Protein Translocation Mutants Defective in the Insertion of Integral Membrane Proteins into the Endoplasmic Reticulum

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Submitted August 30, 1991; Accepted November 22, 1991

Yeast mutants defective in the translocation of soluble secretory proteins into the lumen of the endoplasmic reticulum (*sec61*, *sec62*, *sec63*) are not impaired in the assembly and glycosylation of the type II membrane protein dipeptidylaminopeptidase B (DPAPB) or of a chimeric membrane protein consisting of the multiple membrane-spanning domain of yeast hydroxymethylglutaryl CoA reductase (*HMG1*) fused to yeast histidinol dehydrogenase (*HIS4C*). This chimera is assembled in wild-type or mutant cells such that the His4c protein is oriented to the ER lumen and thus is not available for conversion of cytosolic histidinol to histidine. Cells harboring the chimera have been used to select new translocation defective *sec* mutants. Temperature-sensitive lethal mutations defining two complementation groups have been isolated: a new allele of *sec61* and a single isolate of a new gene *sec65*. The new isolates are defective in the assembly of DPAPB, as well as the secretory protein α -factor precursor. Thus, the chimeric membrane protein allows the selection of more restrictive *sec* mutations rather than defining genes that are required only for membrane protein assembly. The *SEC61* gene was cloned, sequenced, and used to raise polyclonal antiserum that detected the Sec61 protein. The gene encodes a 53-kDa protein with five to eight potential membrane-spanning domains, and Sec61p antiserum detects an integral protein localized to the endoplasmic reticulum membrane. Sec61p appears to play a crucial role in the insertion of secretory and membrane polypeptides into the endoplasmic reticulum.

INTRODUCTION

The first stage in the eukaryotic secretory pathway is the translocation of proteins into, or across, the endoplasmic reticulum (ER)¹ membrane. Deshaies and Schekman (1987) reported the identification and char-

acterization of yeast mutants defective in this first step of secretion. These mutants fall into three complementation groups, thus defining three genes, *SEC61*, *SEC62*, and *SEC63*, whose products are essential for protein translocation into the yeast ER (Deshaies and Schekman, 1987, 1989; Rothblatt *et al.*, 1989). In addition to these three *SEC* genes, two classes of topologically distinct Hsp70-like proteins have been implicated in protein translocation in yeast, namely, the ER luminal Kar2p and the cytosolic products of the *SSA* gene family. In the case of *KAR2*, a yeast homologue of the mammalian immunoglobulin heavy-chain binding protein (BiP; Normington *et al.*, 1989; Rose *et al.*, 1989), mutant alleles have been isolated that exhibit severe defects in the translocation of secretory proteins (Vogel *et al.*, 1990). The *SSA1* and *SSA2* genes encode functionally inter-

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¹ Abbreviations used: DPAPB, dipeptidylaminopeptidase B; HMG, hydroxymethylglutaryl coenzyme A reductase; PGK, phosphoglycerate kinase; CPY, carboxypeptidase Y.

Table 1. Bacterial and yeast strains

Strain	Genotype	Source or reference
<i>Saccharomyces cerevisiae</i>		
FC2-12B	<i>leu2-3, -112 ura3-52 trp1-1 his4-401 HOL1-1 MATα</i>	Parker and Guthrie, 1985
FC2a	<i>leu2-3, -112 ura3-52 trp1-1 his4-401 HOL1-1 MATa</i>	Derived from DYCF2-12B (Deshaies and Schekman, 1987)
JRM156	<i>pep4-3 ura3-52 leu2-3, -112, MATα</i>	Rothblatt <i>et al.</i> , 1989
RDM15-5B	<i>leu2-3, -112 ura3-52 ade2 pep4-3 sec61-2 MATα</i>	Deshaies and Schekman, 1987
RDM43-9C	<i>sec62-1 his4 ura3-52 Δpep4::URA3 MATα</i>	Rothblatt <i>et al.</i> , 1989
JRM151	<i>sec63-1 pep4-3 ura3-52 leu2-3, -112 MATα</i>	Rothblatt <i>et al.</i> , 1989
RDM52-7C	<i>sec61-2 sec 62-1 ura3-52 Δpep4::URA3 MATα</i>	Rothblatt <i>et al.</i> , 1989
JRM164	<i>sec62-1 sec63-1 pep4-3 his4 leu2-3, -112 MATα</i>	Rothblatt <i>et al.</i> , 1989
CSY128	<i>leu2-3, -112 ade2 trp1-1 ura3-52 his3-11 sec65-1 MATα</i>	This study
CSY150	<i>leu2-3, -112 trp1-1 ura3-52 sec61-3 MATα</i>	This study
W303-Leu	<i>leu2-3, -112/LEU2 his3-11, -15/his3-11, -15 trp1-1/trp1-1 ura3-1/ura3-1 ade2-1/ade2-1 can1-100/can1-100 MAT a/MATα</i>	
CSY110	As W303-Leu except <i>sec61::HIS3/SEC61</i>	This study
RSY257	<i>ura3-52, leu2-3, -112, MAT a</i>	This study
RSY607	<i>ura3-52, leu2-3, -112, pep4::URA3, MATα</i>	This study
<i>E. coli</i>		
NK5772	<i>dcm6 dam3 galK2 galT22 metB1 leuY1 tsx78 thi1 tonA31 mt11</i>	Kadonaga, unpublished data
MV1184	<i>araΔ(lac-proAB) rpsL thi (ϕ80LacZΔM15) Δ(srl-recA306::Tn10F' [traD36 proAB lacIq lacZΔM15])</i>	Vieira and Messing, 1987

changeable cytosolic Hsp70s, at least one of which is required for efficient translocation of prepro- α -factor, both in vivo and in vitro (Chirico *et al.*, 1988; Deshaies *et al.*, 1988).

Detailed analysis of the spectrum of protein precursors affected by the *sec61*, *sec62*, and *sec63* mutational blocks has revealed a striking dichotomy between soluble and integral membrane protein precursors. Mutants in all three groups accumulate untranslocated precursors of various secretory and soluble vacuolar proteins (e.g., prepro- α -factor, acid phosphatase, carboxypeptidase Y [CPY]); however, none are appreciably defective in the insertion of the integral membrane protein dipeptidyl-aminopeptidase B (DPAPB). This may indicate that membrane protein insertion occurs via a pathway that is independent of *SEC61*, *SEC62*, and *SEC63* or may simply reflect some inherent bias in the original selection that has enriched for a subset of mutant alleles. To address this question, we developed an alternative selection procedure that is designed to select for conditionally lethal mutants defective in the insertion of integral membrane proteins into the ER. Here we describe this new selection procedure and report the isolation of a new, more restrictive allele of *sec61*, together with the identification of a new translocation mutant, *sec65*. In addition, we report the cloning and sequencing of the *SEC61* gene. We have found it to be an essential gene, encoding a 53-kDa polypeptide that is predicted to be a polytopic integral membrane protein. Immunolocalization studies, using anti-Sec61p antibodies, indicate

that the protein is an integral membrane component of the endoplasmic reticulum.

