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 (ppαF) 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 ppαF 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, 1980*a*, 1980*b*; 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 isolation and characterization of a large number of conditionally lethal, temperature-sensitive (Ts^-) secretion (*sec*) mutants (Novick et al., 1980; Ferro-Novick et al., 1984*a*). 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.

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1. *Abbreviations used in this paper*: AcPase, acid phosphatase; CPY, carboxypeptidase Y; EMS, ethyl methanesulfonate; gpαF, glycosylated pro-α-factor; Hol^+, histidinol prototrophy; MSB, membrane storage buffer; ppαf, prepro-α-factor; SSR, signal sequence receptor; TAME, *Nα-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 *sec6l* 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 *SEC6l*, *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 product is a component of the ER membrane. A preliminary account of these studies was reported elsewhere (Deshaies et al., 1988*b*).

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 FC2-12Bα 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 α factor precursor accumulation phenotypes. 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 *PEP4* 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 *PEP4* gene (Rothman et al., 1986). The *pep4-3* mutation was introduced into the *sec63* mutant by mating with RSY32 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 $[^{35}S]SO_4^{2-}$, 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 KH_2PO_4. 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 (OD_{600}) of dilute cell suspensions was measured in 1-cm quartz cuvettes using a spectrophotometer (PMQII; Carl Zeiss, Inc., Thornwood, NY); 1 OD_{600} 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, ethyl methanesulfonate (EMS), Triton X-100, and nucleoside triphosphates were purchased from Sigma Chemical Co. (St. Louis, MO). Carrier-free $[^{35}S]Na_2SO_4$ was obtained from ICN Radiochemicals (Irvine, CA), and [^{35}S]methionine (>1,000 Ci/mmol), [^{125}I]NaI (highest specific activity), and Amplify were from Amersham Corp. (Arlington Heights, IL). Staphylococcal nuclease S7, SP6 RNA polymerase, m^7GpppG, creatine phosphate, and creatine phosphokinase were obtained from Boehringer-

Table I. Bacterial and Yeast Strains

Strain	Genotype	Source
S. cerevisiae		
FC2-12B	*ura3-52 leu2-3,-112 trp1-1 his4-401 HOL1-1 MATα*	Parker and Guthrie, 1985
RDM15-5B	*sec61-2 pep4-3 ade2 ura3-52 leu2-3,-112 MATα*	Deshaies and Schekman, 1987
RDM33-4A	*sec61-2 ade2 his leu2-3,-112 MATa*	This study
RDM43-9C	*sec62-1 his4 ura3-52 Δpep4::URA3 MATα*	This study
RDM52-7C	*sec61-2 sec62-1 ura3-52 Δpep4::URA3 MATα*	This study
JRM151	*sec63-1 pep4-3 ura3-52 leu2-3,-112 MATα*	This study
JRM156	*pep4-3 ura3-52 leu2-3,-112 MATα*	This study
JRM157	*sec63-1 pep4-3 ade2-1 leu2-3,-112 MATα*	This study
JRM160	*sec63-1 sec18-1 leu2-3,-112 MATα*	This study
JRM163	*sec63-1 ura3-52 leu2-3,-112 MATa*	This study
JRM164	*sec62-1 sec63-1 pep4-3 his4 leu2-3,-112 MATα*	This study
RDB103	*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-Δ9 MATα*	P. Böhni
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		
SE10	*F⁻ pyrF74::Tn5 ara(Δlac pro) rpsL⁻ thi (φ80dlacZΔM15)*	Emr et al., 1986
MC1061	*F⁻ araD139 Δ(araABOIC-leu)7679 Δlacχ74* galU⁻ *galK⁻ rpsL⁻ hsdR⁻*	Casadaban and Cohen, 1980

Mannheim Biochemicals. Protein A-Sepharose CL-4B and Sephadex G-25 (medium) were purchased from Pharmacia Fine Chemicals (Piscataway, NJ); IgG Sorb from the Enzyme Center (Boston, MA); SDS-PAGE reagents and goat anti-rabbit antibody coupled to horseradish peroxidase were from Bio-Rad Laboratories (Richmond, CA); human placental ribonuclease inhibitor from Promega Corp. (Madison, WI); and nitrocellulose membrane filters from Schleicher and Schuell, Inc. (Keene, NH). Lyticase (fraction II, ~60,000 U/ml) was prepared as described by Scott and Schekman (1980). Invertase (Schauer et al., 1985), CPY (Stevens et al., 1982), and α-factor (Rothblatt and Meyer, 1986*a*) antisera were prepared as described previously. Acid phosphatase antiserum was provided by G. Schatz (Biocenter, Basel, Switzerland); ATPase $F_1\beta$ subunit antiserum by Michael Douglas (University of Texas Health Sciences Center, Dallas, TX); and antiphosphoglycerate kinase serum by J. Thorner (University of California, Berkeley, CA).

