Multiple Genes Are Required for Proper Insertion of Secretory Proteins into the Endoplasmic Reticulum in Yeast

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Abstract. Genes that function in translocation of secretory protein precursors into the ER have been identified by a genetic selection for mutant yeast cells that fail to translocate a signal peptide–cytosolic enzyme hybrid protein. The new mutants, sec62 and sec63, are thermosensitive for growth and accumulate a variety of soluble secretory and vacuolar precursors whose electrophoretic mobilities coincide with those of the corresponding in vitro translated polypeptides. Proteolytic sensitivity of precursor molecules in extracts of mutant cells confirms that polypeptide translocation is blocked. Some form of interaction among the SEC61 (Deshaies, R. J., and R. Schekman. 1987. J. Cell Biol. 105:633–645), SEC62 and SEC63 gene products is suggested by the observation that haploid cells containing any pair of the mutations are inviable at 24°C and show a marked enhancement of the translocation defect. The translocation defects of two mutants (sec62 and sec63) have been reproduced in vitro. sec63 microsomes display low and thermolabile translocation activity for prepro-α-factor (ppcF) synthesized with a cytosol fraction from wild type yeast. These gene products may constitute part of the polypeptide recognition or translocation apparatus of the ER membrane. Pulse-chase analysis of the translocation-defective mutants demonstrates that insertion of ppcF into the ER can proceed posttranslationally.

In spite of significant advances that have clarified the structure and function of molecules that mediate targeting of secretory proteins to the endoplasmic reticulum (for review, see Walter and Lingappa, 1986; Hortsch and Meyer, 1986), the actual process of polypeptide transfer across the ER membrane is poorly understood. A common view is that a proteinaceous translocation pore complex within the ER membrane facilitates transfer of the hydrophilic nascent polypeptide across the hydrophobic core of the ER membrane (Blobel and Dobberstein, 1975; Blobel, 1980). The participation of membrane proteins in translocation, as well as in targeting, is indicated by the fact that translocation activity of microsomes is sensitive to proteolysis and chemical alkylation (Walter et al., 1979; Meyer and Dobberstein, 1980a, 1980b; Gilmore et al., 1982; Hortsch et al., 1986). Translocating proteins appear to lie within a polar environment in the bilayer because intermediates interrupted in penetration may be solubilized by agents that disrupt protein structure without solubilizing membrane lipids (Gilmore and Blobel, 1985). A lack of specific probes or inhibitors has frustrated biochemical approaches to identifying functional translocator components. Recently, an ER membrane protein, termed signal sequence receptor (SSR),1 that interacts directly with the signal peptide of nascent proteins has been identified by chemical cross-linking (Wiedmann et al., 1987). The functional role of SSR, however, remains to be determined.

Molecular dissection of the mechanism of protein sorting and intercompartmental transport within the yeast secretory pathway has been facilitated by the identification and characterization of a large number of conditionally lethal, temperature-sensitive (Ts-) secretion (sec) mutants (Novick et al., 1980; Ferro-Novick et al., 1984a). Biochemical analysis of the sec mutants showed that secretion and growth are blocked at the restrictive temperature, leading to the accumulation of soluble secretory and vacuolar precursors, as well as integral membrane proteins, within the secretory pathway (for review, see Schekman and Novick, 1982). Clearly, identification and cloning of genes whose products are required for protein translocation would expedite the functional characterization of ER membrane proteins essential for polypeptide translocation. Among the 25 complementation groups originally defined, none were defective in protein transfer across the ER membrane.

1. Abbreviations used in this paper: AcPase, acid phosphatase; CPY, carboxypeptidase Y; EMS, ethyl methanesulfonate; gpoE, glycosylated pro-α-factor; Hol−, histidinol prototrophy; MSB, membrane storage buffer; ppcF, prepro-α-factor; SSR, signal sequence receptor; TAME, Nct-p-tosyl-L-arginine methyl ester; TPI, triose phosphate isomerase; Ts−, temperature-sensitive; YPD, 1% yeast extract, 2% peptone, 2% dextrose.
To extend our genetic approach to analysis of protein translocation, we devised a genetic selection that permitted isolation of conditionally lethal yeast mutants defective in secretory protein translocation into the ER (Deshaies and Schekman, 1987). The selection strategy demanded cytoplasmic localization of an enzyme (histidinol dehydrogenase) whose normal cytoplasmic location was modified by appending a secretory signal peptide to its amino terminus. By selection for growth on minimal medium containing histidinol and screening mutants for pleiotropic defects in secretion, it was possible to distinguish between two classes of mutants that arose as histidinol prototrophs: signal sequence mutations and mutations in the ER translocation machinery. The sec61 mutant has a defect in the translocation machinery and was described previously. In this report, we describe the isolation and characterization of two novel translocation mutants, sec62 and sec63, and present in vitro data that implicates the SEC63 gene product as a membrane-associated component of the ER translocation apparatus. In addition, the properties of haploid double mutants suggest that the products of the SEC61, SEC62, and SEC63 genes act along the same pathway. In an accompanying paper (Deshaies and Schekman, 1989), in vitro biochemical and molecular genetic analyses provide evidence that the Sec62 protein is a component of the ER membrane. A preliminary account of these studies was reported elsewhere (Deshaies et al., 1988b).

**Materials and Methods**

**Strains and Growth Conditions**

Bacterial and yeast strains used in this study are listed in Table I. Yeast strains were constructed by standard genetic techniques (Sherman et al., 1983). A MATa derivative of FC2-12Ba was obtained by mating-type conversion with the plasmid pHO (Jensen et al., 1983). Original mutant isolates were backcrossed at least three consecutive times to RDB103 to test for cosegregation of the Ts growth and a factor precursor accumulation phenotype. Analysis of procarboxypeptidase Y (CPY) was aided by the use of pep4 mutant strains that are deficient in maturation of vacuole hydrolase precursors (Hemmings et al., 1981). The chromosomal PEM locus in the sec62 mutant was deleted by substitution with the Xho I–Eco RI fragment of pTS15, which carries a URA3 disruption of the PEM gene (Rothman et al., 1986). The pep4-3 mutation was introduced into the sec63 mutant by mating with RSY52 and screening the tetrads for CPY activity (Jones, 1977).

