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Structural and Functional Characterization of Sec66p, a New Subunit of the Polypeptide Translocation Apparatus in the Yeast Endoplasmic Reticulum

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SEC66 encodes the 31.5-kDa glycoprotein of the Sec63p complex, an integral endoplasmic reticulum membrane protein complex required for translocation of presecretory proteins in *Saccharomyces cerevisiae*. DNA sequence analysis of *SEC66* predicts a 23-kDa protein with no obvious NH2-terminal signal sequence but with one domain of sufficient length and hydrophobicity to span a lipid bilayer. Antibodies directed against a recombinant form of Sec66p were used to confirm the membrane location of Sec66p and that Sec66p is a glycoprotein of 31.5 kDa. A null mutation in *SEC66* renders yeast cells temperature sensitive for growth. *sec66* cells accumulate some secretory precursors at a permissive temperature and a variety of precursors at the restrictive temperature. *sec66* cells show defects in Sec63p complex formation. Because *sec66* cells affect the translocation of some, but not all secretory precursor polypeptides, the role of Sec66p may be to interact with the signal peptide of presecretory proteins.

INTRODUCTION

The targeting and translocation of presecretory proteins across the endoplasmic reticulum (ER) membrane requires cytosolic, ER lumenal, and integral membrane proteins (Sanders and Schekman, 1992). In the yeast *Saccharomyces cerevisiae*, the cytosolic components involved in translocation consist of at least two kinds of molecular chaperones and the yeast signal recognition particle (SRP). Cytosolic Hsp70s, the products of the *SSA* gene family, are required for the posttranslational translocation of prepro-α-factor (ppαf) both in vivo and in vitro (Deshaies and Schekman, 1987; Chirico *et al.*, 1988). Ydj1p, a cytosolic DnaJ homologue, is also required for the efficient translocation of ppαf in vivo (Caplan *et al.*, 1992). Strains deficient for the 54-kDa and the 19-kDa homologues of mammalian SRP show strong defects in preprotein translocation (Hann *et al.*, 1989; Hann and Walter, 1991; Stirling and Hewitt, 1992). The ER luminal Hsp70 homologue BiP, the product of the *KAR2* gene, is also required for translocation (Rose *et al.*, 1989). Strains harboring temperature-sensitive mutations in *KAR2* have been isolated and show pronounced translocation defects at the nonpermissive temperature (Vogel *et al.*, 1990). Microsomes prepared from *kar2* strains are temperature sensitive for translocation in vitro, and luminal BiP is required for translocation into reconstituted proteoliposomes (Sanders *et al.*, 1992; Brodsky *et al.*, 1993).

Three ER-localized integral membrane proteins required for translocation have been characterized. Temperature-sensitive mutations in *SEC61*, *SEC62*, and *SEC63* were isolated based on their failure to localize a signal peptide–bearing cytoplasmic enzyme chimera to the lumen of the ER (Deshaies and Schekman, 1987; Rothblatt *et al.*, 1989). Phenotypic analysis showed that these mutations interrupt the translocation of a number of normal presecretory proteins in vivo and in vitro (Deshaies and Schekman, 1989; Rothblatt *et al.*, 1989). Sec61p, a 54-kDa hydrophobic protein predicted to span the ER membrane 7–9 times (Stirling *et al.*, 1992), is intimately associated with preproteins as they are being translocated across the membrane. In vitro translocation intermediates can be crosslinked to Sec61p in an ATP dependent fashion. Mutations in Sec62p or Sec63p inhibited this interaction, suggesting that these proteins act upstream of Sec61p in the process of translocation (Müsch *et al.*, 1992; Sanders *et al.*, 1992). Sec62p is a

30-kDa protein with two membrane-spanning domains oriented such that the amino- and carboxyl-termini are exposed to the cytosol (Deshaies *et al.*, 1991). Sec63p is a 73-kDa protein that spans the bilayer three times (Feldheim *et al.*, 1992). An ER luminal domain of Sec63p is 42% identical to *Escherichia coli* DnaJ (Sadler *et al.*, 1989). Kar2p is 50% identical to the *E. coli* hsp70 homologue DnaK (Normington *et al.*, 1989; Rose *et al.*, 1989). Because DnaK and DnaJ interact to promote phage lambda DNA replication (Yamamoto *et al.*, 1987), we have postulated that the DnaJ domain of Sec63p may be required to target BiP to the translocation apparatus (Feldheim *et al.*, 1992).