Modification of the HIS4 Gene Fusion

A plasmid, pαSHF8, that carries a MFα1-SUC2-HIS4 gene fusion under the control of the MFα1 promoter has been described previously (Deshaies and Schekman, 1987). To express the MFα1-SUC2-HIS4 gene fusion in MATa as well as in MATα yeast strains, it was necessary to replace the MFα1 promoter with another that would allow constitutive, mating type independent expression of the hybrid protein. A new recombinant plasmid (pGD2) that encodes the MFα1-SUC2-HIS4 gene fusion under the control of the triose phosphate isomerase (TPI) promoter was constructed as follows: the plasmid pZV160 (provided by V. MacKay; Zymos Corp., Seattle, WA), which contains the MFα1 structural gene fused to the TPI promoter, was subjected to partial digestion with PvuII and complete digestion with Pst I, giving rise to a 3.4 kilobase (kb) fragment that retained the TPI promoter fused to the first eight codons of MFα1. An incomplete MFα1-SUC2-HIS4 fusion (pGD1) was assembled by inserting an internal 2.7-kb Pvu II–Pst I fragment of the MFα1-SUC2-HIS4 gene from pαSHF8 that lacked the 5′-terminal eight codons of MFα1 and a 3′-terminal domain of HIS4. The missing 3′ portion of HIS4 was restored to the TPI promoter MFα1-SUC2-HIS4 gene fusion by replacing the Bam HI–Xho I fragment of pSHE1 (containing a SUC2-HIS4 fusion; Deshaies and Schekman, 1987) with the Bam HI–Xho I fragment of pGD1, giving rise to plasmid pGD2.

Recombinant DNA manipulations were performed according to standard methods (Maniatis et al., 1982). Plasmids pZV160 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 *sec6l* and *sec62* were described previously (Deshaies and Schekman, 1987). The secretory mutant *sec63* was obtained essentially as described for *sec6l* with the following modifications: FC2-12B cells were transformed with pGD2 by the lithium acetate procedure (Ito et al., 1983). Three separate 5-ml cultures of each FC2-12Bα and FC2-12*a* cells containing pGD2 were grown to stationary phase in minimal medium supplemented with histidine, tryptophan, leucine, and adenine at 30°C. Cells (14 OD_{600} U) were collected by centrifugation, washed once with sterile 50 mM potassium phosphate buffer (pH 7.0), and resuspended at a density of 2 OD_{600} 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 thiosulfate. The cells were collected by centrifugation, washed twice with 50 mM potassium phosphate (pH 7.0), and resuspended in 15 ml of 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 OD_{600}/ml in 50 mM potassium phosphate (pH 7.0), and plated onto minimal medium supplemented with tryptophan, leucine, adenine, and 3 mM histidinol (~5×10^6 viable cells/plate). After incubation at 30°C for 6–17 d, $histidinol^+$ prototrophs (Hol^+) 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 retested for Ts^- growth by streaking onto YPD plates and incubating at 37°C. After curing confirmed Hol^+, Ts^- mutants of the fusion plasmid by growth on YPD plates, the α mating type isolates were screened by immunoblotting for accumulation of intracellular prepro-α-factor (ppαF) 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 $[^{35}S]Na_2SO_4$ (250–300 μCi/OD_{600} 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 $[^{35}S]SO_4^{2-}$ 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, 60 mM Tris-HCl, pH 7.4; Anderson and Blobel, 1983). For α factor and invertase immunoprecipitations, samples were supplemented with 2 mg/ml of an unfractionated lysate of nonradioactive PBY404C cells (*suc2*Δ, *MATa*). Immunoprecipitation samples were cleared of insoluble material by addition of 25 μl of a 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 CL4B 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 AcPase) or 11.5% (α factor) SDS-polyacrylamide gels (Laemmli, 1970).

Pulse-chase Radiolabeling of sec6l Cells

Posttranslational translocation of ppαF was examined in *sec6l* (RDM 15-5B) cells grown to an OD_{600} of 0.8 at 30°C in minimal medium supplemented with 200 μM $(NH_4)_2SO_4$. An aliquot of cells (2.6 OD_{600} U) was shifted to 37°C for 30 min, harvested by centrifugation, and resuspended to 1 OD_{600}/ml in minimal medium lacking sulfate. Cells were radiolabeled with 2.6 mCi of $[^{35}S]Na_2SO_4$ for 33 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 5 ml minimal medium supplemented with chase cocktail. The chase period was initiated by resuspending the washed cells in 2.6 ml minimal medium supplemented with 1× chase cocktail, 200 μg/ml BSA, and 2 mM N_α-*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 NaN_3. 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 NaN_3, 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 mAmp, and 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 ppαF was examined in lysates (Deshaies and Schekman, 1987). Both *sec62 secl8* and *sec63 secl8* cells were grown overnight at 24°C and shifted to 30°C for 60 min before labeling with $[^{35}S]Na_2SO_4$ 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 Elvejhem; Fisher Scientific, Pittsburgh, PA). Lysates were clarified at 660 *g* for 4 min in a rotor (HB-4; Sorvall Instruments Div. [part of Dupont Co., 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 α 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, 1986*b*), except that spheroplasts were formed with lyticase (~25 U/OD_{600} equivalent of cells) in 50% YP (1% bacto-yeast extract, 2% bacto-peptone), 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 (Wheaton 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 KOAc, 1 mM DTT, 20 mM Hepes, pH 7.4) and resuspended in MSB. Membranes were treated with staphylococcal nuclease S7 (250 U/ml) in the presence of 2 mM $CaCl_2$ 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.