Stationary phase yeast cultures were grown at 17°C or 24°C in rich 1% YPD medium containing bacto-yeast extract, 2% bacto-peptone (Difco Laboratories Inc., Detroit, MI) and 5% glucose. YPD-agar and Wickerham's minimal medium agar plates contained 2% glucose and 2% bacto-agar. In preparation for radiolabeling of cells with [35S]SO42-, stationary phase cells were inoculated into low sulfate minimal medium prepared with chloride rather than sulfate salts and supplemented with 200 μM ammonium sulfate) for overnight growth at 17°C or 24°C. Extracellular acid phosphatase (AcPase) synthesis and secretion was derepressed by growing cells overnight in low sulfate minimal medium prepared with phosphate-free salts and supplemented with 100 μM KH2PO4. Liquid cultures were grown in flasks with vigorous agitation, and experiments were initiated with cells in logarithmic growth phase. The optical density at 600 nm (OD600) of dilute cell suspensions was measured in 1-cm quartz cuvettes using a spectrophotometer (PMQII; Carl Zeiss, Inc., Thornwood, NY); 1 OD600 of cells corresponds to 1 × 10^7 cells.

**Reagents**

DNA restriction endonucleases and modification enzymes were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN) and from Bethesda Research Laboratories (Gaithersburg, MD). Histidinol, tunicamycin, proteinase K, amino acid powders, PMSF, cycloheximide, BSA, protein A, and [35S]methionine (>1,000 Ci/mmol), [125I]NaI (highest specific activity), and Amplify were from Amerham Corp. (Arlington Heights, IL). Sulpholysoccal nucleases S7, SP6 RNA polymerase, m7GpppG, creatine phosphate, and creatine phosphokinase were obtained from Boehringer-Ingelheim. Histidine, L-amino acids, tryptophan, uracil, thymine, and adenine were from Sigma Chemical Co. (St. Louis, MO). Histidine, L-amino acids, and arginine were from Sigma Chemical Co. (St. Louis, MO). Histidine, L-amino acids, and arginine were from Sigma Chemical Co. (St. Louis, MO). Histidine, L-amino acids, and arginine were from Sigma Chemical Co. (St. Louis, MO). Histidine, L-amino acids, and arginine were from Sigma Chemical Co. (St. Louis, MO). Histidine, L-amino acids, and arginine were from Sigma Chemical Co. (St. Louis, MO). Histidine, L-amino acids, and arginine were from Sigma Chemical Co. (St. Louis, MO). Histidine, L-amino acids, and arginine were from Sigma Chemical Co. (St. Louis, MO). Histidine, L-amino acids, and arginine were from Sigma Chemical Co. (St. Louis, MO). Histidine, L-amino acids, and arginine were from Sigma Chemical Co. (St. Louis, MO).

**Table I. Bacterial and Yeast Strains**

| Strain  | Genotype | Source |
|---------|----------|--------|
| S. cerevisiae | ura3-32 leu2-3,-112 rpl1-1 his4-401 HOLI-1 MATa | Parker and Guthrie, 1985 |
| FC2-12B | sec61-2 pep4-3 ade2 ura3-52 leu2-3,-112 MATa | This study |
| RDM15-5B | sec61-2 ade2 his leu2-3,-112 MATa | This study |
| RDM33-4A | sec61-2 his4 ura3-52 Δpep4::URA3 MATa | This study |
| RDM43-9C | sec61-2 sec62-1 ura3-52 Δpep4::URA3 MATa | This study |
| RDM52-7C | sec63-1 pep4-3 ura3-52 leu2-3,-112 MATa | This study |
| JRM151 | pep4-3 ura3-52 leu2-3,-112 MATa | This study |
| JRM156 | sec63-1 pep4-3 ade2-1 leu2-3,-112 MATa | This study |
| JRM157 | sec63-1 pep4-3 ade2-1 leu2-3,-112 MATa | This study |
| JRM169 | sec63-1 sec18-1 leu2-3,-112 MATa | This study |
| JRM163 | sec63-1 ura3-52 leu2-3,-112 MATa | This study |
| JRM164 | sec62-1 sec63-1 pep4-3 his4 ura3-52 leu2-3,-112 MATa | This study |
| RDB105 | ade2 leu2-3,-112 MATa | Lab strain |
| RDB142 | sec18-1 ura3-52 leu2-3,-112 MATa | Lab strain |
| RSY32 | pep4-3 ade2 leu2-3,-112 MATa | Lab strain |
| PBY404C | suc2-2Δ9 MATa | P. Böhn |
| MYY220 | mas2-10 Δpep4::LEU2 leu2-3,-112 his3 | Yaffe and Schatz, 1984 |
| MYY238 | mas2-1 Δpep4::LEU2 leu2-3,-112 his3 | Yaffe and Schatz, 1984 |

**E. coli**

| Strain  | Genotype | Source |
|---------|----------|--------|
| SE10 | F' pyrF74::Tn5 ara (Δlac pro) rpsL- thi (Δ80ΔlacZΔM15) | Casadaban and Cohen, 1980 |
| MC1061 | F' araD139 Δ (araAB0C-leu) 7679 ΔlacZ74 galU- galK- rpsL- hsdR | Casadaban and Cohen, 1980 |
and goat anti-rabbit antibody coupled to horseradish peroxidase were from Mannheim Biochemicals. Protein A-Sepharose CL-4B and Sephadex G-25 filters from Schleicher and Schuell, Inc. (Keene, NH). Lyticase (fraction NJ); IgG Sorb from the Enzyme Center (Boston, MA); Invertase (Schauer et al., 1985), CPY (Stevens et al., 1982), and a-factor (lythblatt and Meyer, 1986a) antisera were prepared as described previously. Acid phosphatase antiserum was provided by G. Schatz (Biocenter, CA).