MATERIALS AND METHODS

Strains, Growth Media, and Conditions

Bacterial and yeast strains employed in this study are listed in Table 1. Microbiological culture media were obtained from Difco Laboratories (Detroit, MI). *Escherichia coli* were cultured routinely in Luria-Bertani medium, 2xYT, or M9 minimal medium supplemented with the appropriate nutrients (Maniatis, 1982). Where appropriate, ampicillin was added at 100 μ g/ml and/or kanamycin at 30 μ g/ml. *Saccharomyces cerevisiae* were routinely cultured in either YPD (comprising 1% Bacto yeast extract, 2% Bacto-peptone, and 2% glucose), or in Difco Yeast nitrogen base supplemented with 2% glucose, plus appropriate nutrients. Solid media were supplemented with 2% bacto-agar.

In Vitro DNA Manipulations

Enzymes were purchased from either Boehringer Mannheim (Indianapolis, IN) or Bethesda Research Laboratories (Gaithersburg, MD). Conditions for enzymatic reactions were those recommended by the suppliers, with protocols essentially as described by Maniatis *et al.* (1982). Unidirectional *Exo* III deletions were performed according to the method of Henikoff (1987). Single-stranded DNA templates were prepared after co-infection of MV1190 with the helper phage M13-K07, essentially as described by Vieira and Messing (1987). Single-stranded templates were sequenced using the sequenase modification of the dideoxy chain termination technique of Sanger *et al.* (1977). Sequencing reactions were performed using a Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH), according to the supplier's instructions.

Plasmids and Bacteriophages

The *E. coli* plasmids, pUC118 and pUC119, and the helper bacteriophage M13-K07 have previously been described (Vieira and Messing,

1987), as have the yeast shuttle vectors YIp352, YEp351 (Hill *et al.*, 1986), pRS315 (Sikorski and Hieter, 1989), and YCp50 (Rose and Fink, 1987). p6110 was isolated from a YCp50-based yeast genomic library (Rose and Fink, 1987) by complementation of the *sec61*-2 temperature-sensitive growth phenotype. pCS11 and pCS12 correspond to the 2.4-kbp *HindIII* fragment from p6110 cloned in opposite orientations into pUC119. A *sec61* null allele was constructed by first cloning the 4.3-kbp *Sph I*/*Sst I* fragment from p6110 into pUC119 to generate pCS13. A 1.8-kbp *BamHI* fragment containing the *HIS3* gene (Struhl, 1987) was cut from plasmid pRS4.2 and the ends were filled in with Klenow polymerase plus dNTPs. Then this fragment was cloned into *Xba I*/*Sty I*-restricted pCS13 similarly filled in by Klenow polymerase plus dNTPs to generate pCS14. The 3.2-kb *Sst I*/*Hpa I* fragment from pCS14 was then gel purified and used to transform W303-Leu⁺ to His⁺, thus generating CSY110. Plasmid pCS21 (multicopy *SEC61*) was constructed by cloning the 4.3-kbp *Sph I*/*Sst I* fragment from p6110 into YEp351. The *Sty I** frame shift allele was constructed by cutting pCS12 with *Sty I*, filling in with Klenow polymerase plus dNTPs, and religating to generate pCS12-*Sty I** containing a 4-bp insertion within the *SEC61* coding sequence. The *HindIII* fragment from pCS12-*Sty I** was then cloned into pRS315 to produce pCS43. The authenticity of the cloned *SEC61* gene was tested using the integrating plasmid pCS7, in which the 4.3-kbp *Sph I*/*Sst I* fragment from p6110 was cloned into the integrating vector YIp352.

Construction of pCS4 and pCS5

The precise construction of plasmids pCS4 and pCS5 has been described previously by Sengstag *et al.* (1990), where they were redesignated pA and pAD7, respectively, to conform to the nomenclature of a series of deletion derivatives. The dependence of this work upon pCS4 and pCS5 requires a summary of their construction. Both are derivatives of pCS3, which encodes a gene fusion between the N-terminal 307 codons of the mature form of invertase, and the C-terminal 767 codons of *HIS4* in the multicopy vector YEp352 (Sengstag *et al.*, 1990). Two tripartite *HMG1-SUC2-HIS4* fusions were then constructed by fusing the N-terminus of *HMG1* to *SUC2-HIS4*, at each of two different points within the membrane domain of *HMG1*. In the case of pCS4, the N-terminal 525 codons of *HMG1* were fused upstream of *SUC2-HIS4*, whereas in pCS5, only the N-terminal 463 residues of *HMG1* were included.

Mutagenesis and Mutant Isolation

FC2-12B or FC2a cells containing pCS5 were grown to stationary phase (~10 OD₆₀₀ U/ml) in minimal medium supplemented with histidine, tryptophan, and leucine (30 µg/ml each). The cells were harvested and mutagenized by exposure to the alkylating agent, ethylmethanesulfonate, according to the method of Deshaies and Schekman (1987). After exposure to ethylmethanesulfonate, cells were allowed to recover in YPD overnight at 23°C and then washed in phosphate buffer and plated onto minimal medium supplemented with histidinol (6 mM), tryptophan, and leucine (30 µg/ml each). After 7–14 d incubation at 23°C, histidinol⁺ colonies were patched onto YPD plates, grown up at 23°C, and then screened for temperature-sensitive growth by replica-plating at 37°C. Candidate histidinol⁺, Ts⁻ mutants were cured of pCS5 by successive rounds of unselected growth on YPD. Cured strains were then retransformed with fresh pCS5 and tested for their ability to grow on histidinol, thus eliminating mutants whose histidinol⁺ phenotype was linked to the original plasmid.

Mutant candidates (cured of pCS5) were then screened for the accumulation of precursor forms of the vacuolar membrane protein DPAPB (Roberts *et al.*, 1989) by immunoblotting. Strains were grown to mid-log phase (OD₆₀₀ = 1) in YPD liquid culture at 23°C and then split into two parallel cultures (5 ml), which were incubated at either 23 or 37°C for a further 2 h. Whole cell extracts were prepared by glass-bead lysis, and samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to

nitrocellulose filters essentially as described by Deshaies and Schekman (1987). Filters were probed with a 1:500 dilution of polyclonal anti-DPAPB antiserum (from Chris Roberts and Tom Stevens, University of Oregon), and bound antibodies were detected by decoration with goat anti-rabbit IgG coupled to alkaline phosphatase (Bio-Rad, Richmond, CA).