Protein synthesis reactions were conducted with 100,000 *g* supernatant (S-100) 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, 1986*a*), using ppαF 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 30 μl reaction. Microsomes (0.09 A_{280} equivalents in 5 μl of MSB) were added to a completed translation 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 heated to 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., Rochester, NY) at −80°C. Fluorograms were quantified by scanning with a spectrodensitometer (SD3000; Kratos Analytical Instruments, Ramsey, NJ) 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, *sec61* 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 *a* 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 (pαSHF8; Deshaies and Schekman, 1987) was modified to remove plasmid expression from control of the α mating type–specific MFα1 promoter. A constitutive promoter from the triose phosphate isomerase gene was fused to the signal sequence of MFα1 creating a mating type–independent hybrid protein encoded by plasmid pGD2.

The new selection plasmid, pGD2, allowed growth of *sec61* (strain RDM 15-5B) cells but not Sec^+ cells (strain FC2-12Bα) at 30°C on minimal medium containing histidinol. This result confirmed the utility of pGD2 in the selection of additional translocation *sec* mutants. Immunoblot analysis of extracts prepared from *a* and α cells harboring pGD2 showed that the hybrid protein was expressed in both mating types at approximately equal amounts (data not shown). The Hol growth phenotypes of wild-type FC2-12B (MAT*a* or MATα) and *sec61* cells containing pGD2 correlated with a change in the electrophoretic mobility of the hybrid protein in SDS-polyacrylamide gels. Wild-type cells expressed a higher molecular weight form of the fusion protein, consistent with core glycosylation of the precursor within the ER lumen. *sec61* mutant cells accumulated a hybrid molecule identical in size to the predicted translation product. Thus the Hol^- growth phenotype of pGD2-transformed wild-type cells was because of sequestration of the hybrid protein within the ER lumen.

Mutagenesis of FC2-12Bα and FC2-12B*a* yeast cells transformed with pGD2 produced a large number of Hol^+ isolates among which slightly >10% also showed a Ts^- growth phenotype (Table II). Ts^- α mating type isolates were examined by immunoblotting whole cell extracts for intracellular accumulation of α factor precursor. Around 20% (73) of the Ts^- isolates accumulated a form that co-migrated with ppαF accumulated in *sec61* cells. Standard complementation tests allowed the identification of a large number of new isolates of *sec61* and *sec62* (Table II). Complementation groups among the remaining 20 Ts^- isolates were established by mating each to the collection of MAT*a* Ts^- isolates and scoring for rescue of the growth defect in diploids at 37°C. Several potential new complementation groups were identified, representatives of which were crossed against wild-type strains to establish genetic linkage of the Ts^- and *sec* phenotypes. After four cycles of backcrossing only one new complementation group, *sec63*, demonstrated linkage between the thermosensitive growth and ppαF accumulation phenotypes.

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 $[^{35}S]SO_4^{2-}$ 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 (gpαF) (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, *sec61* (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 ppαF translated in vitro (data not shown). Very little gpαF was apparent at either temperature. At 37°C, only the low molecu-

Table II. Histidinol Selection for Conditionally Lethal Translocation Mutants

Stage of mutant selection	Number of colonies analyzed		
	MATα cells	*MATa* cells	Frequency*
Cells plated on MV-histidinol	5.5×10^7	5.5×10^7	
Histidinol prototrophs (Hol^+)	3,951	1,452	4.9×10^{-5}
Temperature-sensitive for growth	393	216	5.5×10^{-6}
Accumulate unglycosylated prepro-α-factor	73	NE‡	1.3×10^{-6}
sec61 Alleles	14	12	2.4×10^{-7}
sec62 Alleles	39	23	5.6×10^{-7}
sec63 Alleles	7	15	2.0×10^{-7}

* This value represents the number of colonies exhibiting a given phenotype divided by the number of cells plated on histidinol.
‡ NE, not expressed in *MATa* cells.

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 ppαF was apparent after radiolabeling for 30 min at 17°C, but gpαF predominated (Fig. 1, lane *4*). Conversely, when *sec62* cells were shifted to 24°C for 2 h before $[^{35}S]SO_4^{2-}$-labeling, ppαF was abundant and gpαF declined. These results suggested that *sec62* and *sec63* cells, like *sec61* 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 ppαF. 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., 1984*b*).