Modification of the HIS4 Gene Fusion

A plasmid, pooSH8, that carries a MFcd-SUC2-HIS4 gene fusion under the control of the MFcd promoter has been described previously (Deshaies and Schekman, 1987). To express the MFcd-SUC2-HIS4 gene fusion in MATa cells as well as in MATα yeast strains, it was necessary to replace the MFcd promoter with another that would allow constitutive, mating type independent expression of the hybrid protein. A new recombinant plasmid (pGD2) that encodes the MFcd-SUC2-HIS4 gene fusion under the control of the triose phosphate isomerase (TPI) promoter was constructed as follows: the plasmid pZVI60 (provided by V. MacKay; Zymos Corp., Seattle, WA), which contains the MFcd structural gene fused to the TPI promoter, was subjected to partial digestion with PvuII and complete digestion with PstI, giving rise to a 3.4-kb fragment that retained the TPI promoter fused to the first eight codons of MFcd. An incomplete MFcd-SUC2-HIS4 fusion (pGD1) was assembled by inserting an internal 2.7-kb PvuII-Pl fragment of the MFcd-SUC2-HIS4 gene from ppoSH8 that lacked the 5′-terminal eight codons of MFcd and a 3′-terminal domain of HIS4. The missing 3′ portion of HIS4 was restored to the TPI promoter MFcd-SUC2-HIS4 gene fusion by replacing the BamHI-XhoI fragment of pGD1 that contained the MFcd-SUC2-HIS4 fusion and HIS4 with the BamHI-XhoI fragment of pGD2, giving rise to plasmid pGD2.

Recombinant DNA manipulations were performed according to standard methods (Maniatis et al., 1982). Plasmids pZVI60 and pGD1 were propagated in Escherichia coli strain SE10 and pGD2 in strain MC1061.

Mutant Isolation and Screening

The experimental details pertaining to the isolation of yeast mutants sec61 and sec62 were described previously (Deshaies and Schekman, 1987). The secretory mutant sec63 was obtained essentially as described for sec61 with the following modifications: FC2-12B cells were transformed with pGD2 by the lithium acetate procedure (Ito et al., 1983). Three separate cultures of each FC2-12B and FC2-12a cells containing pGD2 were grown to stationary phase in minimal medium supplemented with histidine, tryptophan, leucine, and adenine at 30°C. Cells (14 OD600 U) were collected by centrifugation, washed twice with 0.1 M potassium phosphate (pH 7.0), and resuspended at a density of 20 D600 U/ml in the same buffer. Mutagenesis was initiated with the addition of EMS to a final concentration of 3%, and the cells were incubated with the mutagen for 30 min at 30°C with agitation. These conditions for EMS mutagenesis killed ∼50% of the cells. The mutagen was neutralized by adding an equal volume of 12% sodium thiosulphate. The cells were collected by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.0), and resuspended at a density of 2 OD600 U/ml in the same buffer. After centrifugation, the cells were collected by centrifugation, resuspended to 10 OD600/ml in 50 mM potassium phosphate (pH 7.0), and plated onto minimal medium supplemented with histidine, tryptophan, leucine, and adenine. After a 24-h recovery period at 24°C with agitation, the cells were collected by centrifugation, resuspended to 10 OD600/ml in 50 mM potassium phosphate (pH 7.0), and plated onto minimal medium supplemented with histidine, tryptophan, leucine, and adenine, and 3 mM histidinol (5 × 10⁴ viable cells/plate). After incubation at 30°C for 6-17 d, histidinol protophyetes (Hot+) were picked and patched onto YPD plates. After 2 d at 30°C, these were replica-plated onto YPD plates, and the replicas were incubated at 30°C or 37°C for 2-3 d. Clones that grew at 30°C but not at 37°C were picked and restested for their growth by streaking onto YPD plates and incubating at 37°C. After curing confirmed Hot+, Ts+ mutants of the fusion plasmid grown by YPD plates, the α mating type isolates were screened by immunoblotting for accumulation of intracellular prepro-α-factor (pppαF) as previously described (Deshaies and Schekman, 1987), except that binding of α-factor antibody to cross-reacting bands was visualized with goat anti-rabbit antibody coupled to horseradish peroxidase.

Radiolabeling and Immunoprecipitation

Radiolabeling of wild-type and mutant cells with [35S]Na2SO4 (250-300 µCi/OD600 cells) and immunoprecipitation of denatured proteins from the radiolabeled extracts were carried out, with the minor modifications noted below, as described elsewhere (Deshaies and Schekman, 1987). Asparagine-linked core oligosaccharide addition was inhibited by treating cells with 10 µg/ml tunicamycin (prepared as 10 mg/ml in absolute EtOH) for 15′ before and during [35S]SO42− radiolabeling. Extracts were prepared by resuspending cells in 200-400 µl 1% SDS, 50 mM Tris-HCl (pH 7.4), 1 mM PMSF, followed by addition of ∼0.2 g of 0.5 mm glass beads (BioSpec Products, Bartlesville, OK), and vortexing the samples twice for 30 s at full speed in 13 × 100 mm glass culture tubes. Lysates were heated to 95°C for 5 min. Aliquots of the extracts were diluted to 1 ml with 4 vol of immunoprecipitation dilution buffer (1.25% Triton X-100, 190 mM NaCl, 6 mM EDTA, 50 mM Tris-HCl, pH 7.4). For pulldown and inverse immunoprecipitations, samples were supplemented with 2 mg/ml of an unfractionated lysate of nonradioactive PBY404C cells (suc2Δ, MATa). Immunoprecipitation samples were cleared of insoluble material by addition of 25 µl of 10% IgG Sorb suspension, followed by incubation at RT for 5-10 min and centrifugation in a microcentrifuge for 5 min. The supernatant fraction was transferred to fresh microcentrifuge tubes, and the appropriate antiserum was added in saturating amounts for overnight incubation at 4°C. After a 2-h incubation of the samples with protein A-Sepharose CLAB beads at 22°C, the beads were collected by brief centrifugation and washed twice 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. To reduce immunoprecipitation of nonspecific molecules, the bound antigens were dissociated from the protein A-Sepharose beads by heating in 1% SDS-50 mM Tris-HCl, pH 7.4, at 95°C for 5 min and subjected to a second round of immunoprecipitation as above. Finally, antigens were dissociated from the protein A-Sepharose beads by heating in SDS-PAGE sample buffer at 95°C for 5 min and loaded onto 7.5% (invertase, CPY, and A2-Pase) or 11.5% (α factor) SDS-polyacrylamide gels (Laemmli, 1970).