Radiolabeling and Immunoprecipitation

For the experiments in Figure 1, cells were grown in minimal medium containing 200 µM ammonium sulfate to mid-log phase. Cells were then collected and resuspended in minimal medium lacking sulfate salts 5 min before adding [³⁵S]SO₄²⁻ (carrier-free, ICN Radiochemicals, Irvine, CA). N-linked glycosylation was inhibited by treating cells with 10 µg/ml tunicamycin (10 mg/ml stock in absolute ethanol) (Sigma Chemical Co., St. Louis, MO) for 15 min before and during the period of [³⁵S]SO₄²⁻ labeling. Cells (6 OD₆₀₀ U) were labeled for 30 min at the indicated temperature at a density of 4 OD₆₀₀ cells/ml in minimal medium (–sulfate) containing 1.2 mCi per ml of [³⁵S]SO₄²⁻. Radiolabeling was terminated by chilling the sample to 4°C and by addition of NaN₃ to a final concentration of 10 mM. Radiolabeled cells were resuspended in 0.48 ml of 1% SDS, 50 mM Tris-(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF) and ~0.2 g of 0.5 mm glass beads (Biospec Products, Bartlesville, OK) and agitated twice at top speed on a vortex mixer for 30 s. After lysis, the samples were heated to 80°C for 10 min and diluted to 2.4 ml with 4 vol of immunoprecipitation dilution buffer (1.25% Triton X-100, 190 mM NaCl, 6 mM EDTA, 60 mM Tris-HCl, pH 7.4). Immunoprecipitation samples were precleared of insoluble and nonspecific cross-reacting material by addition of 30 µl of a 10% IgG Sorb (The Enzyme Center, Walden, MA) suspension and unlabeled cell extract (50 µl in 1% SDS, 50 mM Tris-HCl, pH 7.5) prepared from stationary phase cultures of KEX2 deletion mutant cells (BFY113, $\Delta kex2::TRP1$ derivative of W303-1B) or DPAPB deletion mutant cells (JHRY 20-1A, $\Delta dap2::LEU2$), followed by incubation at 22°C for 10 min and centrifugation in a microcentrifuge for 5 min at top speed. Aliquots of the supernatant fraction (equivalent to 2.5 OD₆₀₀ cells) were transferred to fresh microcentrifuge tubes and saturating amounts of antiserum (4 µl Kex2 serum provided by R. Fuller, Stanford University, Stanford, CA; 8 µl DPAPB serum provided by C. Roberts and T. Stevens, University of Oregon, Eugene, OR) was added for overnight incubation at 4°C. Protein A-Sepharose CL4B beads (~1.2 µl packed beads per 1 µl of Ab) (Pharmacia Fine Chemicals, Piscataway, NJ) were added and the mixture incubated for 2 h at 22°C. Beads were sedimented in a microcentrifuge and washed with 1% Triton X-100, 0.2% SDS, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4 (IP buffer); once with IP buffer containing 2 M urea; once with IP buffer containing 500 mM NaCl; and finally with 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4. Antigens were dissociated from the protein A-Sepharose beads by heating in 200 µl SDS-Tris buffer (80°C, 5 min), diluted, and reprecipitated as above. Following addition of SDS-PAGE sample buffer and heating to 80°C for 10 min, samples were applied onto 7.5% SDS polyacrylamide gels. Immunoprecipitated, ³⁵S-labeled Kex2p in vitro translation product, used as a mobility marker in the experiment in Figure 1A, was provided by C. Wilcox and R. Fuller (Stanford University, Stanford, CA).

Other radiolabeling experiments were performed with cells grown in yeast nitrogen base (w/o amino acids or ammonium sulfate) (Difco Laboratories, Detroit, MI), supplemented with 2% glucose, the appropriate amino acids, and 50 µM ammonium sulfate. Cultures were then labeled using Tran³⁵S-label (ICN, Irvine, CA; 30 µCi per OD₆₀₀ U of cells). Immunoprecipitations of α -factor precursor and DPAPB were performed essentially as described by Deshaies and Schekman (1987) using 2 µl of α -factor precursor antiserum or 3 µl of DPAPB antiserum per OD₆₀₀ unit of cell equivalent. However, in the case of the very hydrophobic pCS4 and pCS5 fusion proteins, extracts were prepared by resuspending cells (1–5 OD₆₀₀ U) in 0.3 g glass beads and 300 µl of urea lysis buffer (8 M urea, 1% SDS, 50 mM Tris-HCl,

pH 7.4, 1 mM EDTA, 1 mM PMSF), followed by vigorous agitation on a vortex mixer for 60 s. The extract was then incubated at 37°C for 10 min, centrifuged in a microcentrifuge for 5 min, and the supernatant fraction was diluted to 1 ml to give a final immunoprecipitation buffer comprised of 1.6 M urea, 1% Triton X-100, 0.2% SDS, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4. Anti-invertase antiserum (Schauer *et al.*, 1985) was then added (2 μ l per OD₆₀₀ U), and samples incubated at 23°C for 2 h. Protein-A Sepharose CL4B beads were then added to saturation (5 μ l of a 20% bead suspension per 1 μ l of antiserum), and incubation continued (with constant agitation) for a further 2 h at 23°C. Immunoprecipitates were harvested by brief centrifugation in a microcentrifuge and then washed twice in the same immunoprecipitation buffer, twice in 500 mM NaCl, 1% Triton X-100, 0.2% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, and then twice in 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4. Beads were then resuspended in Laemmli sample buffer (Laemmli, 1970), heated to 60°C for 10 min before loading on an 8% SDS-polyacrylamide gel.

Anti-Peptide Sec61p Antibodies

The peptide CLVPGFSDLM_{COOH} was chemically synthesized (Milligen/Bioscience, San Rafael, CA) and then coupled to keyhole limpet hemocyanin (KLH; Sigma Chemical), using the heterobifunctional crosslinker m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Pierce Chemical Co., Rockford, IL). KLH carrier protein was dissolved in 20 mM sodium phosphate, pH 7.8, 250 mM NaCl at 15 mg/ml and then dialyzed against an excess of the same buffer. The dialyzed KLH solution was diluted to 10 mg/ml and activated with MBS as follows: 500 μ l of MBS (6 mg/ml in dimethyl formamide), was added dropwise to 1 ml of KLH (10 mg/ml in 20 mM NaPi, pH 7.8, 150 mM NaCl), with constant stirring and then incubated at 23°C for 30 min. After incubation, the activated KLH was separated from unreacted MBS by gel filtration (Sephadex G25), with the opalescent KLH-containing fractions pooled, and then was diluted to 5 mg/ml with 50 mM sodium phosphate, pH 7.0, 150 mM NaCl. Peptide (1 mg) was dissolved in dimethyl formamide (25 μ l) and then carefully diluted with 500 μ l 50 mM sodium phosphate, pH 7, 150 mM NaCl. The solubilized peptide was then added to 1 ml activated KLH (5 mg/ml), and the pH was adjusted to 7.0 with 1 N NaOH. Coupling was performed for 3 h at 23°C with constant stirring. KLH-peptide conjugate was separated from free peptide by gel filtration (Sephadex G25) and the pooled fractions stored in aliquots at -70°C. Two rabbits were each injected at multiple, subcutaneous sites with 100 μ g of KLH-peptide conjugate in Freund's complete adjuvant, followed by monthly boosts of 50 μ g KLH-peptide conjugate in Freund's incomplete adjuvant. Bleeds were taken 10 d after each boost and tested for reactivity against Sec61p by immunoblotting. Serum with a titer of 1/2000 dilution on immunoblots of lysates prepared from 1 OD₆₀₀ U of cells was used for the experiments in Figure 8.