Protease accessibility of ppαF 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 sequestered gpαF as an internal control in the proteolysis experiment (Deshaies and Schekman, 1987). Haploid *sec63 sec18* cells were radiolabeled with $[^{35}S]SO_4^{2-}$ for 30 min at 30°C, a temperature at which *sec18* is fully restrictive and *sec63* only partially restrictive. Spheroplasts were formed, gently lysed, and ppαF (19 kD) and gpαF (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, gpαF species was completely degraded within 1 min of adding protease. Digestion of ppαF in the absence or presence of detergent, was com-

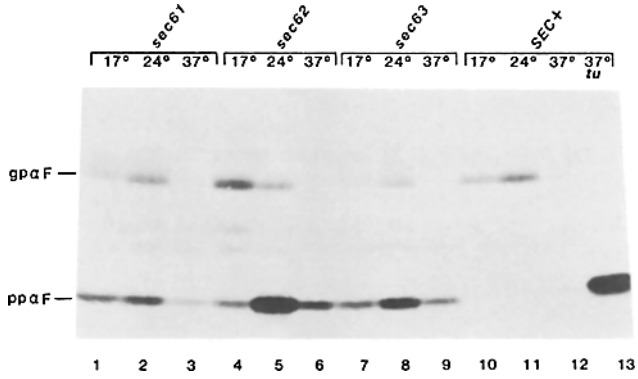

Figure 1. Unglycosylated ppαF accumulates in *sec* mutants. *sec61* (RDM15-5B), *sec62* (RDM43-9C), *sec63* (JRM151), and *SEC* (JRM156) cells were radiolabeled with $[^{35}S]SO_4^{2-}$ at 17°C for 30 min (lanes *1*, *4*, *7*, and *10*, respectively), or, following a 2-h preshift of parallel cultures to 24°C or 37°C, at 24°C (lanes *2*, *5*, *8*, and *11*), or 37°C (lanes *3*, *6*, *9*, and *12*) for 30 min. N-linked core glycosylation of α factor precursor was blocked in *SEC* cells (lane *13*) by incubating cells in the presence of tunicamycin (10 μg/ml) before and during radiolabeling. Cell extracts were prepared by glass-bead homogenization, and α factor antiserum cross-reacting material was immunoprecipitated. Immunoprecipitates were analyzed by electrophoresis on 11.5% SDS polyacrylamide gels. Each lane contained cross-reacting material immunoprecipitated from 0.75 OD_{600} U of cells. Core-glycosylated α factor precursor is designated gpαF and ppαF indicates unglycosylated ppαF.

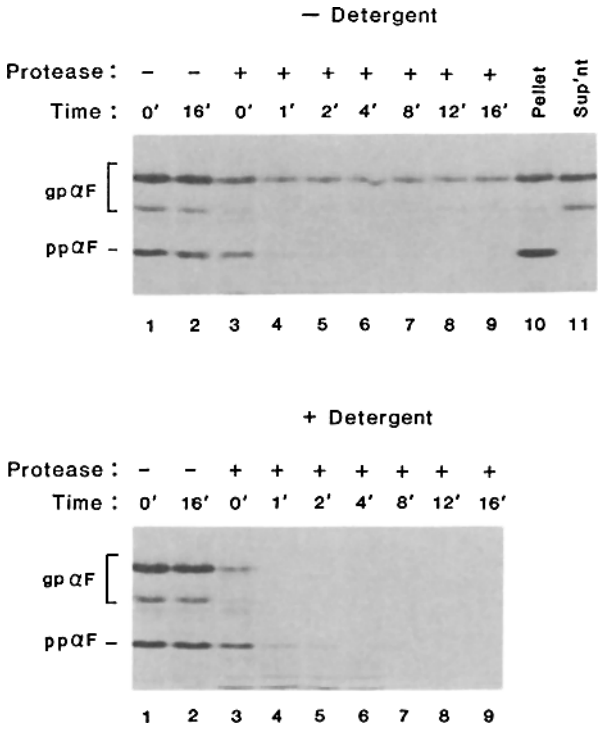

Figure 2. Protease sensitivity of ppαF in a *secl8sec63* cell extract. A lysate was prepared after converting radiolabeled JRM160 cells to spheroplasts, as previously described (Deshaies and Schekman, 1987; see also Materials and Methods). Aliquots of the lysate were mock-digested (lanes *1* and *2*, *top and bottom*) or were treated with 300 μg/ml protease K in the absence (lanes *3–9*, *top*) or in the presence (lanes *3–9*, *bottom*) of 0.4% Triton X-100 for 0–16 min on ice. A separate aliquot (equivalent to 2 OD_{600} U of cells) was fractionated by differential centrifugation into pellet (lane *10*) and supernatant (lane *11*) fractions. Proteolysis was terminated by addition of 20% TCA. All samples were immunoprecipitated with α factor antiserum and electrophoresed on 11.5% SDS polyacrylamide gels. Each proteolysis sample contained α factor precursor immunoprecipitated from 1.35 OD_{600} U of cells. α Factor precursors accumulated by the *secl8* and *sec63* mutations are indicated as gpαF and ppαF, respectively.

plete in 4 min, suggesting that the 19-kD precursor may form a weakly protease-resistant aggregate as a result of accumulation in the cytosol or at the ER membrane. Similar results were obtained with an extract prepared from *sec62 secl8* cells (data not shown).