Sec61p, Sec62p, and Sec63p form a multisubunit protein complex together with a 31.5-kDa glycoprotein and a 23-kDa protein (Deshaies *et al.*, 1991). Antibodies directed against Sec62p coprecipitates Sec63p, Sec61p, a 23-kDa protein, and a 31.5-kDa glycoprotein from detergent solubilized fractions of radiolabeled cells. Antibodies directed against Sec63p coprecipitates these same proteins (Feldheim *et al.*, 1992).

We have purified the Sec63p complex in order to characterize the 23- and 31.5-kDa proteins. This report communicates the molecular characterization of the 31.5-kDa glycoprotein, now called Sec66p, of the Sec63p complex. We find that Sec66p is an integral membrane glycoprotein that is required at 37°C for the translocation of a variety of secretory proteins.

MATERIALS AND METHODS

Strains, Materials, Plasmids, and General Methods

The following strains were used in this study: YPH501 (*ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ63/trp1Δ63 leu2Δ1/leu2Δ1*), RSY151 (*leu2-3, -112 ura3-52 pep4-3 sec63-1 MATα*), YPH500 (*ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 MATα*), RSY925 (*ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ63/trp1Δ63 leu2Δ1/leu2Δ1 sec66::LEU2/SEC66*), RSY926 (*ura3-52 lys2-801 ade2-101 his3Δ 200 leu2Δ1 trp1Δ63 sec66::LEU2 MATα*), RSY607 (*ura3-52 leu2-3, 112 pep4::URA3 MATα*), RSY587 (*ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 sec63::HIS3 MATα*) containing the *SEC63-c-myc* plasmid pDF41. *E. coli* DH5α-harboring plasmid pUV5-G1S was used to isolate lytic β1,3 glucanase (Shen *et al.*, 1991). Yeast cells were grown in rich or minimal medium as described previously (Feldheim *et al.*, 1992). Plasmid pGex2T (Pharmacia, Uppsala, Sweden) was used to generate Sec66p-GST fusion proteins for the production of antiserum (see below). Plasmid pDF63 contains a 0.9-kilobase (kb) *Eco*RI fragment containing *SEC66* inserted into the pBluescript KS (Stratagene, LaJolla, CA) *Eco*RI site. The gene disruption plasmid pDF70 was constructed as follows: A 400-base pairs (bp) *Mun*I fragment was removed from pDF63 which was then converted to a blunt end linear molecule by treatment with Klenow polymerase and dNTPs. A 2-kb *Xba-Hin*dIII *LEU2* fragment of pJJ250 (Jones and Prakash, 1990) was treated with Klenow polymerase and dNTPs, and the product was ligated to the cleaved form of pDF63.

Protein Purification, Protein Fragmentation, and Peptide Sequence Analysis

Purification of the Sec63p complex: RSY587 was grown to midlog phase (between 2–10 OD_{600}/ml) in YPD media (2% bactopeptone, 1% Yeast extract, 2% glucose, Difco laboratories, Detroit, MI). Cells were harvested at 3000 × *g* in a GSA rotor and washed one time in H_2O. Cells were resuspended to 100 OD_{600}/ml in lysis buffer (200 mM mannitol, 100 mM NaCl, 25 mM sodium phosphate, pH 7.4, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μM leupeptin, and 10 μM pepstatin) and lysed by agitation with glass beads at 4°C. The homogenate was centrifuged for 5 min at 370 × *g* to remove unbroken cells followed by 15 min at 12 000 × *g*. The medium speed pellet fraction was resuspended to 50 OD_{600} cell equivalent/ml in lysis buffer containing 20% glycerol. The membranes were solubilized by the addition of Triton X-100 (Tx-100, Sigma, St. Louis, MO) to a final concentration of 1%, rotated for 20 min at 4°C, and clarified at 100 000 ×*g* for 30 min to remove insoluble material. The solubilized material was passed 10 times through a 5-ml human *c-myc* monoclonal antibody column (Evan *et al.*, 1985), made by coupling 10 mg purified human *c-myc* antibody to 5 ml swelled Avid gel resin according to the manufacturer's instructions (BioProbe International, Tustin, CA). The column was washed with 10 column volumes of glycerol lysis buffer plus 0.2% Tx-100 (buffer D), 10 column volumes of buffer D plus 250 mM NaCl and eluted with 0.2 M glycine, pH 2, 0.2% Tx-100. Peak fractions, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining were concentrated by trichloroacetic acid precipitation, washed with cold acetone, resolubilized in 8 M urea, 100 mM $NH_4(CO_3)_2$, reduced with 10 mM β-mercaptoethanol, and separated by reverse phase high pressure liquid chromatography (HPLC) on an RP-300 C8 2.1 × 30 mm column (Applied Biosystems, Foster City, CA). Sec66p-containing fractions were proteolysed with 100 ng LysC in 100 mM $NH_4(CO_3)_2$ (Boehringer Mannheim, Mannheim, Germany) and purified by reverse phase HPLC on a 300 Å C18 column (Vydac, Hesperia, CA). Peptides were sequenced by standard chemistry using fast cycles on a protein peptide 477A sequencer (2.1 × 150 mm, Applied Biosystems). The following peptides were obtained: AALLNRGAESVRRSLK, FSNCGTFFETEEP, KISEQPSIFDEN, and ELAPQINLLYK.