Cell Fractionation and Immunoblotting

RSY607 (4000 OD₆₀₀ U of cells) was grown in YPD containing 5% glucose and lysates were processed as described before (Bernstein *et al.*, 1985), except spheroplasts were suspended at 100 OD₆₀₀ U of cells/ml in a buffer that included leupeptin (1 μ M) and pepstatin (1 μ M). Lysis was performed with a Potter-Elvehjem homogenizer. The low-speed supernatant fraction, prepared for the experiment in Figure 8B, was frozen in liquid nitrogen and stored at -70°C for use in the experiment in Figure 8C. The high-speed pellet fraction was washed once with lysis buffer to further remove soluble proteins. Selective solubilization and immunoblotting were performed as described by Hicke and Schekman (1989), except SDS-PAGE was performed using 10% polyacrylamide gels, and protein-antibody complexes were detected using goat anti-rabbit alkaline phosphatase conjugate, followed by a color detection system as described by the supplier (Bio-Rad). Affinity purified Kar2p antibody was obtained from Mark Rose (Princeton University, Princeton, NJ) and used at 1/5 000 dilution.

Phosphoglycerate kinase (PGK) antiserum was obtained from Jeremy Thorne (this department) and was used at 1/10 000 dilution. Affinity purified Sec21p antibody (Hosobuchi and Schekman, unpublished data) was used at 1/1 000 dilution. Affinity purified Sec23p antibody (Hicke and Schekman, 1989) was used at 1/50 dilution. Sec61p antiserum was used at 1/800 dilution.

RESULTS

Selective Defect in Translocation of Membrane Proteins

Previous work has demonstrated a dramatic effect of mutations in *sec61*, *sec62*, and *sec63* on the translocation of soluble secretory proteins (e.g., α -factor precursor, CPY, acid phosphatase) into the ER lumen (Toyn *et al.*, 1988; Rothblatt *et al.*, 1989). A lesser defect was detected with the secretory protein invertase. We extended the examination of translocation substrates to include two very different membrane proteins: Kex2p, an endopeptidase that is transported to a late compartment of the Golgi apparatus (Redding *et al.*, 1991) and DPAPB, a vacuolar membrane protein (Roberts *et al.*, 1989).

Kex2p is a type I membrane protein (Figure 1A) possessing an amino-terminal signal peptide and a hydrophobic membrane anchor domain adjacent to the carboxyl-terminus (Fuller *et al.*, 1989). Kex2p is predicted to be an ~90-kDa polypeptide, but the primary translation product migrates as an ~110-kDa species on SDS-PAGE (Fuller *et al.*, 1989). Subsequent addition of both N- and O-linked carbohydrate causes the glycoprotein precursor to migrate on 7.5% SDS-PAGE as a 135-kDa species (Fuller *et al.*, 1989). Comparison of in vitro-translated Kex2p (p) (Figure 1A, lanes 1 and 14) with Kex2p immunoprecipitated from [³⁵S]O₄²⁻ radiolabeled cells (Figure 1A, lanes 4–13) demonstrated that among the *sec* mutants, only *sec62* showed a defect in the formation of the glycosylated precursor (m) at 37°C (~50% in cells shifted to 37°C for 2 h). We showed previously that *sec* double mutant strains are more defective in translocation than *sec* single mutants (Rothblatt *et al.*, 1989). Formation of glycosylated Kex2p was completely blocked at the permissive (17°C) and restrictive temperatures (37°C) in *sec61sec62* and *sec62sec63* double mutant strains. We conclude that Kex2p requires the Sec61p, Sec62p, and Sec63p gene products for assembly into the ER membrane.

DPAPB is a type II membrane glycoprotein (Figure 1B) containing no standard signal peptide (Roberts *et al.*, 1989). The mature form of DPAPB (m) migrates as a 120-kDa species, whereas the unglycosylated precursor (p) migrates as a 96-kDa species (Roberts *et al.*, 1989). These forms were detected by radiolabeling and immunoprecipitation from wild-type cells incubated at 17 or 37°C in the absence or presence of the N-linked glycosylation inhibitor tunicamycin (Figure 1B, lanes 1–3). *sec* mutant strains radiolabeled for 30 min at 17 or 37°C showed only a marginal defect in formation of the glycosylated precursor: *sec61* and *sec63* accumulated ~10%

A KEX2 Endopeptidase

Type I

B Dipeptidylaminopeptidase B

Type II

Figure 1. Assembly of membrane proteins in *sec* mutants. Wild-type (JRM156) and mutant (RDM15-5B, RDM43-9C, JRM151, RDM52-7C, and JRM164) cells were radiolabeled for 30 min at 17 or at 37°C after a pre-shift to 37°C for 2 h. Aliquots of the same lysate were immunoprecipitated with Kex2p antiserum (A) or DPAPB antiserum (B). p, precursor form; m, glycosylated mature form. (A) Kex2p synthesized in vitro (lanes 1 and 14) was immunoprecipitated from a wheat germ translation reaction programmed with poly A⁺-mRNA from a Kex2p-overproducing strain. Each lane contains antigen immunoprecipitated from 2.5 OD₆₀₀ U of cells. tu, indicates the presence of 10 µg/ml tunicamycin before and during radiolabeling.

or less of the p form of DPAPB (Figure 1B, lanes 4–9). Unlike the result with Kex2p, *sec* double mutant strains revealed only a marginally more pronounced translocation defect with DPAPB (Figure 1B, lanes 10–13). *sec61sec62* was ≤25% defective in the formation of the m form of DPAPB. Hence, either the translocation Sec proteins are not involved in the assembly of certain proteins, such as DPAPB, or the existing *sec* mutations are incompletely restrictive in regard to these translocation substrates.

An Alternative Selection for Translocation Mutants

The existing *sec* translocation mutants were selected by the use of a hybrid protein consisting of the N-terminal signal peptide of α -factor precursor linked to histidinol

dehydrogenase (His4Cp). Successful translocation of the hybrid sequesters histidinol dehydrogenase activity in the ER lumen and precludes the conversion of histidinol to histidine, which occurs only in the cytosol. Mutants that mislocalize the hybrid protein to the cytosol are selected by their ability to grow on histidinol medium. Because the signal peptide from α -factor precursor was used to localize His4p to the ER, it is not surprising that the *sec* mutants isolated by this procedure are notably defective in the translocation of intact α -factor precursor (Deshaies and Schekman, 1987).