Pulse-chase Radiolabeling of sec61 Cells

Posttranslational translocation of ppF was examined in sec61 (RDM 15-5B) cells grown to an OD600 of 0.8 at 30°C in minimal medium supplemented with 200 µM (NH4)2SO4. An aliquot of cells (2.6 OD600 U) was shifted to 37°C for 30 min, harvested by centrifugation, and resuspended to 1 OD600/ml in minimal medium lacking sulfate. Cells were radiolabeled with 2.6 mCi of [35S]Na2SO4 for 35 min at 38°C. Labeling was terminated by adding 1/100th volume of chase cocktail (0.3% cysteine, 0.4% methionine, 100 mM ammonium sulfate), and the cells were harvested by centrifugation and washed with 0.1 M potassium phosphate (pH 7.0), and plated onto minimal medium supplemented with chase cocktail. The chase period was initiated by resuspending the washed cells in 2.6 ml minimal medium supplemented with 1x chase cocktail, 200 µg/ml BSA, and 2 mM Np-p-tosyl-l-arginine methyl ester (TAME). BSA and TAME were included to inhibit proteolytic degradation of secreted α factor peptide (Ciejek and Thorner, 1979). After 0, 5, 15, 45, 90, and 150 min of chase, 0.4 ml aliquots were withdrawn, chilled on ice and adjusted to 10 mM NaN3. Quenched samples were separated into cell pellet and culture fluid fractions by centrifugation in a clinical centrifuge. Supernatant fractions were supplemented with SDS to a final concentration of 0.4% and heated to 95°C for 5 min. Cell pellets were washed with ice cold 10 mM NaHCO3, converted to spheroplasts (Deshaies and Schekman, 1987), and solubilized directly in SDS-PAGE sample buffer and heated to 95°C for 5 min. Samples were immunoprecipitated with either anti-α factor or antiphosphoglycerate kinase serum, and immune complexes were harvested and washed as described in Radiolabeling and Immunoprecipitation. Immunoprecipitates were evaluated by SDS-PAGE on a 15% polyacrylamide gel. The stacking portion of the gel was run at a standard current (15 mAmps), but the resolving gel was run at 50 mAmps. An aluminum plate was clamped to the gel to allow heat dissipation. The gel was soaked in Amplify without prior fixation and dried onto filter paper for fluorography (Whatman Inc., Clifton, NJ).

Preparation and Proteolysis of Mutant Extracts

Proteolytic accessibility of accumulated ppF was examined in lysates (Deshaies and Schekman, 1987). Both sec62<sup>sec62</sup> sec62<sup>sec62</sup> cells were grown overnight at 24°C and shifted to 30°C for 60 min before labeling with [35S]Na2SO4 for 30 min at 30°C. Spheroplasts were prepared and dis-
rupted in 0.3 M mannitol, 0.1 M KCl, 1 mM EGTA, 50 mM Tris-HCl, pH 7.5, with a motor-driven homogenizer (Potter Elvehjem; Fisher Scientific, Pittsburgh, PA). Lysates were clarified at 660 g for 4 min in a rotor (HB-4; Sorvall Instruments Div., Inc., Newton, CT), and aliquots of the supernatant fraction were treated with proteinase K at a final concentration of 290 μg/ml in the presence or absence of 0.4% Triton X-100 on ice for varying periods of time. Proteolysis was terminated by addition of 20% TCA, and the precipitates were solubilized in 1% SDS and immunoprecipitated with a factor or CPY antisera.

In Vitro Analysis of Protein Translocation

Translocation of α factor precursor was examined with membrane fractions from sec63 and SEC cells grown at 24°C. Membranes were prepared as previously described (Rothblatt and Meyer, 1986b), except that spermidin was replaced by tyrosine (325 μg/mg equivalent of cells) in 50% yeast glucose (YP) (1% bacto-yeast extract, 2% bacto-peptone), CaCl2 (2 mM), and 1.2 M sorbitol, 5 mM DTT, 20 mM Hepes, pH 7.5, for 25 min at 24°C, and then lysed with 10-15 strokes in a Dura-Grind stainless steel homogenizer (Wheatton Instruments Div., Millville, NJ) (clearance = 0.01 in) in ice. The membrane pellet obtained by centrifugation at 25,000 g for 25 min was washed once with membrane storage buffer (MSB: 0.25 M sorbitol, 50 mM KAc, 1 mM DTT, 20 mM Hepes, pH 7.4) and resuspended in MSB. Membranes were treated with staphylococcal nuclease (57 (250 U/ml) in the presence of 2 mM CaCl2 for 5 min at 20°C, and the reaction was terminated by addition of EGTA (final concentration of 4 mM). Microsomes were frozen in liquid nitrogen and stored at −85°C. Pretranslational reactions were conducted with 100,000 g supernatant (S100) fractions prepared from sec63 and SEC cells as described in the accompanying paper by Deshaies and Schekman (1989). Translations were performed at 23°C for 30 min as previously described (Rothblatt and Meyer, 1986b), using pporf mRNA (1 μl/20 μl translation) transcribed in vitro from linearized plasmid pDJ100 (Hansen et al., 1986) with SP6 polymerase (Melton et al., 1984).

Posttranslational translocation of α factor precursor into yeast microsomes was assessed in a 50 μl reaction. Microsomes (0.09 A260 equivalents in 5 μl of MSB) were added to a completed translocation reaction (20 μl) in which further protein synthesis was blocked by addition of cycloheximide to 400 μM. Where indicated, microsomes were incubated at 33°C before addition to the translocation assay. Salts and ATP-regenerating system were readjusted to initial concentrations. Reactions were carried out for 30 min at 23°C and then chilled on ice.

Sequestration of glycosylated α factor species within microsomes was assessed by exposing one-half (15 μl) of the translocation reaction to proteinase K (final concentration = 300 μg/ml) for 45 min on ice. Proteolysis was stopped by addition of PMSF to a final concentration of 1.6 mg/ml. After 5 min on ice, SDS-PAGE sample buffer was added to all samples which were then treated at 95°C for 5 min. The in vitro products were fractionated on 11.5% SDS-polyacrylamide gels, fixed and treated with Amplify for fluorography, and exposed to film (X-OMAT AR; Eastman Kodak Co., New York, NY). Proteins were visualized using a spectrodensitometer (SD3000; Kratos Analytical Instruments, Gilroy, CA) coupled to a density computer (SDS300; Kratos Analytical Instruments) and an integrator (3380A; Hewlett-Packard Co., Palo Alto, CA).