The selective solubilization of partially glycosylated α factor precursor has also been observed in extracts of *sec6l secl8* cells (Deshaies and Schekman, 1987) and *secl8* cells (Hicke and Schekman, 1989). While the *secl8* mutation may increase the fragility of the ER causing both ppαF 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 *sec6l*, *sec62*, and *sec63* cells. First, in *sec6l secl8* 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 ppαF 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 *10*), ppαF was quantitatively recovered in the 100,000 *g* (30-min) pellet, whereas gpαF 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 ppαF 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 ppαF floated along with mutant membranes isolated on a dense sucrose cushion (data not shown).

sec63 Microsomes Are Defective In Vitro

ppαF can be translocated across the yeast ER membrane in vitro after polypeptide translation is complete (Hansen et al., 1986; Rothblatt et al., 1986*b*; Waters et al., 1986). The ability of microsome and cytosol fractions from *sec63* cells to support posttranslational translocation of ppαF was examined in vitro. ppαF 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 ppαF 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 ppαF 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 ppαF made in a wild-type S-100 fraction. Production of sequestered gpαF 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).

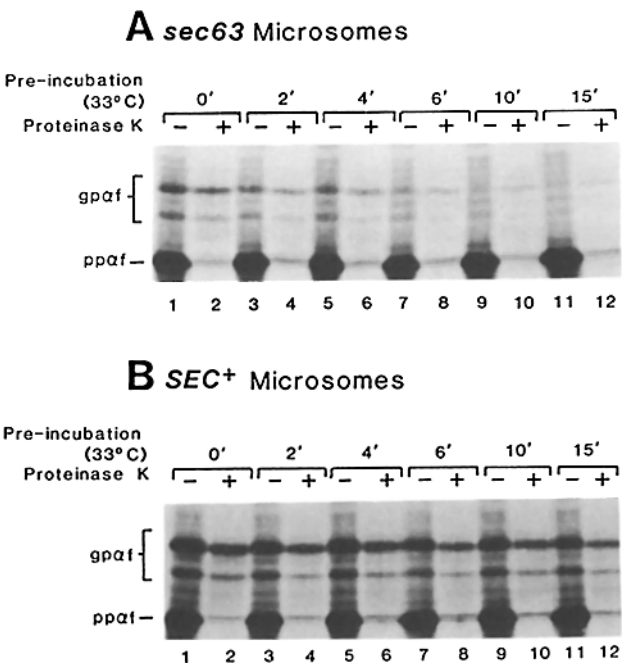

Figure 3. In vitro analysis of α factor precursor translocation into *sec63* microsomes. Microsomes prepared from permissively grown *sec63* (*A*) or wild-type (*B*) cells were preincubated at 33°C for 0–15 min and then added to a cycloheximide-inactivated ppαF translation reaction, to assay precursor 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.).

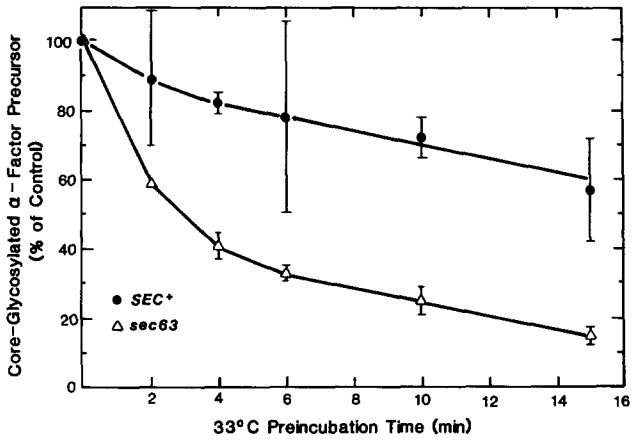

Figure 4. Thermolability of in vitro α factor precursor translocation into *sec63* microsomes. Translocation of ppαF was assayed exactly as described in Fig. 3. For each preincubation time point, the protease-resistant glycosylated species of α factor were quantified by scanning the fluorograms in a spectrodensitometer. Data points indicate the fraction (in percent) of glycosylated α factor detected in *sec63* (-△-) or SEC+ (-●-) in microsomes not exposed to 33°C that was translocated after 2–15 min preincubation of microsomes at 33°C. The range in values from two experiments is given. The efficiency of ppαF translocation into SEC+ microsomes was ~47% and into sec63 microsomes was ~17%.