The *SEC66* gene was cloned by screening a YEp13-based library (10-kb average insert length) from T. Yoshihisa (University of California, Berkeley, CA) using a degenerate oligonucleotide (5′ TTYTTYGARACNGARGARCC 3′ corresponding to the peptide NCGTFFETEEP) that was end labeled by reaction with polynucleotide kinase and $^{32}P\gamma ATP$. Colony hybridization (Ausubel *et al.*, 1987) was performed at 50°C in 6× SSC, 1× Denhardt's solution, 100 μg/ml yeast tRNA, and 0.05% sodium pyrophosphate for 16 h. Filters were washed three times for 5 min at 25°C followed by three times for 5 min at 50°C in 6× SSC, 0.05% sodium pyrophosphate. Filters were exposed to film at −70°C for 24–48 h, and positive colonies were rescreened until purified. Plasmids were grouped into classes by restriction digestion and mapping followed by hybridization. Plasmids that also hybridized with the oligonucleotide 5′ GCTGCTTTGTTGAATAGAGGTGCTGAATC 3′ corresponding to the peptide AALLNRGAESV under the same hybridization conditions were studied further.

DNA Sequencing

To determine the DNA sequence of *SEC66*, pDF63 was sequenced with standard methods using Sequenase (United States Biochemical, Cleveland, OH) following the manufacturer's instructions.

Gene Disruptions

A null allele of *SEC66* was generated in vitro by digesting pDF70 with *Eco*RI, giving a plasmid DNA fragment terminating with ~100 bp of 5′*SEC66* sequence and 300 bp 3′ of the *LEU2* gene. The fragment was gel purified using Geneclean (Bio 101, LaJolla, CA) according to the manufacturer's instructions. The diploid yeast strain YPH501 was transformed with the *Eco*RI fragment using the lithium acetate method (Ausubel *et al.*, 1987), and transformants were selected on minimal medium lacking leucine. Transformants were induced to sporulate on acetate sporulation plates and dissected into tetrads. Genomic DNA

from the heterozygous diploid RSY925 and representative spores were isolated and used as template in polymerase chain reactions (PCR) using the primers 5′ CCGCCAAAAAACGTCAA 3′ and 5′ TGATAACGTCTAGAGAG 3′ (corresponding to nucleotides −1–16 and 689-672 of Figure 1). The PCR products were separated on 1% agarose gels. Genomic DNA from the parent diploid strain or from the Leu$^-$ spores gave rise to an expected 600-bp DNA fragment after amplification, whereas an additional 2.2-kb fragment was seen in the heterozygous diploid. Only the 2.2-kb fragment was observed when genomic DNA from Leu$^+$ spores was amplified.

Radiolabeling and Immunoprecipitation

Radiolabeling of wild-type and mutant cells followed by immunoprecipitation of denatured proteins from extracts was carried out as described (Stirling *et al.*, 1992). Antibodies raised against carboxypeptidase Y (Stevens *et al.*, 1982) were used at 1 μl serum/OD_{600} cell equivalent, invertase (Schauer *et al.*, 1985) 2 μl serum/OD_{600} cell equivalent, α-factor (A. Eun, University of California, Berkeley, CA) 4 μl serum/OD_{600} cell equivalent, Sec66p 5 μl serum/OD_{600} cell equivalent, Kar2p (Jeff Brodsky, University of California, Berkeley, CA) 2 μl serum/OD_{600} cell equivalent. Tunicamycin (Sigma, St. Louis, MO) was added at a final concentration of 10 μg/ml 10 min before radiolabeling. Crosslinking of radiolabeled membranes and immunoprecipitation of the Sec63p complex has been described (Deshaies *et al.*, 1991).

