifuged for 10 min at 12,000 × *g* and for 15 min at 412,160 × *g* in a TLA100.3 rotor (Beckman Instruments, Palo Alto, Calif.). The final supernatant was diluted to 40 ml with lysis buffer, adjusted to 1% Triton X-100, and mixed with 2 ml of IgG-Sepharose 6. Following 3 h of incubation at 4°C, the IgG beads were washed (by centrifugation) two times each with 2 M urea–200 mM NaCl–1% Triton X-100–100 mM Tris hydrochloride (pH 7.5)–2 mM NaN_3 and 150 mM NaCl–1% Triton X-100–0.1% SDS–15 mM Tris hydrochloride (pH 7.5)–2 mM NaN_3. Washed beads were poured into a column and washed with 20 ml of 50 mM Tris hydrochloride (pH 7.6)–150 mM NaCl–0.05% Tween 20, and the protein A hybrid was eluted with 0.2 M glycine (pH 2.5) into tubes containing 1.5 M Tris (pH 8.8). Neutralized fractions containing fusion proteins were dialyzed against 20 mM sodium P_i (pH 7.4)–150 mM NaCl (PBS) and concentrated in a Centriprep-10 concentrator (Amicon, Danvers, Mass.). Two rabbits were each injected at multiple subcutaneous sites with ~150 μg of purified fusion protein in Freund complete adjuvant, followed by monthly boosts (at multiple subcutaneous sites) with ~150 μg of fusion protein in Freund incomplete adjuvant. Blood samples were collected on days 10 and 17 following each boost. Antiserum raised against the protein A-Sec62p fusion protein is referred to as anti-C-terminal domain serum (anti-CTD serum).

A β-galactosidase–Sec62p expression vector (pRD17) was constructed by inserting an ~1,150-bp fragment containing the entire *SEC62* coding region plus four codons upstream of the putative initiator methionine into the *Sma*I site of pEX2. Heat induction of pRD17 transformants of *E. coli* POP2136 revealed a 140-kDa fusion protein. A 4-liter culture was grown at 30°C to an optical density at 600 nm of 0.3 and induced for fusion protein expression by incubation at 42°C for 2 h. Following induction, cells were harvested, washed with STE, and frozen in liquid nitrogen. The thawed cell pellet was suspended in 15 ml of STE supplemented with 1 mM PMSF and 10 μM each leupeptin and pepstatin, followed by sonication and low-speed centrifugation (as described above for the purification of protein A-Sec62p) to sediment inclusion bodies. The 12,000 × *g* pellet was washed consecutively with STE, STE plus 1% Triton X-100, STE plus 1 M urea, and STE plus 0.02% SDS. The final pellet was suspended in 50 ml of SDS-polyacrylamide gel electropho-

resis (PAGE) sample buffer, heated for 7 min at 95°C, and applied to 3-mm-thick 7% SDS-polyacrylamide gels. Following electrophoresis, the LacZ-Sec62p fusion protein was visualized by staining with aqueous Coomassie blue (22), electoeluted from gel slices in an Elutrap (Schleicher & Schuell, Keene, N.H.) in accordance with manufacturer instructions, dialyzed against PBS plus 0.5 mM EDTA and 0.02% SDS, and concentrated in a Centriprep-10 concentrator. Two rabbits were injected, boosted, and bled as described above for the protein A-Sec62p fusion protein, except that 250 μg of LacZ-Sec62p fusion protein was used per injection. Antiserum raised against LacZ-Sec62p is referred to as anti-Z62 serum.

Preimmune and immune sera from all four rabbits reacted with multiple yeast polypeptides; therefore, sera were subjected to affinity purification on a TrpE-Sec62p fusion protein matrix prior to further use. An ~1,150-bp fragment that contained the entire *SEC62* coding region (identical to that used to construct pRD17) was inserted into the *Eco*RI site of pATH3 to generate pRD23. *E. coli* cells (MC1061) transformed with pRD23 expressed a 68-kDa fusion protein upon induction with indoleacrylic acid. A 6-liter culture was induced for fusion protein expression (20). Cells were lysed by sonication, and the inclusion body fraction was isolated and solubilized as described elsewhere (16), except that an additional two wash steps, one with 300 mM NaCl–50 mM Tris hydrochloride (pH 7.5)–0.5 mM EDTA–1% Nonidet P-40 and one with 50 mM NaCl–50 mM Tris hydrochloride (pH 7.5)–0.5 mM EDTA, were included prior to solubilization. The solubilized fusion protein was gel purified and concentrated as described above for the LacZ-Sec62p fusion protein, except that the TrpE-Sec62p polypeptide was visualized by treatment with 3 M sodium acetate following SDS-PAGE (22). Concentrated TrpE-Sec62p fusion protein (2 ml at 5 mg/ml) was dialyzed extensively against 100 mM $NaHCO_3$ (pH 8.3)–150 mM NaCl–0.1% SDS, and 5 mg was coupled to 1 ml (packed volume) of CNBr-activated Sepharose 4B (65% coupling) in accordance with manufacturer instructions. A second immobilized fraction was prepared by mixing 15 mg of total protein from *E. coli* cells transformed with pEX1 (LacZ fusion vector alone) and 1 mg of human keratin (our sera frequently contained antikeratin antibodies) with 3 ml of CNBr-activated Sepharose 4B as described above (84% coupling).

The anti-CTD and anti-Z62 sera were each affinity purified in a two-step procedure. Ten milliliters of serum was diluted fivefold in PBS and applied, at a flow rate of 0.5 ml/min, to a column containing the immobilized *E. coli*-keratin protein mixture. The flowthrough fraction was recycled through the same column and applied, at the same flow rate, in two cycles to the immobilized TrpE-Sec62p. Serum that flowed through the TrpE-Sec62p column no longer detected Sec62p on immunoblots. The TrpE-Sec62p column was washed extensively in succession with 10 mM Tris hydrochloride (pH 7.5), 10 mM Tris hydrochloride (pH 7.5)–500 mM NaCl, and 10 mM Tris hydrochloride (pH 7.5) prior to elution of antibodies with 0.1 M glycine (pH 2.5). The eluate was rapidly neutralized with 1 M Tris hydrochloride (pH 8.0) and added to 0.15 ml of protein A–Sepharose CL-4B. Following overnight incubation at 4°C, the Sepharose suspension was poured into a column and washed with 10 ml of 10 mM Tris hydrochloride (pH 7.5), and bound IgG was eluted with 0.1 M glycine (pH 2.5) into a tube containing 1 M Tris hydrochloride (pH 8). Eluted antibodies (~0.8 ml) were dialyzed against PBS and adjusted to 5 mM NaN_3. Both antibody preparations were essentially free of contaminant protein, as assayed by SDS-PAGE. The protein concentrations of the affinity-purified antibodies (~300 μg/ml each) were determined by measuring the A_{280} with human gamma globulin as a standard. Both antibody preparations were used for immunoblotting at 1/2,500 to 1/10,000 dilutions.