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). Apparently, 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. *sec18-1* 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

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

Cross	Germination temperature	Number of tetrads	Segregation of viable spores in tetrads (live/dead) 4:0	3:1	2:2
sec61 × *sec62*	24°C	19	2	15	2
(RDM 33-4A × RDM 43-9C)	17°C	15	8	5	2
sec62 × *sec63*	24°C	20	1	10	9
(RDM 43-9C × JRM 163-4D)	17°C	20	16	2	2
sec61 × *sec63*	24°C	21	5	13	3
(RDM 15-5B × JRM 163-4D)	17°C	21	2	14	5

1). *pep4* mutant strains, which lack proteinase A activity required for processing of proCPY, were used to prevent the formation of mature CPY that migrates close to the position of preproCPY. *sec62* Cells grown and labeled at 17°C produced p1 and p2 CPY and a small amount of a lower molecular mass form (Fig. 5 *A*, lane *3*). This low molecular mass species migrated more slowly than CPY immunoprecipitated from tunicamycin-treated *SEC* cells (Fig. 5 *A*, lane 2) possibly because of retention of the signal peptide (preproCPY). After 2 h at 37°C, mainly preproCPY was produced (Fig. 5 *A*, lane *4*). Some p1 and p2 CPY was labeled in *sec63* cells at 17°C or 37°C with a modest increase in preproCPY at the high temperature (Fig. 5 *A*, lanes *5–6*). Compared to *sec61*, *sec63* cells were more deficient in CPY maturation at the restrictive temperature (not shown). The translocation block was exaggerated in the two viable double mutant strains (*sec61 sec62*; *sec62 sec63*). Even at 17°C, very little maturation of preproCPY was observed; at 37°C the blocks were absolute (Fig. 5 *A*, lanes *7–10*).

Invertase synthesis was derepressed in minimal medium containing 0.1% glucose for 20–30 min at 24°C or 37°C followed by radiolabeling for 30 min. In the absence of derepression only the constitutive cytoplasmic form of invertase was detected (Fig. 5 *B*, lane *1*). Unlike the substantial block in assembly of preproCPY, the mutants were incompletely restrictive in translocation of invertase (Fig. 5 *B*, lanes *4–5*). Nevertheless, a novel form of invertase (*p*) not detected in derepressed *SEC* cells accumulated in *sec61* at 37°C (Fig. 5 *B*, lane *5*; Deshaies and Schekman, 1987). Likewise, p was labeled in *sec62* (Fig. 5 *B*, lanes *3–4*) and *sec63* (not shown). The p form co-migrated with the in vitro translation product of mRNA encoding secretory invertase (Fig. 5 *B*, lane 2). Thus, p represents unglycosylated preinvertase. Again, in contrast to the effect of double mutants on preproCPY assembly, the *sec62 sec63* strain allowed translocation and glycosylation of invertase at 17°C (not shown). Preincubation of this strain at 37°C for 2 h however resulted in a complete 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 comparable to those produced in tunicamycin-treated wild-type cells (Fig. 5 *C*, lanes *3–5*). Identical results were obtained for AcPase in *sec62* at both temperatures. The double mutant strains were nearly completely deficient in AcPase translocation at all temperatures (Fig. 5 *C*, lanes *6–9*).

The secretion selectivity of *sec* mutant blocks was confirmed by examining the import and processing of a mitochondrial precursor protein, $F_1\beta$ 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 $F_1\beta$-specific antiserum showed only mature (*m*) $F_1\beta$ in the *SEC* and *sec* samples (Fig. 5 *D*, lanes *1–6*), while both mature and precursor (*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.

Posttranslational Translocation of ppαF In Vivo

Since intact, untranslocated secretory precursors accumulated in *sec61* cells, it was possible to test whether these polypeptides could complete translocation into the ER posttranslationally. *sec61* cells were pulse-labeled with $[^{35}S]SO_4^{2-}$ for 33 min at 37°C, and a chase incubation at 30°C was initiated by transferring cells to fresh 30°C medium containing excess unlabeled ammonium sulfate, methionine, and cysteine. Aliquots of the reaction were removed at various time points during the chase, and separated into cell pellet and culture medium fractions. All fractions were assayed for their con-

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

Cross	Tetrad type	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*, 1 *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 *SEC62sec63*, 1 *sec62sec63*‡

* Inviability because of spore germination at 24°C.
‡ Viable mutants obtained by spore germination at 17°C.