Cell Fractionation and Immunoblotting

RSY607 was grown in YPD, and lysates were prepared as described (Feldheim *et al.*, 1992). To determine the nature of the association of Sec66p with the membrane, we lysed 200 OD_{600} cells using glass beads in 2 ml of Buffer G (0.1 M sorbitol, 50 mM KOAc, 2 mM EDTA, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4, 1 mM dithiothreitol [DTT], 1 mM PMSF). Homogenates were cleared at 370 × *g* for 4 min in a HB4 rotor (Beckman Instruments, Palo Alto, CA), and the low speed supernatant fraction was diluted with 1/10 volume of one of the following: 5 M NaCl, 8 M urea, 1 M Na_2CO_3, pH 11, or 10% Tx-100. Mixtures were incubated for 20 min on ice and centrifuged for 30 min at 100 000 × *g* in a TLA100.3 rotor (Beckman Instruments). Pellet fractions were resuspended to an equal volume in Buffer G, and samples were prepared for SDS-PAGE. Aliquots (0.5 OD_{600} cell equivalents) were applied to each lane. Transfer of proteins from SDS-PAGE to nitrocellulose was performed as described previously. Filters were blocked, and all antibody incubations and washes were conducted with 2% nonfat dry milk in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (25 mM Tris, pH 7.4, 150 mM NaCl), 0.1% Tween 20. Detection of filter-bound antibodies was done by the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham, Arlington Heights, IL). Antibodies to Sec63p and Sec23p are described elsewhere (Hicke and Schekman, 1989; Feldheim *et al.*, 1992).

Production of Anti-Sec66p Antiserum

Polyclonal antiserum recognizing Sec66p epitopes was obtained by immunizing rabbits with glutathione-S-transferase (GST)-Sec66p hybrid proteins expressed in *E. coli*. GST fusions were constructed by inserting the 350-bp *Mun* I-*Eco* RI fragment of pDF67 into plasmid pGEX2T creating plasmid pDF73. To purify the Sec66p-GST fusion protein, 50 ml of *E. coli* DH5α containing pDF67 was grown to stationary phase in Luria-Bertaini (LB) broth plus 100 μg/ml ampicillin and diluted 1/100 into 1 l of LB at 37°C. After 1 h, 0.5 mM isopropylthiogalactopyranoside was added to induce expression of the fusion protein, and cells were grown 3–7 h at 37°C. Cultures were centrifuged at 370 × *g* for 5 min, and the pellets were washed once in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4), resuspended in 25 ml of PBS, and lysed by sonication. Tx-100 was added to a final concentration of 1%, and the lysate was rotated for 20 min at 4°C and cleared at 12 000 × *g* for 10 min in a SS34 rotor (Beckman). The supernatant fraction was added to 1 ml swelled Glutathione agarose (Sigma) and rotated at 4°C for 1 h. The affinity matrix was washed with 50 column volumes of PBS plus 0.2% Tx-100 and eluted with PBS, 0.2% Tx-100, and 5 mM glutathione. Peak protein fractions, as judged by A_{280}, were pooled and used for injections. Rabbits were injected subcutaneously with 200 μg of protein in an emulsion containing Freund's complete (first injection) and incomplete (subsequent injections) adjuvant.