Immunofluorescence. Immunofluorescence microscopy was performed essentially as described by Pringle et al. (40) with the following modifications. Lyticase was used to remove the cell wall from formaldehyde-fixed cells, and spheroplasts were washed with 1.2 M sorbitol–0.1 M sodium phosphate (pH 6.5). Fixed spheroplasts were suspended in wash buffer supplemented with 1% SDS and immediately washed four times in sorbitol-sodium phosphate wash buffer lacking SDS (41). The SDS extraction step was essential for visualizing single-copy Sec62p with either anti-CTD or anti-Z62 affinity-purified antibodies, whereas overproduced Sec62p was easily detected in cells prepared for immunofluorescence by the standard procedure (40). Although the affinity-purified anti-Sec62p antibodies yielded a low background signal with wild-type cells prepared by the standard procedure, substantial nonspecific staining was observed with SDS-treated cells. Further purification of both anti-Z62 IgG and anti-CTD IgG (~25 μg of each) on 1-cm^2 pieces of nitrocellulose coated with ~80 μg of β-galactosidase–Sec62p fusion protein (40) reduced background staining and resulted in specific immunofluorescence detection of Sec62p in SDS-treated cells. Doubly-purified anti-Sec62p antibodies (5 to 10 μg of IgG per ml) were used at dilutions of 1/5 to 1/25, and anti-Kar2p serum was used at a 1/1,000 dilution. Bound primary antibodies were decorated with FITC-conjugated goat anti-rabbit IgG, and slides were mounted in Citifluor supplemented with 1 μg of 4′,6-diamino-2-phenyl-indole (DAPI). Cells were originally examined at a magnification of 400× on a Nikon Optiphot microscope. Images were recorded on Kodak TMAX P3200 black and white film (exposure time, 3 s) with a Nikon FX-35WA camera attached to an AFX-IIA shutter controller.

Preparation, fractionation, and proteolysis of cell extracts. Extracts of *sec18* cells (RSY271) were prepared by homogenization of spheroplasts as described elsewhere (14), except for the following modifications. Cells were shifted to 33°C for 60 min in YP plus 0.1% glucose, harvested, and incubated for 10 min in 100 mM Tris (pH 9.4)–10 mM dithiothreitol–10 mM NaN_3 prior to spheroplast formation. Lysis buffer consisted of 200 mM sorbitol, 100 mM NaCl, 5 mM $MgCl_2$, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4). Fractionation of the resulting extract was performed as described by Bernstein et al. (7). To determine the nature of the association of Sec62p with the membrane fraction, the 370 × *g* supernatant was diluted with a 1/5 volume of 2.5% Triton X-100, 0.5 M Na_2CO_3 (pH 12.5), 8 M urea, or 3 M NaCl. Following 15 min of incubation at 4°C, samples were centrifuged for 25 min at 96,600 × *g* in a TLA100 rotor (Beckman). The resulting pellets were suspended in the appropriate extraction buffer, and both the supernatant and pellet fractions were diluted with SDS-PAGE sample buffer, heated at 95°C, and applied to 12.5% SDS-polyacrylamide gels. Protease treatments of the 370 × *g* supernatant fraction were conducted as described elsewhere (24), except that digestions were terminated by the addition of a 1/2 volume of 4 mM PMSF in lysis buffer. Following 10 min of incubation on ice, samples were processed for SDS-PAGE as described above.

Construction and analysis of *SEC62-SUC2* gene fusions. A set of in-frame *SEC62-SUC2* gene fusions was constructed by ligating different exonuclease III-generated 3′ deletions of

SEC62 (originally used for DNA sequence analysis [15]) to the 5′ end of *SUC2*. In this manner, seven gene fusions containing incrementally larger deletions of the 3′ end of *SEC62* fused to the third codon of mature *SUC2* (52) were generated. Fusion constructs were named according to the number of amino acids deleted from the carboxy terminus of Sec62p. All of the fusions were constructed in the multicopy *SUC2* fusion vector pSEY304 (Δ9, Δ96, Δ122, and Δ146) or the closely related pCS29 (Δ15, Δ62, and Δ256). Several fusions were also constructed in the centromere-containing *SUC2* fusion vector pSEYC306 (Δ9, Δ15, and Δ62).

The presence or absence of N-linked carbohydrate on various hybrid proteins was used to assess the membrane orientation of Sec62p. BF-1 cells transformed with different *SEC62-SUC2* fusion plasmids were grown to the mid-log phase in minimal medium lacking uracil. Portions of each culture were pretreated with 10 μg of tunicamycin per ml, and then all samples were pulse-radiolabeled for 18 min at 30°C as described previously (44), except that Tran^{35}S-label (30 μCi per unit of optical density at 600 nm) was used instead of $Na_2{}^{35}SO_4$. Labeled cells were lysed by agitation with glass beads and immunoprecipitated with antiinvertase serum (44).

The membrane association of Sec62p was evaluated by invertase assays of fractions obtained from cells harboring the hybrid proteins. Wild-type (BF-1) cells transformed with multicopy *SEC62-SUC2* fusion plasmids were grown at 30°C in minimal medium lacking uracil and containing 5% glucose (to repress the synthesis of secretory invertase encoded by the chromosomal *SUC2* gene). Cells were converted to spheroplasts and lysed by gentle agitation with glass beads (7). The homogenate was centrifuged for 4 min at 370 × *g* in an HB-4 rotor (Sorvall, Newton, Conn.) to remove unbroken cells, and the resulting supernatant was centrifuged at 103,040 × *g* for 15 min in a TLA100.3 rotor to generate high-speed pellet and supernatant fractions. Invertase activity was assayed by a protocol devised by B. Koch (our laboratory). A sample of each fraction (0.3 to 10 μl) was mixed with 1 ml of assay cocktail (250 mM mannitol, 85 mM potassium phosphate [pH 6.5], 25 μg of glucose oxidase per ml, 2.5 μg of peroxidase per ml, 100 mM sucrose, 100 μg of 3,3′,5,5′-tetramethylbenzidine per ml) with or without 0.1% Triton X-100. Samples were incubated for 15 min at 30°C, and reactions were terminated with 1 ml of 6 N HCl. The A_{450} was compared with that obtained with 0.5 to 8.0 mU of invertase (400 U/mg; Sigma) as a standard.

RESULTS

Detection of Sec62p in yeast cell extracts. Analysis of the *SEC62* DNA sequence revealed an open reading frame capable of encoding a 32-kDa protein with two potential alpha-helical membrane-spanning domains (15). To characterize further the polypeptide encoded by *SEC62* (Sec62p), we elicited antisera directed against different portions of the *SEC62* coding sequence. A fragment encoding the carboxy-terminal 62 amino acids of Sec62p was appended in frame to the 3′ end of the coding sequence for *Staphylococcus aureus* protein A, and the entire *SEC62* open reading frame was fused to the 3′ end of *lacZ*. *E. coli* cells containing these constructs were induced for fusion protein expression, hybrid proteins were isolated by either preparative SDS-PAGE (LacZ-Sec62p) or IgG affinity chromatography (protein A-Sec62p), and the purified antigens were injected into rabbits. Antiserum elicited against the protein A-Sec62p fusion is referred to as anti-CTD serum whereas antiserum elicited against the LacZ-Sec62p fusion is referred to as anti-Z62 serum. Both immune sera were affinity purified by adsorption to an immobilized form of the TrpE-Sec62p fusion protein. Antibodies eluted from this matrix were tested for their specificities by immunoblotting of crude yeast cell extracts (Fig. 1). Both the anti-CTD (lane 1) and anti-Z62 (lane 4) antibodies detected a single polypeptide of 30 kDa. Cells containing the multicopy *SEC62* plasmid pRD6 (15) overproduced the 30-kDa protein 10-fold (lanes 2 and 3), and *sec62* cells cultured at 24°C (lane 6) or shifted to 37°C for 2 h (lane 5) contained 3- to 6-fold-less antigen. A minor 32-kDa species (*) was also overproduced in pRD6 transformants. This form may have arisen by a low level of post-translational modification of the 30-kDa Sec62p or by translational initiation at a distinct ATG codon.