To obtain mutants defective in the insertion of integral membrane proteins, a new selection was devised in which His4p was directed to the ER lumen via the insertion of the integral membrane domain of hydroxymethylglutaryl CoA reductase (Hmg1p, Figure 2A)

Figure 2. Structural and functional analysis of the HMG1-SUC2-HIS4 fusion proteins. (A) Diagrammatic representation of the predicted topologies of the pCS4 and pCS5 hybrid proteins. O-, N-linked core oligosaccharide. (B) The pCS5 hybrid protein acquires N-linked carbohydrate. Wild-type (FC2a) cells transformed with either pCS4, pCS5, or the control vector YEpl352, were grown in minimal selective medium, radiolabeled for 20 min at 30°C using Trans ³⁵S label, either in the presence or absence of tunicamycin (10 µg/ml, 20 min pretreatment). Lysates were immunoprecipitated with invertase antiserum which recognizes the linker domain between His4 and Hmg1. Immunoprecipitates were resolved by SDS-PAGE on 6% gels and exposed by fluorography. Each lane contains antigen immunoprecipitated from 2 OD₆₀₀ U of cells. (C) Histidinol growth phenotypes conferred by various plasmids in wild-type and *sec* mutant strains. pGD2 encodes a Suc2- His4 hybrid that contains the signal peptide coding region of the α -factor precursor gene and was used in previous selections for *sec* translocation mutants (Deshaies and Schekman, 1987; Rothblatt *et al.*, 1989).

(Sengstag *et al.*, 1990). His4Cp was fused to a site within the predicted luminal domain of Hmg1 located between transmembrane domains six and seven (Figure 2A, pCS5 fusion). Wild-type cells expressed a hybrid protein from the multicopy plasmid pCS5 that was extensively glycosylated, as expected from the luminal orientation of

the His4Cp segment (Figure 2B, pCS5, + or - tuni). Again, as expected, pCS5- transformed cells were unable to grow on histidinol medium. In contrast, cells transformed with a control plasmid, pCS4 (Figure 2A), in which His4Cp was fused to a cytosolic domain of Hmg1, expressed a hybrid protein that was not ex-

tensively glycosylated (Figure 2B, pCS4, + or – tunic), and grew on histidinol medium. Significantly, pCS5 failed to confer the histidinol growth phenotype when introduced into isolates of the original *sec* translocation mutant strains even though pGD2, the plasmid used to select the original *sec* isolates, worked properly (Figure 2C). Thus, the Hmg1-His4C hybrid protein represents a class of translocation substrates, such as DPAPB, that are not as severely affected by the original *sec* isolates.

The failure of pCS5 transformants to grow on histidinol medium presented an opportunity to select new translocation mutants deficient in membrane protein assembly. Haploid cells carrying pCS5 were mutagenized to 30% killing with ethylmethanesulfonate and permitted to renew growth on rich medium before selection on histidinol-defined medium as before (DeShaies and Schekman, 1987). As a defect in the translocation of membrane proteins was expected to be lethal, conditional mutants were selected at an arbitrary semi-permissive temperature (23°C) and then screened for failure to grow at 37°C. The intention was to identify alleles that are sufficiently defective at 23°C to allow cytosolic retention of some His4Cp but not so defective as to preclude membrane assembly. Mutants solely defective in the translocation of the pCS5 hybrid protein were not expected to be conditionally lethal when grown in the presence of histidine. Nevertheless, to ensure the identification of pleiotropically defective mutations, we evaluated extracts of Ts^- lethal strains for accumulation of a precursor form of DPAPB by SDS-PAGE and immunoblotting.

His⁺ mutants arose at a frequency of 1.9×10^{-6} , among which 9% were Ts^- . Among 118 His⁺, Ts^- mutants analyzed, two (CSYa42 and CSYa59) accumulated DPAPB precursor (see Figure 3). These mutants were crossed to existing translocation mutants (*sec61*, *sec62*, *sec63*, and *kar2*), and the resultant diploids scored for complementation of the Ts^- growth phenotype. CSYa42 was identified as an allele of *sec61* (*sec61-3*), whereas CSYa59 contains a mutation in a newly identified gene, *SEC65*. *sec61-3* was both Ts^- and cold sensitive (Cs^- , no colonies formed at 17°C), but grew normally in the range of 23–30°C. The isolation of a new, presumably broader spectrum conditional allele of *sec61* suggests that Sec61p is required for assembly of membrane and secretory proteins.

Translocation defects were evaluated by radiolabeling backcrossed and plasmid-free mutant cells at permissive and nonpermissive temperatures, followed by immunoprecipitation of DPAPB and α -factor precursor using specific antibodies. Mutant *sec61-3* cells were grown at 30°C, and an aliquot was transferred to 17°C for 2 h, followed by labeling of both cultures with ³⁵S-met for 20 min at the same temperature. Figure 3 shows mature (~120 kDa) forms of DPAPB immunoprecipitated from wild-type, *sec61-2*, and *sec61-3* cells labeled at 30°C (lanes 2, 4, and 7). Approximately 25–40% of the

Figure 3. Mutants accumulate precursor form of DPAPB. Wild-type cells (FC2 α) were labeled with Trans ³⁵S label for 20 min at 30°C in the presence or absence of tunicamycin (10 μ g/ml; 20 min pretreatment [lanes 2, 3]). Strain RDM15-5B (*sec61-2*) was labeled for 20 min at 30°C (lane 4). Strain CSY128 (*sec65-1*) was labeled for 20 min at 24°C or after a 1 h transfer to 37°C (lanes 5 and 6). Strain CSY150 (*sec61-3*) was labeled at 30°C or after a 2 h transfer to 17°C (lanes 7 and 8). CSY150 cells transformed with p6110 (see Figure 5) were labeled for 20 min at 30 or 17°C (lanes 9 and 10). Lanes contain DPAPB immunoprecipitated from 1 OD₆₀₀ U of cells and resolved on 8% polyacrylamide SDS-PAGE followed by fluorography. Molecular weight standards: myosin heavy chain, 212 kDa; phosphorylase b, 97.4 kDa; bovine serum albumin, 69 kDa (lane 1).

DPAPB labeled in *sec61-3* at 17°C migrated at the position of the unglycosylated precursor (lanes 3 and 8). A somewhat less restrictive block in DPAPB assembly (~10–20%) was seen in *sec61-3* labeled at 37°C. However, no accumulation of unglycosylated DPAPB was seen in *sec61-2* cells shifted to 17 or 37°C for 1 h before labeling. Mutant *sec65-1* cells produced mature DPAPB at 24°C but were nearly completely defective in DPAPB assembly at 37°C (lanes 5 and 6).