Results

Selection of Secretory Protein Translocation Mutants

The strategy for isolating thermosensitive, conditionally lethal yeast mutants that fail to translocate secretory and vacuolar precursors into the endoplasmic reticulum was described previously (Deshaies and Schekman, 1987). Two genes, sec63 and sec62, were defined among 10 mutant isolates. We judged that a larger-scale isolation of Ts+ histidinol prototrophs (Hol+) would lead to the identification of additional translocation-defective yeast mutants. This was accomplished by altering the protocol so that mutants could be selected in both α and α mating type strains, which allowed arrangement of mutants into complementation groups by scoring diploids for rescue of the Ts+ growth phenotype. The MFα1-SUC2-HIS4 fusion plasmid (pS¢F8F; Deshaies and Schekman, 1987) was modified to remove plasmid expres-

Unprocessed α Factor Precursor Accumulates in sec62 and sec63 Cells

The biogenesis of α factor was examined by radiolabeling mutant and wild-type cells at various temperatures. SEC (JRM156) cells, radiolabeled with 35S]SO42- for 30 min at 17°C or at 24°C, contained only a 26-kD species corresponding to the core-glycosylated precursor form of α factor (pporf) (Fig. 1, lanes 10–11). Rapid intracellular transport or reduced synthesis of the precursor in wild-type cells at 37°C precluded detection of the core-glycosylated species at this temperature (Fig. 1, lane 12). Unglycosylated precursor was detected in wild-type cells only in samples treated with tunicamycin (Fig. 1, lane 13). In contrast, sec63 (RDM 15-5B) or sec63 (JRM151) cells labeled for 30 min at 17°C or 24°C contained a low molecular mass species (Fig. 1, lanes 1–2, 7–8) which co-migrated on SDS-PAGE with pporf translated in vitro (data not shown). Very little pporf was apparent at either temperature. At 37°C, only the low molecu-
lar mass species was present (Fig. 1, lanes 3 and 9). However, a shift in temperature from 17°C to 37°C did not alter the extent of precursor accumulation. Since tunicamycin-treated wild-type cells accumulated a large amount of unglycosylated precursor in the ER lumen, the most likely explanation for this result is that the precursor form of α factor is unstable if retained in the cytoplasm of cells growing at elevated temperatures. sec62 cells (RDM43-9C) were more thermosensitive (Fig. 1, lanes 4–6). A low level of ppoF was apparent after radiolabeling for 30 min at 17°C, but gpoF predominated (Fig. 1, lane 4). Conversely, when sec62 cells were shifted to 24°C for 2 h before [35S]SO42- labeling, ppoF was abundant and gpoF declined. These results suggested that sec62 and sec63 cells, like sec56 cells (Deshaies and Schekman, 1987), accumulated a precursor form of α factor that was not modified by addition of N-linked core oligosaccharides or removal of the signal peptide.

sec62 and sec63 Mutations Result in Accumulation of α Factor Precursor in the Cytosol

The processing deficient phenotype demonstrated in Fig. 1 could result from a defect in polypeptide targeting to or translocation across the ER membrane. In this case, the precursor would accumulate in the cytosol or in association with the cytoplasmic face of the ER membrane, such as has been demonstrated in sec61 (Deshaies and Schekman, 1987). Alternatively, a defect in precursor processing with no effect on translocation could account for intracellular accumulation of ppoF. In this instance, the precursor would remain within the ER lumen. Such behavior has been documented for sec53 and for tunicamycin-treated wild-type cells (Feldman et al., 1987; Ferro-Novick et al., 1984b).

Protease accessibility of ppoF in homogenates of sec62 and sec63 was used to assess the disposition of the precursor. The sec18 mutation, which blocks protein transport from the ER, was introduced into these strains to allow the accumulation of unglycosylated ppoF (19 kD) and gpoF (26 kD) were detected by immunoprecipitation from samples incubated in the absence of protease (Fig. 2, upper and lower sections, lanes 1 and 2). Addition of proteinase K (290 μg/ml) in the absence of detergent, resulted in a rapid and complete degradation of the 19-kD species (Fig. 2 A, lanes 3–9). In contrast, the 26-kD glycosylated form was largely resistant to proteolysis, indicating protection within the ER lumen. Approximately 40% of this core-glycosylated species was degraded during the first minute of proteolysis (Fig. 2, compare lanes 3 and 4), consistent with the observation that ~50% of the 26-kD form is released into the supernatant fraction during the preparation of the cell homogenate (Fig. 2; compare lanes 10 and 11). After 16 min of proteolysis, no further degradation of the 26-kD species was detected. The sensitivity of a partially glycosylated precursor (migrating at a position consistent with the presence of two asn-linked core oligosaccharides) appeared to result from preferential release into the cytosol fraction during cell lysis (Fig. 2 A, lane 11). In the presence of detergent, gpoF species was completely degraded within 1 min of adding protease. Digestion of ppoF in the absence or presence of detergent, was complete.
The selective solubilization of partially glycosylated α-factor precursor has also been observed in extracts of sec18 and sec63 cells (Deshaies and Schekman, 1987) and sec62 cells (Hicke and Schekman, 1989). While the sec18 mutation may increase the fragility of the ER causing both ppeF and the partially glycosylated form to be released and susceptible to protease digestion, other lines of evidence indicated that secretory precursors accumulate in the cytoplasm of sec61, sec62, and sec63 cells. First, in sec61 sec68 cell extracts preproCPY is protease-sensitive in the absence of detergent, while the core-glycosylated forms are entirely resistant to protease (Deshaies and Schekman, 1987). In addition, the precursor forms of α factor, invertase, CPY, and AcPase accumulating in the three sec mutant strains co-migrate with in vitro translation products on SDS-polyacrylamide gels, indicating that no signal peptide processing has occurred. In other sec mutants, such as sec53, displaying a pleiotropic glycosylation mutation, signal peptide processing is not affected (Feldman et al., 1987).

The partition of accumulated ppeF between the sedimentable and supernatant fractions of the yeast lysate was examined by differential centrifugation. As shown for sec63 cells in Fig. 2 A (lane J0), ppeF was quantitatively recovered in the 100,000 g (30-min) pellet, whereas ppaF was equally distributed between the pellet and supernatant fractions. This outcome suggested that the glycosylated precursors remained soluble once released from microsomes during homogenization while the accumulated ppeF either was firmly bound to the ER membrane surface or aggregated in the cytosol. Some form of membrane association was indicated by the observation that both CPY and ppeF floated along with mutant membranes isolated on a dense sucrose cushion (data not shown).