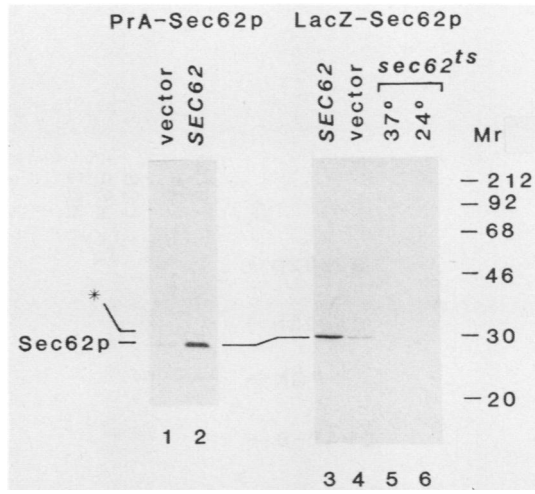

FIG. 1. Specific detection of a 30-kDa protein in yeast cell extracts by affinity-purified anti-Sec62p antibodies. Glass bead extracts of wild-type cells (W303-1B) transformed with either the multicopy vector YEp351 (lanes 1 and 4) or YEp351 containing *SEC62* (pRD6, lanes 2 and 3) were fractionated by SDS-PAGE and immunoblotted with affinity-purified anti-CTD (lanes 1 and 2) or anti-Z62 (lanes 3 to 6) antibodies. Lanes 5 and 6 represent a glass bead lysate of RDM50-94C (*sec62*) cells cultured at 24°C (lane 6) or shifted to 37°C for 2 h before lysis (lane 5). *, Minor, ~32-kDa species that is co-overproduced with the 30-kDa form of Sec62p in pRD6 transformants. M_rs are shown in thousands.

Sec62p is an integral protein of the ER membrane. To test whether Sec62p behaves as an integral membrane protein, as predicted by the sequence analysis, we fractionated cell homogenates by differential centrifugation. Samples from different subcellular fractions were resolved by SDS-PAGE and immunoblotted with antisera directed against Sec62p and various marker proteins (Fig. 2A). Sec62p present in the total extract (lane 1) partitioned almost equally into the 370 × *g* pellet (containing unbroken cells; lane 2) and the supernatant (lane 3) fractions. Upon further fractionation, all of Sec62p sedimented at 96,600 × *g*. In contrast, the peripheral membrane protein Sec23p (24) partitioned into both the 96,600 × *g* pellet and the supernatant fractions. As expected, the cytoplasmic enzyme phosphoglycerate kinase was enriched in the soluble fraction and the vacuolar membrane protein DPAP-B (41) partitioned exclusively into the particulate fraction.

The physical nature of the association of Sec62p with the particulate fraction was explored by treating the 370 × *g* supernatant fraction derived from the experiment shown in

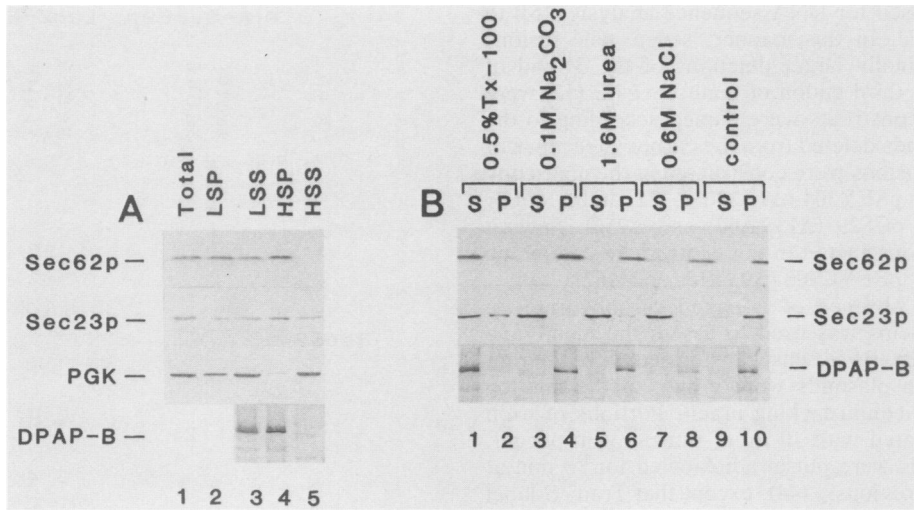

FIG. 2. Behavior of Sec62p as an integral membrane protein. (A) RSY271 (*sec18*) cells incubated at the restrictive temperature of 33°C for 1 h were converted to spheroplasts and lysed by homogenization. The initial lysate (Total) was centrifuged at 370 × *g* yielding low-speed pellet (LSP) and supernatant (LSS) fractions. The LSS was centrifuged at 96,600 × *g* to generate high-speed pellet (HSP) and supernatant (HSS) fractions. Equal portions of each fraction (percent of total) were resolved by SDS-PAGE and immunoblotted with anti-Sec62p (anti-CTD plus anti-Z62 mixture), anti-Sec23p, anti-phosphoglycerate kinase (PGK), or anti-DPAP-B antibodies. (B) Portions of the same LSS fraction as that used in panel A were diluted with lysis buffer (control) or adjusted to final concentrations of 0.5% Triton X-100 (Tx-100), 0.1 M Na_2CO_3 (pH 11), 1.6 M urea, or 0.6 M NaCl as indicated. After 15 min of incubation on ice, all samples were separated into supernatant (S) and pellet (P) fractions by centrifugation at 96,600 × *g*, followed by SDS-PAGE and immunoblotting with anti-Sec62p, anti-Sec23p, or anti-DPAP-B antibodies. The relevant portion of each immunoblot is combined in a montage.

Fig. 2A with various reagents, followed by centrifugation at 96,600 × *g*. Samples of the resulting pellet and supernatant fractions were resolved by SDS-PAGE and immunoblotted with anti-Sec62p, anti-Sec23p, and anti-DPAP-B sera (Fig. 2B). Both Sec62p and the integral membrane protein DPAP-B were efficiently solubilized by nonionic detergent but were not released from the particulate fraction by a high salt concentration, urea, or sodium carbonate (pH 11), which is commonly used to strip nonintegral proteins from membranes (19). In contrast, Sec23p was efficiently eluted from membranes by all of the treatments used (Fig. 2B) (24).