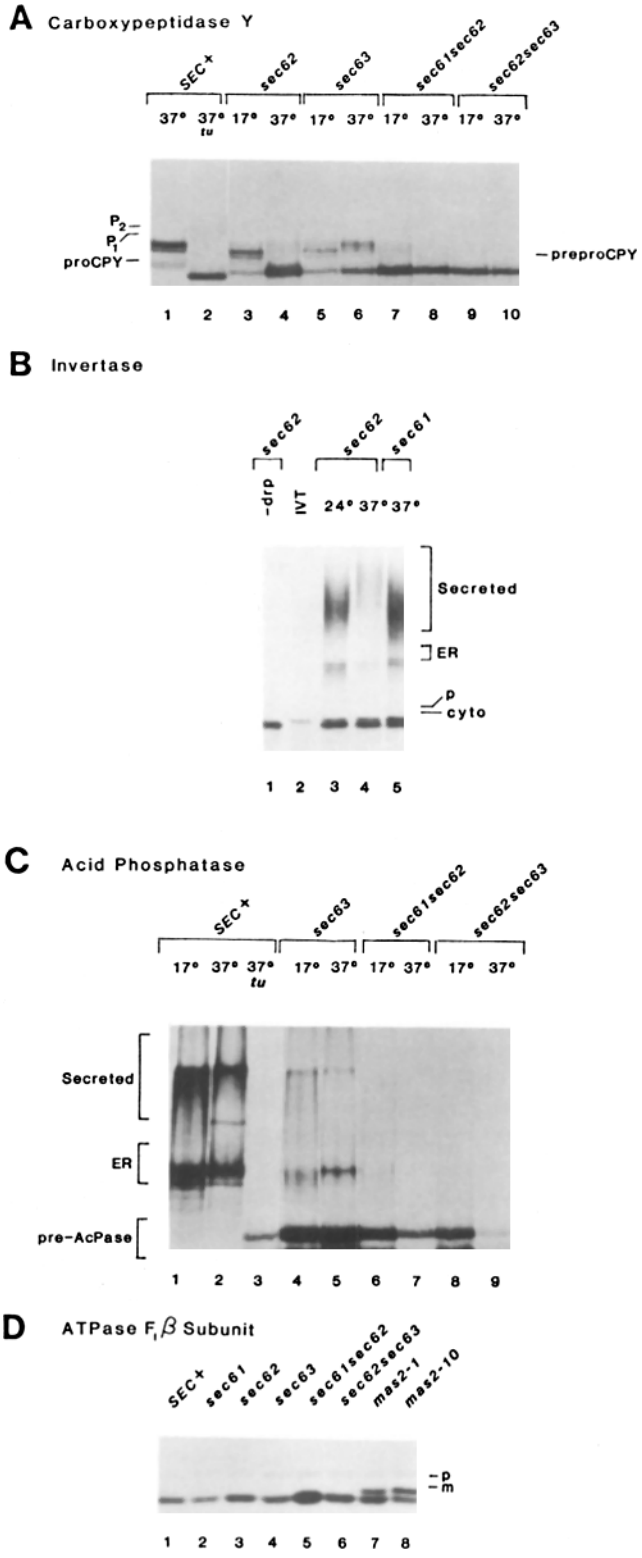

Figure 5. Unglycosylated secretory and vacuolar precursors accumulate in *sec* mutants. (*A*, *B*, and *C*) Stationary phase cells were inoculated into low-sulfate minimal medium and grown overnight at 17°C (*A* and *C*) or 24°C (*B*). Cells were radiolabeled in sulfate-free minimal medium with $[^{35}S]SO_4^{2-}$ at a concentration of 250–300 μCi/OD_{600} for 30 min at the indicated temperature. N-linked core glycosylation of CPY (*A*) and AcPase (*C*) was blocked by addition of 10 μg/ml tunicamycin (*tu*) to the culture medium 15 min before and during radiolabeling. After quenching radiolabel incorporation by addition of ice-cold NaN_3 to 10 mM, extracts were prepared by glass-bead lysis, and immunoprecipitated with the appropriate antiserum. Immunoprecipitates were analyzed by SDS-PAGE on 7.5% polyacrylamide gels. (*A*) *SEC* (JRM156), *sec62* (RDM43-9C), *sec63* (JRM151), *sec61sec62* (RDM52-7C), and *sec62sec63* (JRM164) cells were labeled for 30 min at 17°C (lanes *3*, *5*, *7*, and *8*) or 30 min at 37°C after a 2-h preincubation at 37°C (lanes *1*, *2*, *4*, *6*, *8*, and *10*). Each lane contains anti-CPY cross-reacting material immunoprecipitated from 0.3 OD_{600} U of cells. *preproCPY*, accumulated CPY precursor; *p1*, core-glycosylated proCPY; *p2*, Golgi-modified proCPY; *proCPY*, signal peptide processed form of unglycosylated CPY precursor. (*B*) Before radiolabeling, cultures of *sec61* (RDM15-5B) and *sec62* (RDM43-9C) cells were derepressed for invertase expression by transferring the cells into low-sulfate minimal medium containing 0.1% glucose at 24°C or 37°C for 30 min. *sec62* cells were radiolabeled for 30 min at 24°C (lane *3*) or 30 min at 37°C after a 60-min preincubation at 37°C (lane *4*). A companion culture of *sec62* cells were radiolabeled under repressing conditions (5% glucose) for 30 min at 24°C (lane *1*, *−drp*). Derepressed *sec61* cells were shifted to 37°C for 60 min before radiolabeling for 30 min at 37°C (lane *5*). Secretory invertase precursor produced by cell-free translation of in vitro transcribed *SUC2* mRNA (Rothblatt et al., 1987) in wheat germ lysate was included as a molecular mass standard (lane *3*, *IVT*). Lanes *1*, *3*, *4*, and *5* contain invertase immunoprecipitated from 0.5 OD_{600} U of cells. *cyto*, cytoplasmic invertase; *p*, preinvertase; *ER*, core-glycosylated secretory invertase; *secreted*, outer chain-glycosylated periplasmic invertase. (*C*) Expression of extracellular acid phosphatase was derepressed as described in Materials and Methods. Cells were radiolabeled for 30 min at 17°C (lanes *1*, *4*, *6*, and *8*) or 30 min at 37°C following a 2 h preincubation at 37°C (lanes *2*, *3*, *5*, *7*, and *9*). Each lane contains AcPase immunoprecipitated from 0.5 OD_{600} U of cells. preAcPase, unglycosylated precursor forms of acid phosphatase; *ER*, core-glycosylated AcPase; *secreted*, outer chain-glycosylated periplasmic AcPase. (*D*) Wild-type and mutant strains were grown overnight at 17°C and shifted to 37°C for 2 h. Extracts were prepared from unlabeled cells by glass-bead lysis and aliquots (equivalent to 0.5 OD_{600} U of cells) of the cell extracts were fractionated on a 7.5% SDS polyacrylamide gel. Following transfer to a nitrocellulose membrane, the forms of mitochondrial ATPase $F_1\beta$ subunit were identified by probing the immunoblot with anti-F1β subunit serum and [^{125}]protein A. *p*, precursor form of ATPase F1β subunit; *m*, protease-processed mature form.