RESULTS

Cloning of SEC66

To isolate large quantities of the Sec63p complex for protein microsequence analysis, we produced a strain in which a chromosomal Δ*sec63::HIS3* was complemented by Sec63p epitope tagged with a *c-myc* epitope (Feldheim *et al.*, 1992). Membranes isolated from this strain were solubilized in Tx-100 and chromatographed on a human monoclonal *c-myc* antibody column (see MATERIALS AND METHODS). The Sec63p complex was eluted with 0.2 M glycine, pH 2, and neutralized with 1 M Tris·HCl to pH 7.5. The protein profile as judged by SDS-PAGE was similar to the complex described previously (Deshaies *et al.*, 1991), except that no Sec61p was detected in the eluate. The 31.5-kDa protein was separated from the rest of the Sec63p complex by reverse phase HPLC. A portion of the 31.5-kDa protein was subjected to proteolysis with *Lys-C*, and the proteolytic fragments were separated by reverse phase HPLC. One such peptide of amino acid sequence FSNCGTFFETEEP was used to design a degenerate 32-mer oligonucleotide corresponding to all codon possibilities of NCGTFFETEEP (see MATERIALS AND METHODS). This oligonucleotide hybridized predominately to one band on a total yeast genomic Southern blot. A yeast multicopy chromosomal library propagated in *E. coli* was screened by colony hybridization, and positive clones were colony purified. Plasmids from positive clones were isolated and further characterized by restriction mapping. Five independent clones had identical 3.3-kb *Eco*RI fragments that hybridized with the original oligonucleotide and with an oligonucleotide derived from another peptide generated from the microsequencing (see MATERIALS AND METHODS).

Nucleotide Sequence of SEC66

The DNA sequence surrounding the hybridizing fragment was determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The nucleotide sequence containing 974 bp surrounding the hybridized fragment is shown in Figure 1. A single open reading frame (ORF) of 585 bp was found between nucleotides 195–739. Potential transcription initiation signals (TATA) boxes are noted at positions 57 and 80. These fall within the usual range of −20 to −200 for TATA boxes in yeast (Struhl, 1985). Putative transcriptional termination signals are found at position 793–820. The ORF is the gene encoding gp31.5 because all three pep-

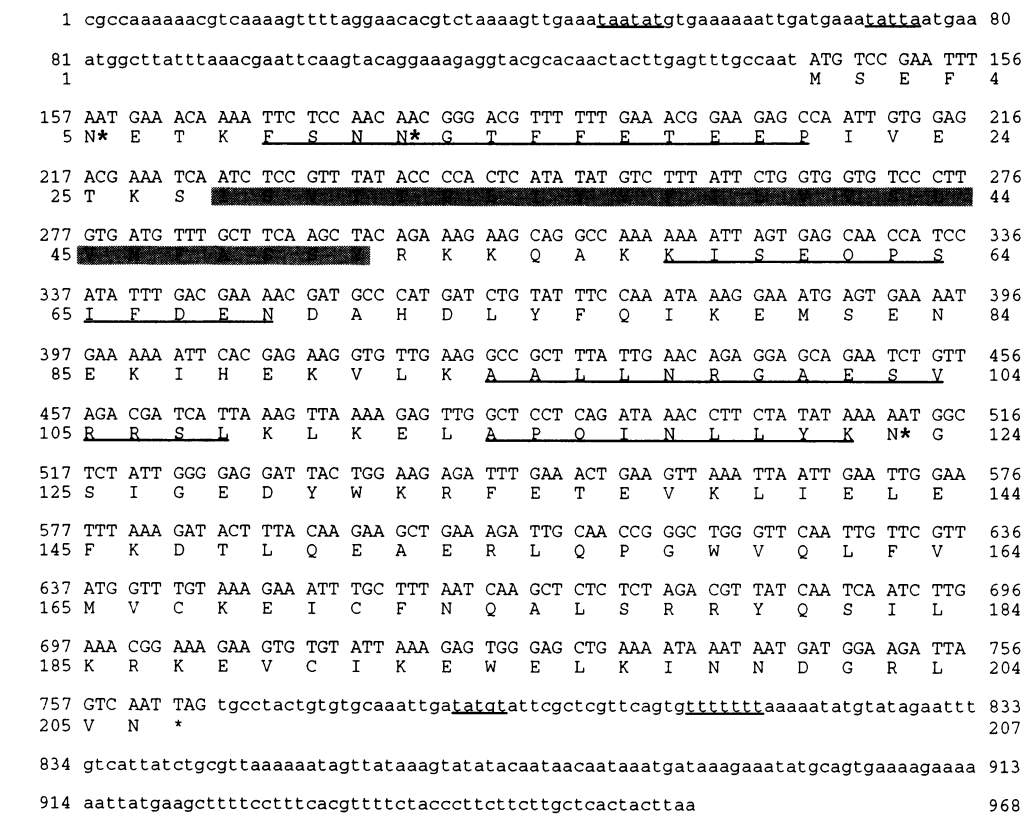

Figure 1. Nucleotide sequence of the *SEC66* gene and the predicted amino acid sequence of Sec66p. Sequences potentially involved in the initiation (TATA) boxes or termination of transcription are underlined. Peptides generated from the microsequencing of purified Sec66p are underlined. The three potential N-linked glycosylation acceptor sites are noted with asterisks and the potential transmembrane domain is shaded. The *sec66* disruption allele replaces bp 215-634 with the LEU2 gene of *S. cerevisiae*. The sequence accession number is U00797.