Although Sec62p behaves as an integral membrane protein, it apparently does not contain Asn-linked carbohydrate. The migration of Sec62p on SDS-polyacrylamide gels was not detectably altered either by digestion with the enzyme endoglycosidase H (D. Feldheim and R. J. Deshaies, unpublished results) or by synthesis in the presence of the drug tunicamycin (B. Koch, unpublished results), which inhibits the formation of oligosaccharide donors.

To assess the intracellular distribution of Sec62p in more detail, we performed indirect immunofluorescence microscopy with wild-type, *sec62*, and *SEC62*-overproducing strains (Fig. 3). Cells were probed with anti-Z62 IgG followed by FITC-conjugated goat anti-rabbit antibodies, and nuclei were labeled with the DNA-binding dye DAPI. In wild-type cells transformed with a multicopy vector lacking an insert (YEp351), Sec62p appeared to be localized primarily in a continuous circumnuclear band and in a segmented strip at the periphery of the cell (Fig. 3C). In addition, fine strands passing through the cytoplasm, connections between the nuclear envelope and the cell perimeter, and punctate cytoplasmic dots were occasionally observed. This localization pattern is reminiscent of that observed for the yeast homolog of the immunoglobulin heavy-chain-binding protein, BiP (Kar2p) (43). In parallel experiments, we observed similar staining patterns with both anti-Kar2p and anti-Z62 antibodies (data not shown). This pattern is thought to represent the distribution of ER membranes in yeasts for the following reasons: Kar2p participates in ER-localized functions (55); BiP is localized to the lumen of the ER in mammalian cells (10, 34); and core-glycosylated mutant invertase molecules that fail to exit the ER accumulate in a similar compartment (27).

Immunofluorescence staining of the ER-nuclear envelope network was directly attributable to Sec62p. Overproduction of Sec62p in cells transformed with pRD6 resulted in a more intense but qualitatively similar staining pattern (Fig. 3H), which was also seen with anti-CTD antibodies (data not shown). Conversely, the intensity of ER-like fluorescence was markedly reduced in *sec62* cells cultured at 24°C (Fig. 3L); Kar2p staining was not altered in these cells (data not shown).

Topology of Sec62p in the ER membrane. From an analysis of the Sec62p sequence, we predicted two closely positioned membrane-spanning segments in the middle of Sec62p flanked by hydrophilic amino (~20-kDa)- and carboxy (~7-kDa)-terminal domains (15). If this model is correct, the bulk of Sec62p must be displayed on either the cytosolic or the lumenal side of the ER membrane. These alternate topologies suggest distinct predictions concerning the sensitivity of Sec62p to digestion by proteases added to a cell homogenate. If the hydrophilic domains of Sec62p are oriented towards the cytosol, they might be sensitive to added proteases, whereas in the converse orientation, protease-resistant amino- and carboxy-terminal fragments of Sec62p might be detected. *sec18* cells were cultured at the nonpermissive temperature for 60 min, and a homogenate was prepared and cleared of unbroken cells by centrifugation at 370 × *g*, yielding a low-speed supernatant fraction. The *sec18* mutation causes secretory precursors to accumulate in the ER lumen, thereby providing a marker for this compartment (7, 37). Samples of the 370 × *g* supernatant fraction

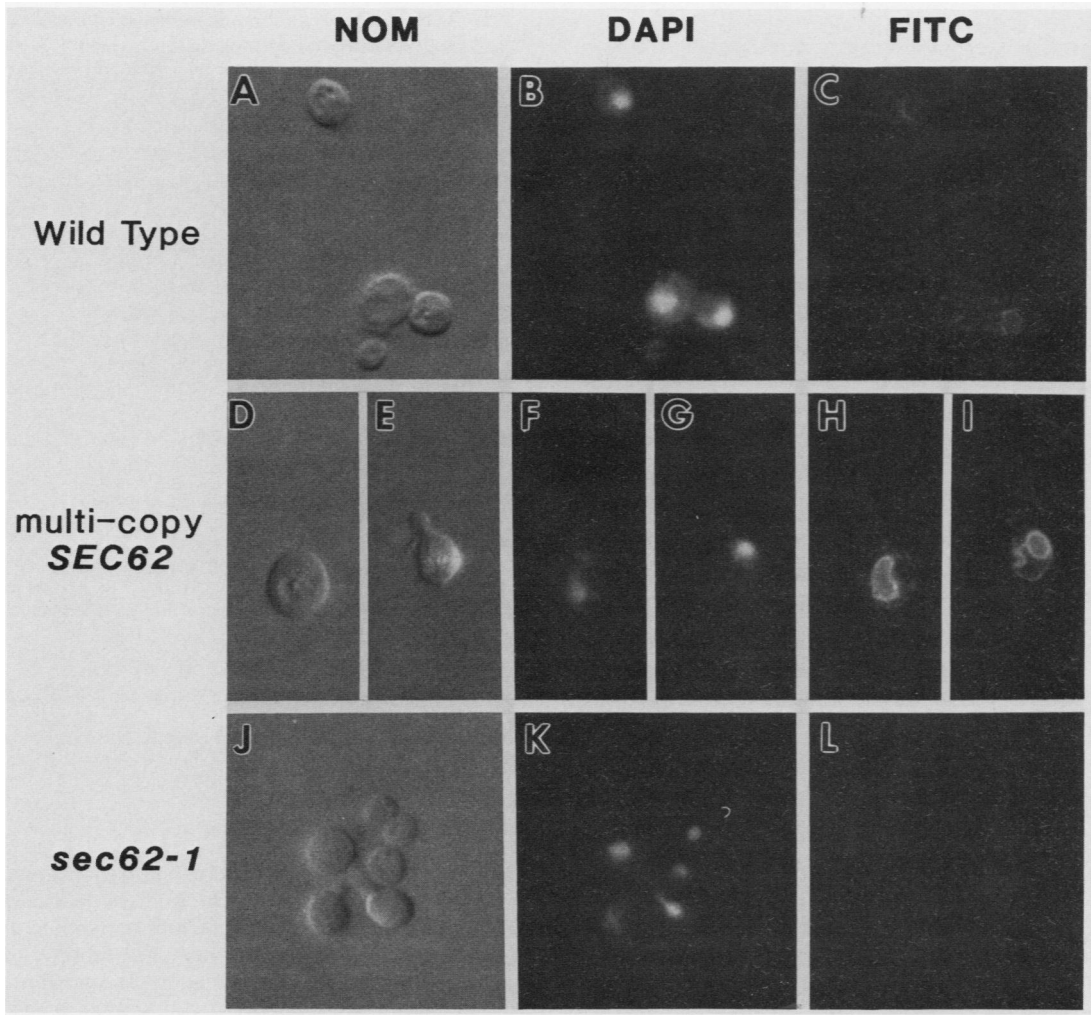

FIG. 3. Localization of Sec62p to the ER. Wild-type cells (BF-1) transformed with the multicopy vector YEp351 (panels A to C) or YEp351 containing *SEC62* (pRD6; panels D to I) and RDM50-94C (*sec62*) cells grown at 24°C (panels J to L) were fixed with formaldehyde and probed with affinity-purified anti-Z62 antibodies followed by secondary decoration with FITC-conjugated goat anti-rabbit IgG. The cells shown were magnified 320 times, and the images in panels C, H, I, and L were exposed for equivalent amounts of time (~3 s). Nomarski images are presented in panels A, D, E, and J; DAPI staining of nuclei is shown in panels B, F, G, and K; and fluorescein fluorescence is shown in panels C, H, I, and L.