**sec63 Microsomes Are Defective In Vitro**

ppcF can be translocated across the yeast ER membrane in vitro after polypeptide translation is complete (Hansen et al., 1986; Rothblatt et al., 1986b; Waters et al., 1986). The ability of microsome and cytosol fractions from sec63 cells to support posttranslational translocation of ppeF was examined in vitro. ppeF was synthesized in a cell-free system using the 100,000 g supernatant (S-100) fraction prepared from sec63 or wild-type cells. After 30 min of protein synthesis at 23°C, cycloheximide (final concentration of 400 μM) and then wild-type or mutant microsomes were added to the reactions. Translocation and core glycosylation of ppeF were assessed at the end of a 30-min incubation at 23°C by exposing a portion of each reaction to 300 μg/ml proteinase K for 45 min on ice and examining the radioactive products after SDS-PAGE and fluorography. Half of the α-factor precursor translated in a wild-type S-100 fraction was imported into and glycosylated by microsomes from a SEC strain (JRM156) (Fig. 3). Microsomes from sec63 (JRM151) cells exhibited reduced (<20%) translocation activity. Localization of the defective component to the membrane fraction was supported by the observation that ppeF translocated into wild-type microsomes with equal efficiency after synthesis in either a wild-type or sec63 S-100 fraction (data not shown).

A direct connection between the sec63 mutation and the translocation mechanism was established by duplicating thermosensitive precursor assembly within mutant membranes. Wild-type and sec63 microsomes were preincubated at 33°C (the maximum temperature that wild-type membranes tolerated) for varying lengths of time and then mixed with ppeF made in a wild-type S-100 fraction. Production of sequestered ppeF at 23°C with membranes prepared from sec63 cells decayed by 50% after exposure to 33°C for 3 min (Fig. 3). In contrast, 50% inactivation of wild-type microsomes required longer than 20 min at 33°C. The loss of activity by sec63 microsomes was bimodal (Fig. 4). A rapid initial decrease in activity was followed by a gradual loss that paralleled the rate of inactivation observed for wild-type microsomes. Microsomes prepared from sec62 cells also displayed reduced translocation activity in vitro (Deshaies and Schekman, 1989).
Figure 3. In vitro analysis of α factor precursor translocation into sec63 microsomes. Microsomes prepared from permissively grown sec63 (A) or wild-type (B) cells were preincubated at 33°C for 0–15 min and then added to a cycloheximide-inactivated ppa2 translation reaction, to assay precursor translocation and glycosylation. After 30 min at 23°C, the translocation reaction was cooled on ice and aliquots of each sample were proteolyzed with 300 μg/ml proteinase K for 45 min on ice. Samples were fractionated on 11.5% SDS polyacrylamide gels, which were then dried and fluorographed onto preflashed film (X-OMAT, Eastman Kodak Co.).

The SEC61, SEC62, and SEC63 Gene Products Act along the Same Pathway

Two independent mutant loci that affect a common process may exhibit a more restrictive phenotype when combined in a haploid strain. Analysis of sec mutants affecting processes late in the secretory pathway for evidence of genetic interaction has shown decreased viability at the permissive temperature in haploid strains carrying two mutations acting at the same step, whereas progeny of a cross between a late acting mutant and one affecting an early step showed no exaggeration of the Ts- growth phenotype (Salminen and Novick, 1987). The relationship between the products of the SEC61, SEC62, and SEC63 loci in the process of polypeptide translocation was examined by performing genetic crosses that placed pairs of mutations in the same haploid strain.

The segregation pattern for spore viability at 24°C of tetrads obtained from a sec61 × sec62 cross indicated that haploid sec61 sec62 double mutants were inviable under conditions that were permissive for growth of either sec61 or sec62 strains (Table III). Genetic complementation analysis confirmed that all viable progeny of the cross were either wild-type or carried only a single sec mutation (Table IV). Viable double mutants could be obtained by germinating the spores at 17°C. The exaggerated temperature sensitivity of spores bearing two of the sec mutations was also demonstrated in the segregation pattern of viable sec62 × sec63 spores germinated at 17°C or 24°C. Most (95%) of the tetrads analyzed gave rise to 1 or 2 spores that were inviable at 24°C. In contrast, 80% of the tetrads germinated at 17°C produced four viable colonies. In the latter group, 17 of 20 tetrads subjected to complementation analysis contained at least one sec62 sec63 progeny (Table IV). An even more dramatic effect of a double mutation was observed in the progeny of a sec61 × sec63 cross, from which viable sec61 sec63 could not be obtained at either 17°C or 24°C (Table III).

Apparent, absence of fully functional copies of both SEC61 and SEC63 confers a lethal disability on such cells. These results suggest that the sec61, sec62, and sec63 mutations result in a partial loss of function and define components acting in tandem in the process of polypeptide translocation.

No such exaggeration of growth deficiency was seen in other double sec mutant combinations. sec62Δ is growth restrictive at 30°C, yet haploid double mutations with sec61, sec62, or sec63 sporulated and grew normally at 24°C. sec11 mutations affect a subunit of the ER signal peptidase (Böhni et al., 1988), yet were not more sickly when combined with any of the translocation mutations (P. Böhni and R. Schekman, unpublished observations).

sec62 and sec63 Cells Accumulate Various Secretory and Vacuolar Precursors

A common mechanism for transfer of soluble secretory protein precursors across the ER membrane requires that sec62 and sec63 mutants accumulate multiple unprocessed protein precursors. Biogenesis of the vacuolar protease CPY, and the periplasmic enzymes invertase and AcPase was examined by immunoprecipitation of radiolabeled precursors from wild-type, single, and double mutant cells.