Although the mutant selection was based on ER localization mediated by a membrane anchor domain, it was relevant to assess the translocation of soluble secretory proteins in the new isolates. Figure 4 shows the results of an experiment in which forms of α -factor were radiolabeled for 7 min at various temperatures and immunoprecipitated from wild-type (lanes 7–9), *sec61-3* (lanes 3–6), or *sec65-1* (lanes 10 and 11) cells. This period of labeling is short with respect to the transit time of α -factor precursor in wild-type cells, particularly at 17 and 24°C, where significant levels of core glycosylated pro- α -factor were observed (gpaF, lanes 7 and 8). The *sec61-3* mutant accumulated unglycosylated prepro- α -factor at 17, 24, and 37°C, with a maximal accumulation (>90%) observed at 17°C (lane 4). As for DPAPB, *sec65-1* was permissive for α -factor at 24°C (lane 10) and nearly completely blocked at 37°C (lane 11). Under similar labeling conditions the maximal defect observed in *sec61-2* cells closely resembled that of *sec65-1* at 24°C. The translocation of preinvertase was also severely blocked in both *sec61-3* (>50%), and *sec65-1* (>75%)

Figure 4. Mutants accumulate α -factor precursor. Various strains were pulse-labeled (7 min) with Trans ^{35}S -label at either 24°C or after a 1-h shift to 37°C or a 2-h shift to 17°C. Wild-type (FC2 α) cells were labeled at 17°C (lane 7), 24°C (lane 8), 37°C (lane 9), or finally at 24°C in the presence of tunicamycin (lane 2). Mutant *sec61-3* cells (CSY150) were labeled at either 17°C (lanes 3 and 4), 24°C (lane 5), or 37°C (lane 6). The *sec65-1* mutant cells (CSY128) were labeled at 24°C (lane 10) or 37°C (lane 11). The various forms of α -factor were immunoprecipitated from 1 OD₆₀₀ U of cells and resolved by SDS-PAGE on a 12.5% polyacrylamide gel. The primary translation product of α -factor mRNA is included as a marker for unglycosylated prepro- α -factor (lanes 1 and 12; Deshaies and Schekman, 1987). The observed reduction in the level of synthesis of prepro- α -factor at 37°C has previously been noted (Deshaies and Schekman, 1987). Note that lanes 3 and 4 in the above figure correspond to different autoradiographic exposures of the same physical sample. p α f, prepro- α -factor; p α f, pro- α -factor; gp α f, glycosylated pro- α -factor; α f, proteolytically processed forms of α -factor.

cells compared with the relatively weak effect observed in previous *sec* isolates.

The experiment in Figure 4 also shows that new *sec* mutant isolates accumulated a species identified as prepro- α -factor based on co-migration with the primary translation product of α -factor mRNA (lanes 1 and 12; Deshaies and Schekman, 1987). This behavior is consistent with a translocation rather than a glycosylation defect. The signal peptide-processed, unglycosylated form that accumulated in cells treated with tunicamycin exhibited a mobility anomaly and migrated more slowly than the intact precursor in this gel system (lane 2). Protease protection experiments, such as those which confirm a translocation defect in *sec61* (Deshaies and Schekman, 1987), were performed with *sec65-1* with similar results.

Translocation deficiency was fully recessive in *sec61-3* and *sec65-1*. A single-copy plasmid containing *SEC61*

(see Figure 5) complemented the DPAPB assembly defect in *sec61-3* (Figure 3, lanes 9 and 10) and a *sec65/Sec*⁺ heterozygous diploid displayed no defect in the translocation of DPAPB and α -factor precursor.

SEC61 Gene

The *SEC61* gene was cloned from a YCp50 genomic library (Rose *et al.*, 1988) by complementation of the temperature-sensitive growth phenotype of RDM 15-5B (*sec61-2, ura3-52*). Plasmid DNA was recovered from Ura⁺, Ts⁺ transformants, amplified in *E. coli* and then retested for the ability to complement *sec61-2*. Plasmid p6110 was isolated by this procedure and then tested for complementation of the translocation defect in *sec61-2* and *sec61-3* strains. The experiment in Figure 3 (lanes 9 and 10) shows that p6110 restored normal translocation of DPAPB at 17°C in strain CSY150 (*sec61-3*).

Figure 5. Subclones of *SEC61*. A restriction map of the 12-kbp insert found in p6110 is shown. Subclones in single-copy (CEN) vectors were tested for complementation of the *sec61-3* Ts⁺ phenotype. The extent and orientation of the *SEC61* open reading frame is indicated by a shaded arrow. A, *Acc* I; B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sst* I; Sy, *Sty* I; X, *Xba* I.

Restriction digest analysis of p6110 indicated a 12-kbp insert whose map is shown in Figure 5. Subclones were obtained and evaluated for complementation of the Ts⁻ growth defect of *sec61-3* (Figure 5). A 2.4-kbp *HindIII* fragment contained full complementing activity.

The complementing fragment cloned in p6110 may represent the authentic *SEC61* gene or an extragenic suppressor. To distinguish these possibilities, we used the cloned fragment to direct the integration of a selectable marker *URA3* into the yeast genome, enabling the genetic linkage between the site of integration and the *sec61-2* locus to be determined. An integrating plasmid pCS7 was constructed by cloning the 4.3-kbp *SphI*-*SstI* fragment from p6110 into the yeast vector YIp352 (*URA3*; Hill *et al.*, 1986). pCS7 was linearized at a unique *SmaI* site and used to transform RSY257 (Sec⁺, *ura3-52*). Three stable Ura⁺ transformants were crossed to RDM15-5B (*sec61-2,ura3-52*) and the resultant diploids were sporulated to score segregation of the Ura and Ts markers among haploid progeny. Fifteen asci were dissected from each diploid, and in each case the Ts⁻ and Ura⁺ phenotypes segregated 2:2. No Ts⁻, Ura⁺ spores were recovered. We conclude that the *URA3* marker integrated at the *SEC61* locus and that p6110 contains the *SEC61* gene.

SEC61 Encodes a Membrane Protein That Is Essential for Cell Growth

The nature of the *SEC61* gene product was determined by nucleotide sequence analysis of the minimum complementing fragment. Computer-aided analysis of the DNA sequence identified a single, long open reading frame that encodes a 480-amino acid residue polypeptide with a predicted molecular mass of 52 943 Da (Figure 6). The *SEC61* reading frame extends to coordinate 1886 bp, 51 bp beyond a frameshift site created by cleavage with *StyI* as described in the METHODS section. The *StyI** frameshift allele partially complemented *sec61-2* (Figure 5). It appears that the C-terminal 16-amino acid residues of Sec61p that are removed by the *StyI** frameshift and the 6 new amino acids introduced by this mutation are compatible with at least partial Sec61p activity.

Potential transcription initiation elements (TATA boxes) are located at coordinates 9 and 396 (Figure 6), corresponding to positions -437 and -50 bp, relative to the initiation codon (coordinate 446 bp). TATA boxes are necessary for transcriptional initiation in yeast and usually are located 40–120 bp upstream of the initiation site (Struhl, 1987). A putative transcription termination element similar to that of the *CYC1* gene (Zaret and Sherman, 1982) is present between co-ordinates 1932 and 2060 (Figure 5), corresponding to 47–175 bp downstream of the *SEC61* reading frame. Northern hybridization analysis, using a strand-specific probe equivalent to the *SEC61* sense strand, identified a single

species of RNA of ~1.7 kb. The size of this RNA is sufficient to contain the entire *SEC61* reading frame and is consistent with the transcription elements identified in 5' and 3' flanking sequences. The *SEC61* sequence has no consensus splicing signals, consistent with the prediction of an uninterrupted coding region.