were treated with different proteases for 2 to 20 min at 4°C. Protease digestions were terminated with PMSF, and the quenched reactions were analyzed by SDS-PAGE and immunoblotting with anti-Z62, anti-Sec23p, and anti-pro-α-factor antibodies (Fig. 4). As a control a sample of the 370 × *g* supernatant fraction was sedimented at 96,600 × *g* to determine the fraction of the lumenal marker protein pro-α-factor that was enclosed within ER vesicles (lanes 11 to 13). When proteinase K, trypsin, or a cocktail of both proteases was added to the homogenate in the presence (lanes 7 to 10) or absence (lanes 2 to 5) of Triton X-100, both Sec62p and Sec23p (a marker for the cytosolic surface of intracellular membranes [24]) were rapidly and completely degraded. Pro-α-factor accumulated in the ER lumen by the action of the *sec18* mutation (37), was resistant to added proteases (lanes 2 to 5), but was rapidly degraded when the ER bilayer was solubilized by detergent treatment (lanes 7 to 10). Our inability to detect protease-resistant fragments of Sec62p suggests that its hydrophilic amino- and carboxy-terminal domains face the cytosol.

Since the proteolysis experiments provided only crude information on the topology of Sec62p and did not directly reveal which portions of Sec62p span the ER membrane, we studied the topology of Sec62p by an independent method. To map the location and polarity of transmembrane domains in Sec62p, we constructed a set of hybrid molecules containing progressively smaller segments of the Sec62p amino terminus fused to the mature portion of the secretory glycoprotein invertase (encoded by the *SUC2* gene [52]). The rationale underlying this approach is that fusion of invertase to segments of Sec62p normally resident in the cytosol will result in fusion proteins that lack Asn-linked carbohydrate, whereas invertase fused to lumenal portions of Sec62p will become extensively core-glycosylated, resulting in aberrant migration of the fusion proteins on SDS-polyacrylamide gels. The endpoints of the deletions used to construct these chimeric genes are indicated on the top half of Fig. 5. All of these fusions were transformed into wild-type cells, and the glycosylation state of the expressed fusion proteins was examined by pulse-radiolabeling in the presence or absence of tunicamycin. Extracts of radiolabeled cells were treated with anti-invertase serum, and the immunoprecipitates were

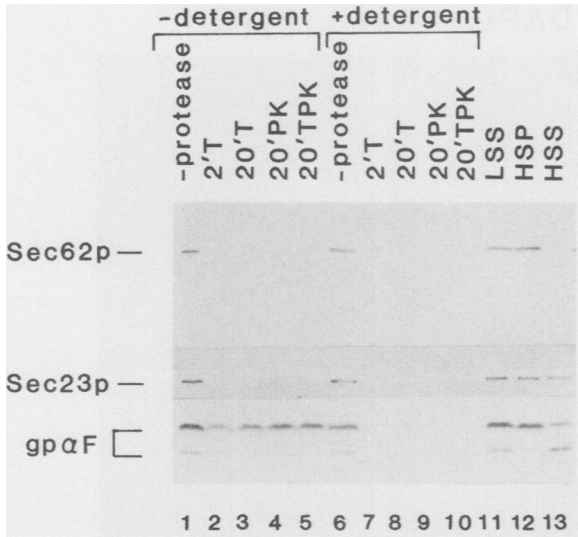

FIG. 4. Accessibility of membrane-associated Sec62p to exogenously added proteases. The same LSS fraction as that used in Fig. 2 was either mock digested (lanes 1 and 6) or treated with 500 μg of trypsin per ml (lanes 2, 3, 7, and 8), 500 μg of proteinase K per ml (lanes 4 and 9), or 250 μg of each protease per ml in combination (lanes 5 and 10) for 2 min (lanes 2 and 7) or 20 min (lanes 3 to 5 and 8 to 10) at 4°C. Protease digests were conducted in the absence (lanes 1 to 5) or the presence (lanes 6 to 10) of 0.4% Triton X-100. All digests were terminated with PMSF and assessed by SDS-PAGE (12.5% gel) and immunoblotting with anti-Sec62p (a mixture of both anti-Z62 and anti-CTD), anti-Sec23p, and anti-pro-α-factor antibodies. The relevant portion of each immunoblot is combined in a montage. The LSS, HSP, and HSS fractions shown in lanes 11 to 13 are as described in the legend to Fig. 2A. gpαF, Glycosylated pro-α-factor accumulated in the lumen of the ER by imposition of the *sec18* block.

TABLE 1. Fractionation and compartmentation of invertase activity associated with Sec62p-invertase hybrid proteins

Fusion	% Invertase activity (sp act)[a] with the following fractionation and enrichment[b]:			Latency[c] (mU/mg)	
	LSS	HSS	HSP	Without Triton X-100	With 0.1% Triton X-100
pCS29	100 (0.4)	117 (0.3)	6 (0.06)	ND[d]	ND
Sec62p (Δ9)-invertase[e]	100 (0.7)	4 (0.04)	96 (1.6)	1.6	1.8
Sec62p (Δ96)-invertase[e]	100 (2.4)	3 (0.09)	84 (4.4)	2.7	5.4
Sec62p (Δ122)-invertase[e]	100 (3.8)	35 (1.8)	45 (4.0)	3.5	4.0
SUC2-4H11-invertase[f]	100 (2.2)	14 (0.4)	66 (3.9)	3.2	5.2

[a] Specific activity is reported as milliunits of invertase activity per milligram of protein. One milliunit of invertase hydrolyzes 1 μmol of sucrose per min at 55°C.

[b] LSS, Low-speed supernatant (370 × *g*); HSS, high-speed supernatant (103,040 × *g*); HSP, high-speed pellet (103,040 × *g*). Percent invertase activity in the HSS and HSP fractions was normalized to the amount detected in the LSS fraction.

[c] Latency measurements were performed with the HSP fraction.

[d] ND, Not determined.

[e] *SEC62-SUC2* gene fusions were carried on a 2μm circle-based *URA3* plasmid.

[f] *SUC2-4H11* encodes a mutant form of secretory invertase that fails to exit the ER (27). The *suc2-4H11* allele is under the transcriptional control of the phosphoglycerate kinase promoter and is contained on a single-copy *URA3* plasmid.

analyzed by SDS-PAGE (Fig. 5, bottom). Cells containing the *SUC2* fusion vector (which lacks both a promoter and an initiation codon) did not express any immunoreactive species (lane 1). Cells expressing the Sec62p (Δ9)-invertase, Sec62p (Δ62)-invertase, Sec62p (Δ122)-invertase, and Sec62p (Δ146)-invertase fusion proteins contained immunoreactive species that were of the expected molecular masses and were not altered by the inclusion of tunicamycin during pulse-labeling, suggesting that they lacked Asn-linked carbohydrate. Immunoprecipitation of the vacuolar glycoprotein carboxypeptidase Y from each sample indicated that the addition of tunicamycin effectively blocked Asn-linked glycosylation (data not shown). Similarly, the Sec62p (Δ15)-invertase and Sec62p (Δ256)-invertase fusion proteins were not modified by core glycosylation (data not shown). In contrast, the chimeric polypeptide encoded by the *sec62-Δ96* allele migrated unexpectedly slowly upon SDS-PAGE (lane 7) and was reduced in apparent molecular mass by ~25 kDa in the presence of tunicamycin (lane 8). These data support the results obtained in the proteolysis experiment (Fig. 4) and suggest that the amino- and carboxy-terminal domains of Sec62p face the cytosol, whereas the loop that separates the transmembrane domains protrudes into the ER lumen.