tides generated from the microsequencing of gp31.5 are contained within this reading frame (underlined amino acids in Figure 1). Sequence analysis of the ORF predicts a polypeptide consisting of 195 amino acids, corresponding to a molecular weight of 22.7 kD, and a single stretch of amino acids capable of spanning a lipid bilayer (amino acids 28–50) (Kyte and Doolittle, 1982). A cluster of positively charged amino acids (4/6 lysine) C-terminal to the hydrophobic domain may serve to terminate insertion of the transmembrane domain (von Heijne, 1990). The predicted *SEC66* amino acid sequence contains three N-linked glycosylation consensus sites (NXS/T), two upstream and one downstream of the predicted transmembrane domain. Searches of databases revealed no significant homologies to other proteins. There is no predicted N-terminal signal peptide in Sec66p suggesting that the first transmembrane domain acts as an insertion signal which is a common feature of the assembly of membrane proteins (von Heijne, 1990).

Sec66p Topology Based on Glycosylation

To characterize the *SEC66* gene product further, we raised polyclonal antibodies to Sec66p fused to GST. The hybrid gene was transformed into *E. coli* and induced for expression. Hybrid proteins were purified by glutathione agarose affinity chromatography, and the antigen was injected into rabbits. The resultant antiserum was affinity purified by binding to and elution from an immobilized form of the original Sec66p-GST hybrid protein. Deshaies *et al.* (1992) showed that Sec66p is an endoglycosidase H sensitive glycoprotein. These results were confirmed and extended using wild-type or *sec66* strains (see below) radiolabeled with ^{35}S Translabel (ICN, Costa Mesa, CA) and immunoprecipitated with anti-Sec66p antibody in the absence (Figure 2A, lane 1) or in the presence of tunicamycin (Figure 2B, lane 2). The immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. Immunoprecipitates from wild-type cells contained two proteins of apparent molecular weight 31 kDa and 28 kDa that were absent in the *sec66* null strain (Figure 2A, lanes 1 and 3). Only a single 25-kDa species was observed in cells treated with tunicamycin (Figure 2A, lane 2). Pulse chase radiolabeling showed that the 28-kDa protein diminished and the 31-kDa species increased in the course of a 45-min chase period (Figure 2B, lane 4). Treatment of the immunoprecipitates at time zero with endogly-

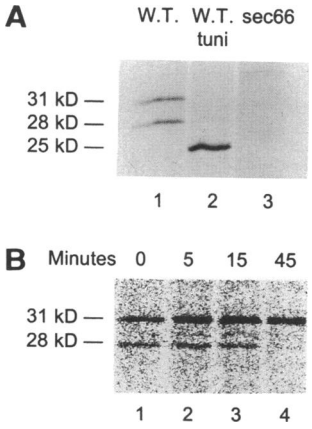

Figure 2. Sec66p is a glycoprotein that appears to be modified at two sites. (A) Pulse: wild-type cells (lanes 1), wild-type cells treated with 10 μg/ml tunicamycin (lane 2), or *sec66* cells (lane 3) were pulse labeled at 30°C with ^{35}S-Translabel for 20 min, and glass bead extracts were immunoprecipitated with anti-Sec66p antibodies. Immunoprecipitates were subjected to SDS-PAGE and fluorography. (B) Chase: wild-type cells were pulse labeled at 30°C for 20 min and incubation was continued in the presence of cold methionine and cysteine (1 mg/ml) for the indicated times. Glass bead extracts were immunoprecipitated with anti-Sec66p antiserum. The precipitates were subjected to SDS-PAGE and fluorography.

cosidase H collapsed both bands to a common 25-kDa species. Thus, Sec66p appears to be glycosylated at two sites in the protein. As there are two glycosylation sites N-terminal to the predicted transmembrane domain and only one C-terminal, we propose that Sec66p is a type 3 membrane protein (von Heijne, 1990) with the amino terminus localized to the ER lumen.