The *SEC61* sequence predicts an extremely hydrophobic polypeptide. A mean hydropathy profile, calculated over a window of 19 residues, predicts 8 hydrophobic segments of sufficient length to span a lipid bilayer (Figures 6 and 7, domains I–VIII). According to the criteria of Kyte and Doolittle (1982), four of these segments (I, III, IV, and V) exhibit the extreme mean hydropathy values typical of transmembrane domains. The remaining four potential membrane segments (II, VI, VII, and VIII) fall only marginally short of the threshold values required to make confident predictions. Hartmann *et al.* (1989) noted that the net charge difference between residues flanking the first signal/anchor domain of transmembrane proteins correlates strongly with the orientation of that domain within the membrane. Accordingly, the first transmembrane domain of Sec61p is predicted to be oriented such that the N-terminus faces the cytosol.

The isolation of conditionally lethal *sec61* mutations is consistent with the notion that *SEC61* encodes an essential function. However, several cases of temperature-sensitive growth resulting from null alleles are documented (Craig and Jacobsen, 1984; Novick *et al.*, 1989). We constructed a *sec61* null mutation and tested its viability under various conditions. The null allele was constructed in vitro by replacing the 1370-bp *XbaI*-*StyI* fragment, representing 95% of the *SEC61* coding sequence, with a fragment containing the entire *HIS3* gene (Struhl, 1987). The *sec61::HIS3* allele was transformed into diploid strain W303-leu, and His⁺ transformants were selected. Two stable His⁺ transformants (CSY110 and CSY111) were found by Southern hybridization to possess one wild-type and one disrupted copy of *SEC61* each. Twenty tetrads of each diploid strain were dissected after sporulation at 22°C. No more than two viable spores/tetrad were obtained; 36 out of 40 tetrads segregated 2:2 for viability, with the rest segregating 1:3. Among 76 viable spores, none were His⁺. Similar results were obtained with tetrads allowed to germinate at 17 or 30°C.

In viable *sec61* null mutant spores germinated but did not progress beyond the emergence of a small bud. No further growth was observed, even after 8 d on germination plates. To confirm an absolute requirement for *SEC61* during vegetative growth, CSY110 transformed with p6110 was sporulated and tetrads evaluated for plasmid segregation. Six out of 19 viable spores were His⁺, and all His⁺ spores were also Ura⁺, hence p6110 complemented the *sec61::HIS3* null mutation. No His⁺ transformants could be cured of p6110 by selection on 5-fluoroorotic acid (5-FoA)-containing me-

dium (Boeke *et al.*, 1987). In contrast, His⁻Ura⁺ progeny, which contain a normal *SEC61* locus, were readily cured of p6110 by the 5-FoA selection.

A decapeptide comprising the C-terminal nine amino acids of Sec61p and an N-terminal cysteine was synthesized for use in raising a polyclonal antiserum. The peptide was coupled via its free sulfhydryl to KLH using the heterobifunctional crosslinker MBS. After an initial injection and several boosts, a polyclonal serum was obtained that recognized a single, 38-kDa polypeptide on an immunoblot of SDS-PAGE-resolved yeast proteins (Figure 8A, lanes 3 and 4). Preimmune serum failed to detect this protein (Figure 8A, lanes 1 and 2), while the level was elevated in cells transformed with the *SEC61* gene on a multicopy plasmid (pCS21) (Figure 8A, lane 3). The mobility of Sec61p in SDS-PAGE is anomalous and may be due to the unusual hydrophobic

Cell fractionation experiments were performed to test the membrane localization of Sec61p. A series of fractions generated by differential centrifugation were evaluated by immunoblotting using antibodies against a cytosolic protein (phosphoglycerate kinase [PGK]), two cytosolically oriented peripheral membrane proteins (Sec23p, Hicke and Schekman, 1989; Sec21p, Hosobuchi and Schekman, unpublished data), an ER luminal protein (Kar2p, Rose *et al.*, 1989), and Sec61p. Figure 8B demonstrates that Sec61p fractionated exclusively in a pellet fraction (washed high-speed pellet [HPW]), which also contained the luminal ER marker. A crude lysate fraction was also treated with agents that strip peripheral proteins from membranes (urea, Na_2CO_3 ,

Figure 7. Mean hydropathy profile of Sec61p. The mean hydropathy index was calculated across a window of 19 residues according to the method of Kyte and Doolittle (1982). A dashed line is used to indicate the threshold value of +1.6, which represents the mean hydropathic value typically exceeded by transmembrane segments. Potential transmembrane domains are indicated by shaded boxes (I–VII).

and KOAc) or solubilize integral proteins (Triton X-100). Treated fractions (total [T]) were centrifuged and the particulate (P) and soluble (S) material evaluated by immunoblot (Figure 8C). Among the treatments, only Triton X-100 solubilized Sec61p.

Immunofluorescence microscopy using Sec61p antibody revealed a concentration of antigen in the nuclear rim and in tubules radiating from the nucleus (not shown). A similar pattern of immunofluorescent staining was seen with Sec62p and Sec63p antibodies (Deshaies and Schekman, 1990; Feldheim, unpublished data). This pattern is characteristic of the ER in yeast and confirms the expected membrane localization of Sec61p.

DISCUSSION

Translocation defective *sec* mutant cells isolated on the basis of mislocalization of a secretory protein chimera display selective defects in the translocation of various polypeptides into the ER. Some soluble proteins such as α -factor precursor, CPY precursor, acid phosphatase (Rothblatt *et al.*, 1989), and at least one membrane protein, Kex2p (Figure 1A) are blocked in single- or double-mutant combinations of the original isolates of *sec61*, *sec62*, and *sec63*. Other soluble proteins, such as invertase (Rothblatt *et al.*, 1989), or membrane proteins, such as DPAPB (Figure 1B), are much less severely affected by these mutations. It is not entirely evident how these molecules are distinguished; however, we have sug-

gested elsewhere that an elevated hydrophobicity of the signal peptide may diminish the dependence of translocation on the Sec61, Sec62, and Sec63 gene products (Rothblatt *et al.*, 1989). The two new examples presented in this report continue this pattern: Kex2p has a signal peptide that is on the same order of hydrophobicity as those of α -factor precursor and CPY precursor (Fuller *et al.*, 1989) and much less hydrophobic than the invertase signal peptide; DPAPB is a type II membrane protein with a near N-terminal membrane anchor domain that would constitute an extremely hydrophobic signal peptide.