Additional evidence for the compartmentation of the invertase moieties of the Sec62p invertase fusion polypeptides was provided by an analysis of the latency of invertase activity in membrane fractions prepared from transformants expressing the different fusion constructs (Table 1). Sucrose, the substrate for invertase, does not readily penetrate membranes. Fusion proteins which display invertase on the cytoplasmic face of the ER membrane should exhibit maximal invertase activity in the absence of detergent, whereas the activity of invertase molecules sequestered in the ER lumen should show apparent stimulation in the presence of detergent. Invertase activities associated with the Sec62p (Δ9)-invertase and Sec62p (Δ122)-invertase fusion proteins were not appreciably altered by the addition of Triton X-100, whereas the activities of lumenal invertase (encoded by *SUC2-4Hll* [27]) and the Sec62p (Δ96)-invertase fusion protein were consistently stimulated approximately twofold by the detergent. Thus, the invertase moiety of the nonglycosylated fusion proteins was exposed to the cytosolic compartment, whereas at least 50% of the invertase molecules encoded by the *SEC62* (*Δ96*)-*SUC2* fusion were sequestered by an intact membrane.

Failure to glycosylate the Sec62p (Δ9)-invertase hybrid may have arisen from defective insertion into the ER membrane. Three lines of evidence argue against this possibility. First, invertase activity exhibited by this hybrid protein, as well as that associated with Sec62p (Δ96)-invertase, was enriched in the particulate fraction of wild-type cells (Table 1). In contrast, cytoplasmic invertase (pCS29 transformants) was enriched in the soluble fraction and invertase activity associated with Sec62p (Δ122)-invertase, which lacks both predicted transmembrane domains, was distributed in both the 103,040 × *g* supernatant and the pellet fractions (Table 1). Perhaps some of the soluble Sec62p (Δ122)-invertase molecules are bound to a component of the ER membrane translocation apparatus. Second, immunofluorescence microscopy with anti-invertase antibodies indicated that the Sec62p (Δ9)-invertase hybrid exhibited an ER localization pattern (data not shown) similar to that seen for authentic

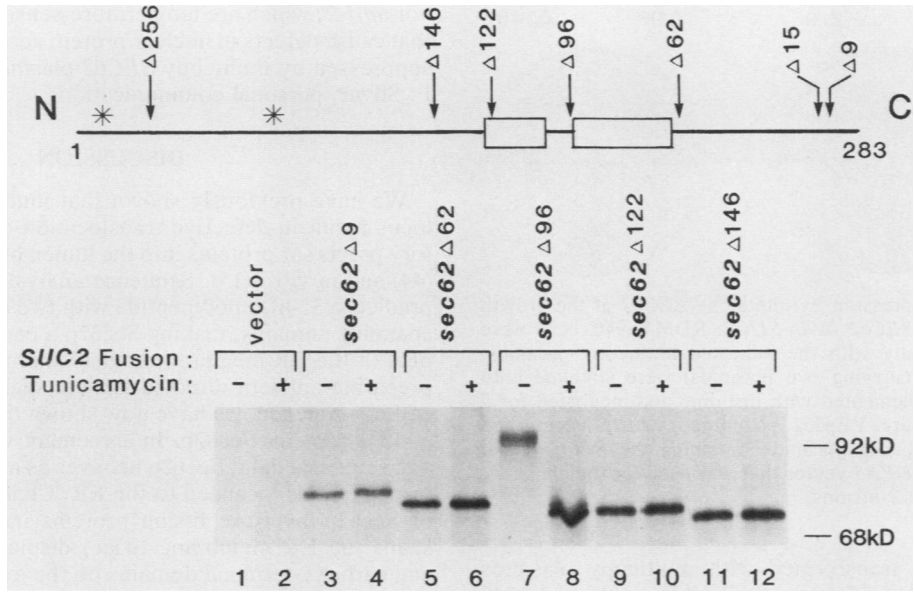

FIG. 5. Analysis of the topology of Sec62p-invertase fusion proteins. (Top) Schematic diagram of Sec62p. Boxed regions represent predicted transmembrane domains, and asterisks indicate potential acceptor sites for oligosaccharides. The *SEC62* open reading frame encodes 283 amino acids. Endpoints of carboxy-terminal deletions used to construct *SEC62-SUC2* fusions are indicated above Sec62p. Deletion alleles are named according to the number of amino acids deleted from the carboxy terminus of Sec62p. (Bottom) Wild-type cells (BF-1) transformed with the *SUC2* fusion vector pCS29 (lanes 1 and 2) or with the indicated *SEC62-SUC2* hybrid constructs (lanes 3 to 12) were pulse-labeled for 18 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-invertase serum, and the immunoprecipitates were subjected to SDS-PAGE and autoradiography. kD, Kilodaltons.

Sec62p (Fig. 3). Third, the *SEC62* (Δ9)-*SUC2* chimeric gene complements both temperature-sensitive and null alleles of *SEC62* when present on either a multicopy or a centromeric plasmid (Table 2).

Genetic analysis of *SEC62-SUC2* fusions. The ability of *SEC62* (Δ9)-*SUC2* to complement *sec62* mutations indicated that the carboxy-terminal nine amino acids of Sec62p were not essential for its activity. To test whether other sequences in the carboxy-terminal portion of Sec62p were required for activity, we introduced all of the *SEC62-SUC2* fusions into RDM50-94C [*sec62-1*(Ts)]. Multicopy and centromeric plasmids bearing the Δ9 and Δ15 alleles complemented the temperature-sensitive growth phenotype of RDM50-94C, whereas neither multicopy nor centromeric plasmids containing the Δ62 allele were able to sustain growth at 37°C (Table 2). Multicopy plasmids bearing each of the subsequent deletions (Δ96, Δ122, Δ146, and Δ256 alleles) were likewise unable to complement *sec62-1*(Ts).

TABLE 2. Complementation of and dominant interference with *sec62*1 (Ts) by *SEC62-SUC2* gene fusions

Fusion plasmid[a]	Complementation of *sec62*(Ts) growth[b]	Growth of *sec62* transformants at 24°C[c]
pCS29	−	+++
pSEYC306	−	+++
SEC62 (Δ9)-*SUC2* CEN	+	+++
SEC62 (Δ9)-*SUC2* 2μm	+	+++
SEC62 (Δ15)-*SUC2* CEN	+	+++
SEC62 (Δ15)-*SUC2* 2μm	+	+++
SEC62 (Δ62)-*SUC2* CEN	−	+++
SEC62 (Δ62)-*SUC2* 2μm	−	+/−−[d]
SEC62 (Δ96)-*SUC2* 2μm	−	+/−[d]
SEC62 (Δ122)-*SUC2* 2μm	−	+[d]
SEC62 (Δ146)-*SUC2* 2μm	−	+++
SEC62 (Δ256)-*SUC2* 2μm	−	+++

[a] CEN, Centromere plasmid pSEYC306. 2μm, Multicopy plasmids pCS29 and pSEY304.