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-

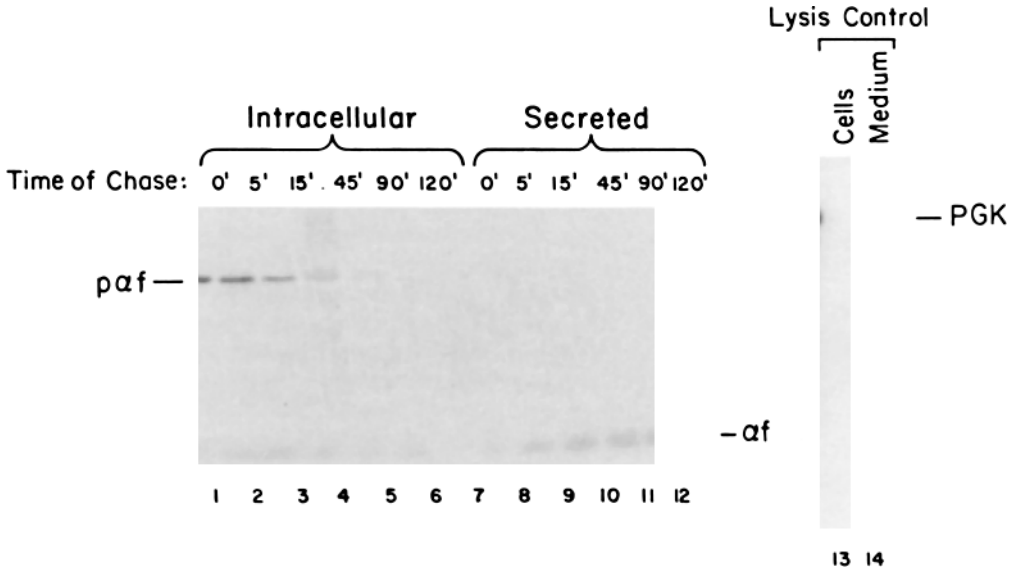

Figure 6. ppαF can be post-translationally translocated across the ER membrane in vivo. *sec6l* (RDM15-5B) cells were pulse-labeled with [^{35}S]SO_4^{2-} for 33 min at 37°C, and then chased for up to 120 min in the presence of excess unlabeled cysteine, methionine, and ammonium sulfate. At various time points during the chase period, aliquots of the culture were removed, separated into cell pellet (*Intracellular*, lanes *1–6*) and culture medium (*Secreted*, lanes *7–12*) fractions, and analyzed for their content of α-factor species by immunoprecipitation and SDS-PAGE on a 25% polyacrylamide gel. The distribution of phosphoglycerate kinase in cell pellet (*Cells*, lane *13*) and culture medium (*Medium*, lane *14*) fractions prepared after 120 min of chase allowed an estimate of the extent of cell lysis during the pulse-chase. prepro-α-factor, accumulated at the sec6l block; *αf*, mature α factor peptide; *PGK*, phosphoglycerate kinase.

tein synthesis inhibitor cycloheximide was present during the chase incubation. Therefore, secretion of accumulated ppαF did not require full activity of the defective *sec6l* 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, *SEC6l*, *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 *ptl1* 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* (*npl1*; 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., 1988*a*). 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, *sec6l*, *sec62*, and *sec63* appear to block protein translocation at a similar point in the pathway. All three accumulate unglycosylated precursor forms of four 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 ppαF 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 ppαF radiolabeled in a pulse of *sec6l* 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 *sec6l* 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 ppαF translocation using fractions prepared from mutant cells. *sec63* membranes display thermolabile translocation activity with ppαF 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 NH_2 terminus (Perlman and Halvorson, 1983; von Heijne, 1985); invertase lacks this feature. In addition, the invertase signal is considerably more hydrophobic (16 hydrophobic residues of a total 19) than the signals of preAcPase (9:14 after the lys residue), ppαF (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.

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