The pattern of selective translocation deficiency is reproduced with a chimeric protein that contains an N-terminal membrane anchor domain with six of the seven potential membrane-spanning segments in the ER membrane protein *Hmg1*. Histidinol dehydrogenase fused to the last putative ER lumenal loop of *Hmg1* becomes localized to the ER lumen and fails to produce histidine from histidinol in the cytosol (Sengstag *et al.*, 1990). As predicted from the hydrophobic nature of the membrane anchor segment of *Hmg1*, assembly of the *Hmg1*-His4C hybrid protein is not blocked by the original isolates of *sec61*, *sec62*, and *sec63*.

New mutants, isolated by selection for histidinol prototrophy using the *HMG1-HIS4C* fusion, are defective in the translocation of DPAPB, invertase, and α -factor precursor. One of the new isolates, a mutant that is both Ts⁻ and Cs⁻, simply represents a more restrictive

Figure 8. Detection and fractionation of Sec61p. (A) Whole cell extracts prepared from wild-type cells (FC2 α) transformed either with the control vector YEp351 (lanes 2 and 4) or a multicopy *SEC61* plasmid (pCS21; lanes 1 and 3) were resolved by SDS-PAGE on an 8% polyacrylamide gel and immunoblotted with pre-immune (lanes 1 and 2) or Sec61p antiserum (lanes 3 and 4). Serum was used at 1/2000 dilution. Each lane corresponds to the contents of 1 OD₆₀₀ U of cells. (B) Fractions of strain RSY607 were prepared as before (Bernstein *et al.*, 1985), resolved by SDS-PAGE on 10% polyacrylamide gels, and immunoblotted with antisera at dilutions specified in the Methods section. CL, cell lysate; LP, low-speed pellet; LS, low-speed supernatant; HP, high-speed pellet; HPW, washed high-speed pellet; HS, high-speed supernatant. Each lane contains the contents of 0.5 OD₆₀₀ U cells. (C) The LS fraction from above was treated as before (Hicke and Schekman, 1989), resolved by SDS-PAGE on 10% polyacrylamide gels, and immunoblotted with sera as above. T, total sample; P, pellet; S, supernatant. Each lane contains the contents of 1 OD₆₀₀ U of cells.

allele of *sec61*. The other mutant *sec65* was not obtained in our previous selections (Deshaies and Schekman, 1987; Rothblatt *et al.*, 1989), but nevertheless it is quite defective in the translocation of DPAPB, invertase, and α -factor precursor. We conclude that the various translocation substrates use common components for membrane assembly or penetration but exhibit different thresholds of requirement for the *SEC* gene products. The failure to obtain more restrictive alleles of *sec62* and *sec63* in the new selection procedure should not be taken as evidence that they are required for the translocation of only a subset of secretory proteins. This selection procedure has not yet been saturated and may prove able to identify mutations in additional genes involved in membrane assembly.

Although the new selection procedure may simply have demanded more restrictive alleles to allow cell growth on histidinol medium, the question remains why proteins such as DPAPB and α -factor precursor display such dramatically different requirements for the translocation *Sec* proteins. Perhaps molecules such as Sec61p possess multiple domains each designed to interact with a different substrate-selective component of the translocation apparatus. Mutations that affect one such domain would produce a selective translocation defect, whereas other mutations could render Sec61p pleiotropically deficient.

Sec61p is the most hydrophobic of the three *Sec* translocation proteins characterized in yeast (Deshaies and Schekman, 1989; Sadler *et al.*, 1989); thus it could

represent the core subunit of a translocation channel or translocase in the ER membrane. In several ways the Sec61p resembles the *E. coli* SecY (PrfA) protein that is part of the bacterial secretory protein translocase (Cerritti *et al.*, 1983; Akiyama and Ito, 1987). Both proteins are of similar size and hydrophobicity, display anomalous SDS-PAGE mobility, and even share a limited level of sequence identity. In the segment from amino acid position 70–200, the two proteins are ~20% identical and 49% similar. However, this region includes several membrane-spanning domains that may constrain the potential variability. An alignment made after shuffling the sequence within the region of homology shows no significant change in the level of similarity.

More striking similarity is seen in a comparison of Sec61p with a SecY homologue obtained by hybridization from the archaeobacterium, *Methanococcus vanneilli*. An alignment of these two sequences shows 34.6% identity spread throughout the molecules (Driessen, personal communication). This rather more significant identity suggests that both eubacterial SecY and eukaryotic Sec61p derive from a common archaeobacterial progenitor.

Like *E. coli* SecY, Sec61p shows genetic and physical interaction with other membrane proteins required for protein translocation. Chemical cross-linking identified a complex that includes Sec61p, Sec62p, and Sec63p, as well as two genetically unidentified subunits (gp31.5 and p23) (Deshaies *et al.*, 1991). Association of Sec61p with the other members of this complex is tenuous and apparently substoichiometric. Immunoprecipitation from detergent solubilized membrane using Sec62p antibody failed to precipitate Sec61p, and even after chemical cross-linking only a fraction of the Sec61p is recovered in the immunoprecipitate. Sec61p could be in some dynamic state of association with other members of the complex such that only a fraction is at any instant in the bound form. Sec62p and Sec63p are oriented such that large N- and C-terminal domains of the proteins project into the cytosol (Deshaies and Schekman, 1990; Feldheim, unpublished data). Secretory protein precursors may engage these molecules in the cytosol either directly or through the intervention of Hsp70 or signal recognition particle and its receptor (Amaya and Nakano, 1991; Deshaies *et al.*, 1991; Hahn and Walter, 1991). Upon binding to the Sec62p/63p complex, secretory precursor polypeptides may engage Sec61p to begin the membrane penetration event. A cycle of assembly and disassembly of Sec62p/63p from Sec61p may govern the activity of the translocase.

Sec65p must be involved intimately in the translocation of a wide variety of protein substrates as the *sec65* mutation displays the most conditional and restrictive quality of the existing *sec* translocation mutations. Recent molecular cloning evidence demonstrates that SEC65 encodes a 30-kDa homologue of the 19-kDa

subunit of mammalian SRP (Stirling, unpublished data). This evidence strongly supports a role for yeast SRP in the translocation of molecules, such as α -factor precursor, that are capable of post-translational membrane insertion.

ACKNOWLEDGMENTS

We gratefully acknowledge the material assistance of C. Wilcox and R. Fuller (Kex2p antibodies, $\Delta kex2$ strains, and in vitro translated Kex2p), C. Roberts and T. Stevens (DPAPB antibody and *dap2* strain), J. Thorner (PGK antibody), M. Rose (Kar2p antibody), T. Yoshihisa (Sec23p antibody), and C. Sengstag (*HMG1* gene). We thank D. King for confirming the sequence of the Sec61p C-terminal peptide, D. Feldheim and S. Sanders for informative discussions, and P. Smith for preparing this manuscript. C. Stirling was supported by an SERC/NATO Fellowship and J. Rothblatt by an American Cancer Society senior postdoctoral fellowship. The work was supported by grants from the NIH (GM-26755) and the Howard Hughes Medical Research Institute.

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