[b] Complementation of RDM50-94C [*sec62-1*(Ts)] was assayed at 34 and 37°C on both YPD and selective minimal medium.

[c] Colony sizes of RDM50-94C [*sec62-1*(Ts)] transformants were scored at 24°C on selective minimal medium.

[d] +, +/−, and +/−−, Increasingly severe reductions in the rate of growth of these transformants relative to pCS29-bearing cells.

Some of the fusion proteins, when overproduced, severely inhibited the growth of *sec62* (RDM50-94C) but not wild-type (BF-1) cells at 24°C (Table 2). The *SEC62* (Δ62)-*SUC2*, *SEC62* (Δ96)-*SUC2*, and *SEC62* (Δ122)-*SUC2* fusions inhibited the growth of *sec62* cells to various extents; inhibition by the Δ96 allele was severe, whereas the Δ122 allele was moderately inhibitory. In contrast, neither the smaller deletions (Δ9 and Δ15) nor the larger deletions (Δ146 and Δ256) had a deleterious effect on the growth rate, suggesting that the toxicity of the Δ62, Δ96, and Δ122 alleles was not merely due to the presence of invertase.

We considered the possibility that the inhibitory fusion proteins lacked a function associated with the carboxy-terminal domain of Sec62p but retained the capacity to interact with and thereby titrate some component of the translocation machinery. Two types of genetic interaction have been observed between *sec62* and *sec63*: synthetic lethality (44) and allele-specific copy number suppression of the temperature-sensitive growth associated with the *sec63-1* mutation by *SEC62*-containing plasmids (see below). As these genetic interactions may portend some form of biochemical interaction between the SEC62 and SEC63 gene products, it seemed reasonable to propose that the inhibitory Sec62p-invertase fusion proteins sequester, either directly or indirectly, the SEC63 protein. To address this possibility, we introduced the multicopy vector YEp351 or YEp351 containing the *SEC63* gene (pDF14) into RDM50-94C. Cells bearing

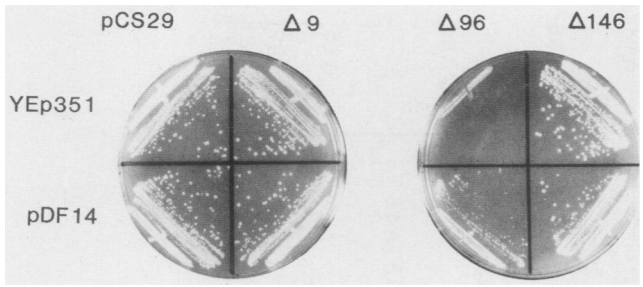

FIG. 6. Partial suppression by multicopy *SEC63* of the growth inhibition caused by *SEC62* (Δ96)-*SUC2*. RDM50-94C cells were transformed sequentially with the indicated multicopy plasmids. Transformants (each carrying two plasmids) were streaked onto minimal medium supplemented with histidine and incubated for 5 days at room temperature. YEp351 is a multicopy *LEU2* vector, and pDF14 is a YEp351-based plasmid containing the *SEC63* gene. pCS29 is a multicopy *URA3* vector that was used for the construction of the *SEC62-SUC2* fusions.

each plasmid were transformed with multicopy plasmids bearing inhibitory (Δ96 allele) or noninhibitory (Δ9 and Δ146 alleles) *SEC62-SUC2* hybrids or with the *SUC2* fusion vector alone (pCS29). Clones harboring both YEp351 and *SEC62* (Δ96)-*SUC2* grew slowly, whereas cells doubly transformed with YEp351 and pCS29, *SEC62* (Δ9)-*SUC2*, or *SEC62* (Δ146)-*SUC2* grew normally (Fig. 6). RDM50-94C transformants containing pDF14, however, were partially immune to the inhibitory effects of *SEC62* (Δ96)-*SUC2*. Although increased *SEC63* gene dosage reproducibly provided some protection against the toxic effects of all three inhibitory fusions (data not shown), full restoration of normal growth rates was never observed.

Overexpression of Sec62p suppressed the temperature-sensitive growth phenotype of *sec63* cells (Fig. 7). The extent of suppression was related to the level of Sec62p expression; multicopy plasmids bearing *SEC62* corrected the growth defect more effectively than did centromeric plasmids harboring the same *SEC62* insert (data not shown). Conversely, multicopy *SEC63* plasmids failed to suppress the *sec62-1* mutation. Overproduction of Sec62p did not bypass a requirement for Sec63p function, since the multicopy *SEC62* plasmid pRD6 did not suppress the lethal phenotype caused by deletion of the *SEC63* gene (D. Feldheim, unpublished results). Copy number suppression of *sec63-1* by *SEC62* plasmids was allele specific; neither *npl1-1* nor *npl1-2*, which are temperature-sensitive alleles of *SEC63* that cause defects in nuclear protein accumulation (46), were suppressed by multicopy *SEC62* plasmids (data not shown; P. Silver, personal communication).

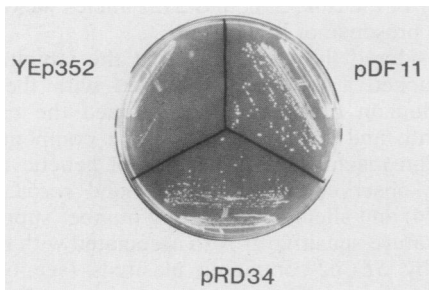

FIG. 7. Suppression by increased *SEC62* gene dosage of the temperature-sensitive growth phenotype of *sec63-1* cells. RSY151 (*sec63-1*) cells transformed with the indicated plasmids were streaked onto minimal medium supplemented with leucine and incubated at 37°C for 2 days. YEp352 is a multicopy *URA3* vector. pDF11 and pRD34 are YEp352-based plasmids containing the *SEC63* and *SEC62* genes, respectively.

DISCUSSION

We have previously shown that mutations at the *SEC62* locus result in defective translocation of a subset of secretory precursor proteins into the lumen of the ER both in vivo (44) and in vitro (15). Sequence analysis of the *SEC62* gene predicts a 32-kDa polypeptide with two potential membrane-spanning domains, making Sec62p a candidate for a component of the ER membrane translocation machinery (15). By preparing antisera directed against segments of the *SEC62* coding sequence, we have now shown that *SEC62* encodes a 30-kDa protein, Sec62p. In agreement with the biochemical and sequence data, Sec62p behaves as an integral membrane protein and is localized to the ER. Characterization of a set of Sec62p-invertase fusion proteins indicated that Sec62p spans the ER membrane twice, displaying soluble amino- and carboxy-terminal domains on the cytoplasmic face. The carboxy-terminal domain of Sec62p is essential for Sec62p function, and the amino-terminal domain acts as a *trans*-dominant growth inhibitor of *sec62* cells, suggesting that the amino-terminal domain may interact with other components of the translocation machinery independently of the carboxy-terminal domain.