Two forms of CPY representing ER- and Golgi-modified species (p1 and p2, respectively, Stevens et al., 1982) were observed in SEC cells radiolabeled at 37°C (Fig. 5 A, lane...
A, lane 4). Some pl and p2 CPY was labeled in
Nevertheless, a novel form of invertase (p) not detected in
restrictive in translocation of invertase (Fig. 5 B, lanes
expression only the constitutive cytoplasmic form of invertase
was detected (Fig. 5 B, lane 1). Unlike the substantial block
containing 0.1% glucose for 20-30 min at 24°C or 37°C fol-
sec63
high temperature (Fig. 5 A, lanes
lar mass form (Fig. 5 A, lane 3). This low molecular mass
duced pl and p2 CPY and a small amount of a lower molecu-
were comparable to those produced in tunicamycin-treated
wild-type cells (Fig. 5 A, lanes 3-5). Identical results were
obtained for AcPase in sec62 at both temperatures. The double
mutant strains were nearly completely deficient in Ac-
Pase translocation at all temperatures (Fig. 5 C, lanes 6-9).
The secretion selectivity of sec mutant blocks was con-
ferred by examining the import and processing of a mito-
chondrial precursor protein, F1β ATPase. Extracts were
prepared from wild-type, and single and double sec mutant
cells that had been incubated for 2 h at 37°C. Two isolates
of the mas2 mutant, which is defective in mitochondrial
precursor processing (Yaffe and Schatz, 1984), were used as
a control. After SDS-PAGE, immunoblotting with F1β-spe-
cific antiserum showed only mature (m) F1β in the
core-glycosylated
and outer chain-glycosylated
block in invertase maturation.
AcPase from wild-type cells migrated on SDS-PAGE as
core-glycosylated (ER) and outer chain-glycosylated (secreted) species (Fig. 5 C, lanes 1-2). sec63 cells accumulated lower molecular mass forms of AcPase at 17°C or 37°C that were
identical results were
obtained for AcPase in sec62 at both temperatures. The double
mutant strains were nearly completely deficient in Ac-
Pase translocation at all temperatures (Fig. 5 C, lanes 6-9).
The secretion selectivity of sec mutant blocks was con-
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prepared from wild-type, and single and double sec mutant
cells that had been incubated for 2 h at 37°C. Two isolates
of the mas2 mutant, which is defective in mitochondrial
precursor processing (Yaffe and Schatz, 1984), were used as
a control. After SDS-PAGE, immunoblotting with F1β-spe-
cific antiserum showed only mature (m) F1β in the SEC and
sec samples (Fig. 5 D, lanes 1-6), while both mature and
preceptor (p) were detected in the mas samples (Fig. 5 D, lanes 7-8). From this, we conclude that the sec mutations
do not affect assembly of the mitochondrion.

**Table III. Haploid Double Mutants Are Inviable at 24°C**

| Cross      | Germination temperature | Number of tetrads | Segregation of viable spores in tetrads (live/dead) |
|------------|-------------------------|-------------------|-----------------------------------------------------|
| sec61 × sec62 | 24°C                   | 19                | 4:0 3:1 2:2                                         |
| (RDM 33-4A × RDM 43-9C) | 17°C                   | 15                | 5 4 3                                             |
| sec62 × sec63 | 24°C                   | 20                | 1 10 9                                            |
| (RDM 43-9C × JRM 163-4D) | 17°C                   | 20                | 1 16 2                                            |
| sec61 × sec63 | 24°C                   | 21                | 5 13 3                                            |
| (RDM 15-5B × JRM 163-4D) | 17°C                   | 21                | 2 14 5                                            |

Table IV. Segregation Analysis of Tetrads from sec61 × sec62 and sec62 × sec63 Crosses

| Cross | Tetratype | Number of tetrads | Genotype of spores |
|-------|-----------|-------------------|--------------------|
| sec61 × sec62 | Parental ditype | 2 | 2 sec61SEC62, 2 SEC61sec62 |
|        | Nonparental ditype | 2 | 2 SEC61SEC62, 2 inviable (sec61sec62)* |
|        | Tetratype | 15 | 1 SEC61SEC62, 1 sec61SEC62, 2 SEC61sec62, 1 inviable (sec61sec62)* |
| sec62 × sec63 | Parental ditype | 3 | 2 sec62SEC63, 2 SEC62sec63 |
|        | Nonparental ditype | 5 | 2 SEC62SEC63, 2 sec62sec63 |
|        | Tetratype | 12 | 1 SEC62SEC63, 1 sec62SEC63, 1 inviable (sec61sec62)* |

* Inviability because of spore germination at 24°C.
† Viable mutants obtained by spore germination at 17°C.
tent of radiolabeled α factor by immunoprecipitation followed by SDS-PAGE.

ppαF in the pulse label gradually disappeared during the chase period (Fig. 6, lanes 1–6). Mature α factor accumulated in the culture medium in parallel with the decline in intracellular ppαF (lanes 7–12). Cell lysis did not account for α factor release since sec61 cells subjected to a 120-min chase retained the cytosolic phosphoglycerate kinase (lanes 13 and 24). Radiolabeled sec61 cells contained a low molecular mass species that persisted throughout the chase period (lanes 1–6). This molecule migrated more rapidly than secreted α factor on SDS polyacrylamide gels, and possibly was generated by intracellular degradation of ppαF accumulated at the sec61 block.

Though sec61 cells were radiolabeled at 37°C and chased at 30°C in the experiment described above, ppαF was also secreted by sec61 cells retained at 37°C during the chase period (data not shown). In addition, untranslocated ppαF was converted to mature α factor and secreted even if the pro-
protein synthesis inhibitor cycloheximide was present during the chase incubation. Therefore, secretion of accumulated ppaF did not require full activity of the defective sec61 gene product or replacement with newly synthesized counterparts.

**Discussion**

A large scale genetic selection and screen for yeast mutants defective in protein translocation into the ER has identified three genes, SEC61, SEC62, and SEC63. Considering the number and distribution of mutant isolates among the three complementation groups, it seems unlikely that new genes would be identified by repeated application of the same selection protocol. A modification of our scheme in which the TRP1 gene replaced the HIS4 gene as a selectable cytoplasmic marker yielded a translocation mutant pill that appears to be allelic to sec63 (Toyn et al., 1988). Surprisingly, a different genetic selection designed to identify genes required for protein import into the yeast nucleus has uncovered another mutation allelic to sec63 (npull; Sadler et al., 1989).