Predicting the topology of membrane proteins through the characterization of chimeras assumes that the information specifying membrane assembly is decoded in a linear, amino- to carboxy-terminal fashion. Numerous *E. coli* and eucaryotic membrane proteins with hydrophobic alpha-helical membrane anchor domains apparently adhere to this paradigm. Analysis of alkaline phosphatase fusions correctly predicted the orientation of several *E. coli* cytoplasmic membrane proteins with a well-established topology, including the tsr receptor (31, 32) and leader peptidase (47), even though the first hydrophobic domains of these two proteins adopt opposite transmembrane orientations. The pattern of membrane assembly of hybrid proteins containing multiple iterations of the asialo-glycoprotein receptor membrane-spanning region suggests that transmembrane segments act as independent topogenic domains during the insertion of a multispanning membrane protein into the ER bilayer in vitro (56). The orientation of a given transmembrane domain in these chimeras is not influenced by downstream sequences, suggesting that topogenic information is decoded linearly from the amino terminus to the carboxy terminus.

It is apparent for some proteins, however, that a simple extrapolation of transmembrane topology is not possible. Analysis of HMG-CoA reductase topology with *HIS4* fusions suggests that the third and fourth hydrophobic anchor segments of this multispanning membrane protein act in concert during its assembly into the ER membrane (50). Proteins that span membranes in an amphipathic alpha-helical (29) or β-strand (28) configuration may also assemble via nonvectorial pathways. The membrane-spanning segments of mitochondrial and *E. coli* porins are short β-strands arranged in an antiparallel β-sheet structure (28). Small deletions at either the amino or the carboxy terminus of the mitochondrial porin block its insertion into the outer membrane (17), suggesting that the antiparallel β-sheets may assemble into the membrane in a cooperative manner. These examples stress the limitations of the hybrid protein approach.

Independent evidence supports our proposed orientation of Sec62p. The carboxy terminus of Sec62p is proposed to face the cytoplasm, since fusion of invertase at three different positions in the carboxy-terminal domain resulted in unglycosylated hybrid proteins that exhibited nonlatent invertase activity. Furthermore, the Sec62p (Δ9)-invertase hybrid complemented a null allele of *SEC62*, suggesting that it reflects the authentic topology of Sec62p. Three lines of evidence support a cytoplasmic location for the amino-terminal domain of Sec62p. First, invertase fused at three different positions in the amino-terminal domain is cytoplasmic, as assayed by both glycosylation and enzymatic criteria. Second, the cytosolically localized Sec62p (Δ122)-invertase fusion acts as a dominant inhibitor of the growth of *sec62* cells, implying that it interacts specifically with some component of the translocation apparatus. Third, two potential acceptor sites for Asn-linked oligosaccharides in the amino-terminal domain of Sec62p are not used. A lumenal disposition for the loop between the transmembrane domains is supported by the observation that invertase fused to this segment is extensively core glycosylated. Besides the evidence derived from the characterization of the Sec62p-invertase fusion proteins, the protease sensitivity of Sec62p in wild-type microsomes suggests that both hydrophilic domains are exposed to the cytosol. The topology that we propose for Sec62p conflicts with the topology suggested by an algorithm that uses the sum of charged residues flanking putative transmembrane domains to predict the orientation of integral membrane proteins (23). Perhaps this disagreement reflects a distinctive mechanism for the assembly of yeast membrane proteins or components of the translocation machinery. The signal recognition particle receptor, for example, binds tightly to and properly assembles in dog pancreas membranes by a signal recognition particle-independent pathway (1).

Genetic analysis of the *SEC62-SUC2* gene fusions suggested that the amino- and carboxy-terminal domains of Sec62p may function independently to promote protein translocation. Hybrid proteins that lacked large portions of the carboxy-terminal domain of Sec62p failed to complement *sec62-1*(Ts), suggesting that these portions are required for Sec62p function. It is unlikely that the invertase moiety interferes with the function of Sec62p, since both *SEC62* (Δ9)-*SUC2* and *SEC62* (Δ15)-*SUC2* were able to complement *sec62-1*(Ts).

Unexpectedly, the overproduction of chimeric proteins lacking the carboxy-terminal domain of Sec62p severely inhibited the growth of *sec62-1*(Ts) strains at 24°C. Similar *trans*-dominant effects exerted by protein fragments or mutant polypeptides have been observed with components of the *S. cerevisiae CDC25-RAS*-adenylate cyclase signal transduction pathway (18, 39). Several possible models may be invoked to explain this phenomenon. By analogy with the toxic effect of overproducing maltose-binding protein–LacZ fusion proteins in *E. coli* (4), the inhibitory chimeric proteins may jam translocator sites during assembly into the ER membrane. This scenario is unlikely, because growth inhibition was observed with a fusion protein [Sec62p (Δ122)-invertase] that apparently lacked membrane insertion signals. Alternatively, the inhibitory hybrid proteins may interfere with the function of some critical pathway unrelated to translocation. However, as the inhibitory constructs affected *sec62-1* but not wild-type cells, we suspect that the inhibition arose by exacerbation of the translocation defect imposed by the *sec62* mutation. Perhaps the inhibitory Sec62p-invertase chimeras sequestered mutant Sec62p or competitively inhibited interactions between mutant Sec62p and other translocation components. The differential sensitivity of *sec62-1* cells to the toxic effect of the hybrid proteins may have been due to a reduced level of Sec62p in mutant cells or to a reduced affinity of the interaction between mutant Sec62p and another translocation factor(s). *SEC63* overexpression antagonized the action of inhibitory Sec62p-invertase chimeras, suggesting that Sec63p may be one of the translocation factors whose effective concentration is diminished by overproduction of the Sec62p C-terminal deletion proteins. Since multicopy *SEC63* conferred only partial immunity, genes encoding other components of the translocation apparatus may be identified in this manner.

The Sec62p-invertase fusion proteins generated in this study may prove useful for addressing various aspects of ER function. Sec62p (Δ9)-invertase, for example, appears to be properly targeted to the ER, contains an active invertase domain exposed to the cytoplasm, and is fairly stable (half-life, >75 min; unpublished results). These properties make this chimera a useful marker for conventional fractionation or immunoaffinity isolation of ER membranes. Since *SEC62* (Δ9)-*SUC2* complemented a null allele of *SEC62*, the fusion protein can also be used to study the solubilization and fractionation properties of Sec62p itself. Sec62p (Δ96)-invertase, which projects the invertase moiety into the lumen of the ER, may be useful for dissecting *cis*- and *trans*-acting mechanisms responsible for the segregation of ER membrane proteins from exported proteins. Cells transformed with *SEC62* (Δ96)-*SUC2* expressed less than 2% of the total invertase activity at the cell surface (unpublished results), suggesting that the invertase domain is efficiently retained within the cell by the attached segment of Sec62p. Further characterization of the structures of individual ER membrane proteins is likely to generate valuable reagents for studying the functional properties of the ER and to provide useful information about the structural and functional organization of the multicomponent assemblages that promote translocation, posttranslational modification, and intercompartmental transport of secretory proteins.

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