Additional genes required in the translocation process exist and may require other approaches for their detection. One clear example is the 70-kD heat shock protein (hsp70), which is represented by four isozymes in yeast encoded by the SSA gene family. These isozymes serve interchangeable roles in facilitating protein translocation into the ER and the mitochondrion (Deshaies et al., 1988a). No single mutation in any one of the SSA genes yields a secretion defective phenotype. Therefore, the selection of a single recessive mutation would not have revealed this important participant in the assembly process. Our selection scheme also demands that a mutation exert only a partial defect at a temperature compatible with growth, and a more complete defect at a higher, restrictive temperature. Such alleles may not be obtained in certain genes. Variations of the procedure employing other signal peptides or low temperature (cold sensitive) restrictive mutations may uncover new genes.

Phenotypically, sec61, sec62, and sec63 appear to block protein translocation at a similar point in the pathway. All three accumulate unglycosylated precursor forms of different secretory or vacuolar proteins. For α factor precursor and preinvertase, it is clear that translocation is blocked before the signal peptide processing step. Defective translocation is confirmed with the demonstration that precursors accumulate outside the protective barrier of the ER membrane. The precursors appear to be membrane bound as judged by sedimentation and flotation along with membranes isolated from mutant cells. Perhaps a limited number of precursor molecules bind specifically to mutant membranes and cause a backlog of additional precursors to aggregate on the cytoplasmic surface of the ER.

Accumulation of ppaF in the cytoplasm allows an in vivo test of the suggestion that this molecule may be translocated posttranslationally (Hansen et al., 1986). At least half of the ppaF radiolabeled in a pulse of sec61 cells interacted productively with the translocation machinery during a chase period and emerged from the cell as mature-sized pheromone. The kinetics of this chase are much slower than the rate of secretion seen in wild-type cells. It appears that the sec61 defect represents a severe kinetic delay rather than an absolute block because the chase is equally slow but complete at 30°C or 37°C. A comparable test with sec63 cells showed qualitatively the same efficiency of α factor secretion.

The participation and localization of Sec gene products in polypeptide import can be directly tested by in vitro analysis of ppaF translocation using fractions prepared from mutant cells. sec63 membranes display thermolabile translocation activity with ppaF synthesized either in mutant or wild-type S-100 fractions. The simplest explanation of this result is that the Sec63 protein (Sec63p) is a membrane protein and the sec63 mutation creates a thermally inactivated form. Direct
sequence analysis of the SEC63 gene supports an integral membrane location for the gene product (Sadler et al., 1989). Similar biochemical and molecular cloning results suggest that Sec61p and Sec62p are also integral membrane proteins (Deshaies and Schekman, 1989; C. Stirling and R. Schekman, unpublished results). These Sec proteins are likely candidates for the polypeptide translocation apparatus.

A simple genetic test supports the notion that Sec61p, Sec62p, and Sec63p act together to facilitate translocation. Combination of any two of the three mutant loci in a haploid strain is lethal (sec61 sec63) or results in a lowered restrictive growth temperature (sec61 sec62; sec62 sec63). Viable double mutant strains are more severely deficient in translocation of each of the four glycoproteins tested. The effect is specific because double mutants that include one of these loci together with a mutation that affects another step in the secretory process are not more growth restrictive than either haploid parent strain. One interpretation of these results is that Sec61p, Sec62p, and Sec63p are arranged in a complex or act on each other so that the presence of two partially functional members exaggerates the overall deficiency. The alternate interpretation that the gene products function in parallel pathways of protein import seems less likely since a null mutation in any one of these genes is lethal (C. Stirling and R. Schekman, unpublished observation; Deshaies and Schekman, 1989; Sadler et al., 1989). Synthetic lethality, inferring genetic interaction, has also been observed among sec mutants blocking secretory protein transport from the ER to the Golgi apparatus (C. Kaiser and R. Schekman, unpublished observation) and among mutants affecting protein delivery to the cell surface (Salminen and Novick, 1987). In each case, there is a complete concordance between the stage in the secretory pathway that is blocked and the double mutant combinations that generate enhanced lethality.

While it is clear that the translocation mutants affect all four glycoproteins tested, invertase assembly seems to be the least severely affected. Even in the viable double mutants, invertase translocation and secretion occur at temperatures (17°C–24°C) where the other glycoproteins are completely blocked. Invertase differs from the other proteins in the composition and structure of the signal peptide portion. Most secreted proteins that contain a cleaved signal peptide possess a basic amino acid residue near the NH₂ terminus (Perlman and Halvorson, 1983; von Heijne, 1985); invertase lacks this feature. In addition, the invertase signal is considerably more hydrophilic (16 hydrophobic residues of a total 19) than the signals of preAcPase (9:14 after the lys residue), ppAcF (12:17 after the arg residue), or preproCPY (10:20 after the lys residue). Less hydrophobic signals, such as that from preproCPY, function poorly in a heterologous mammalian system, yet can be converted to a functional form by introduction of additional hydrophobic amino acids (Bird et al., 1987). Conversely, hybrid protein containing the preproCPY signal fused to the invertase gene produces a hybrid that is completely blocked in sec62 cells (Johnson et al., 1987; R. Deshaies, unpublished results). Perhaps the invertase signal peptide partitions more readily into the ER bilayer and displays a less stringent requirement for a putative signal peptide receptor.

At least part of the function of the translocation Sec proteins may be to recruit or affix secretory precursors to the ER membrane. This model predicts that integral membrane proteins with hydrophobic domains that mediate membrane assembly will be less severely blocked by the translocation sec mutants.

We would like to thank Drs. Michael Douglas, Jeff Schatz, and Jeremy Thorner for providing antibodies, and Michael Yaffe and Jeremy Toyn for sending yeast strains. Pam Silver and members of her group very generously provided us with information about the nip1 mutants before publication. We are also grateful to Linda Hibke and Chris Kaiser for their comments on the manuscript. Many thanks to Peggy Mc-Cushan Smith for her expert secretarial help during the preparation of this manuscript. J. Rothblatt has been supported by a Postdoctoral Fellowship from the National Institutes of Health (GM-11791) and a Senior Postdoctoral Fellowship from the American Cancer Society, California Division. S. Sanders is a recipient of a National Science Foundation Graduate Minority Fellowship. G. Daum was supported by the Max Kade Foundation. This work was supported by research grants from the National Institutes of Health (GM-26755 and GM-36881) to R. Schekman.

Received for publication 13 June 1989 and in revised form 26 July 1989.

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