Sec66p Is an Integral Membrane Protein

To test if Sec66p behaves as an integral membrane protein, as predicted from its amino acid sequence, we prepared membrane fractions from wild-type yeast cells and extracted them under conditions that either solubilize peripheral membrane proteins or solubilize all membrane proteins. The experiment in Figure 3 demonstrates that >90% of the Sec66p was not removed from membranes treated with 0.5 M NaCl, 0.1 M Na_2Co_3 (pH 11), or 0.8 M urea but was released into the supernatant fraction by treatment with 1% Tx-100. This behavior is consistent with the prediction, based on sequence analysis, that Sec66p is an integral protein. As controls, the integral ER membrane protein Sec63p fractionated exclusively as a membrane protein, whereas the peripheral membrane protein, Sec23p, was partially solubilized under all conditions (Hicke and Schekman, 1989; Feldheim *et al.*, 1992).

Sec66p Is Required for Viability at High Temperatures

To determine the role of Sec66p in vegetatively growing cells, we explored the effects of deletion of the chromosomal copy of *SEC66*. A 416-bp *Mun* I fragment between bp 215-634 of *SEC66* was replaced with the *LEU2* gene of *Saccharomyces cerevisiae*. This deletion removes 139 amino acids of Sec66p including all of the predicted transmembrane domain. An *Eco*RI fragment containing 125 bp 5′ and 200 bp 3′ of surrounding *SEC66* sequence was used to transform the yeast diploid strain YPH501 to leucine prototrophy. PCR analysis, using oligonucleotide primers designed to flank the *Mun* I fragment, was performed using chromosomal DNA as a template to confirm integration of the disruption construct at the *SEC66* locus (see MATERIALS AND METHODS). Leucine prototrophic heterozygous diploids were sporulated, and asci were dissected into tetrads and germinated at 25°C on rich media. Under these conditions all four spores were viable. Leucine prototrophy segregated 2 Leu^+:2 Leu^- and PCR analysis as described above using genomic DNA from spores from a representative tetrad confirmed that the disruption construct segregated 2:2 as well. At 37°C, spores containing the *sec66::LEU2* gene were inviable. The ability of the *sec66* null mutant to grow at 25°C is in contrast to null mutations in the other subunits of the Sec63p complex.

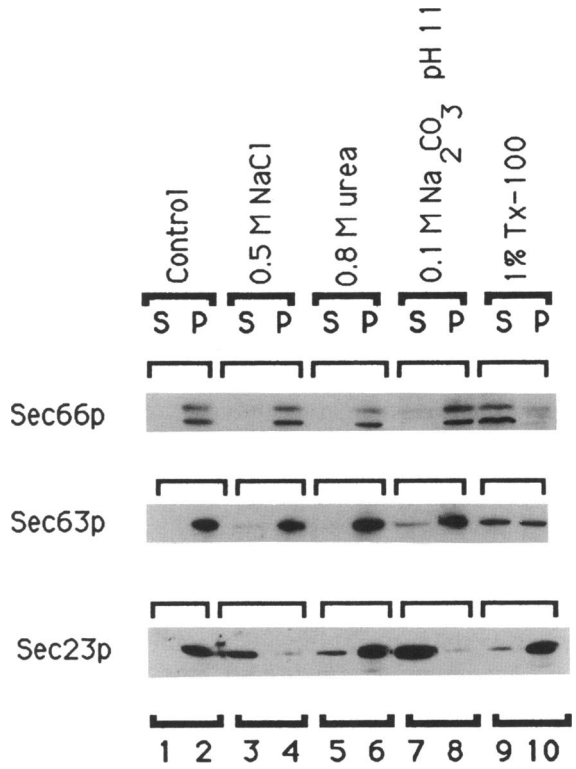

Figure 3. Evidence that Sec66p is an integral membrane protein. Membrane fractions were prepared and treated with either 0.5 M NaCl, 0.1 M Na_2CO_3 (pH 11), 0.8 M urea, or 1% Tx-100. After incubation on ice for 20 min, all samples were separated into supernatant (S) or pellet (P) fractions by centrifugation at 100 000 × *g*, subjected to SDS-PAGE, and immunoblotted with anti-Sec66p, anti-Sec63p, or anti-Sec23p antiserum.

SEC61, *SEC62*, and *SEC63* are essential for cell viability at all temperatures tested (Deshaies and Schekman, 1989; Rothblatt *et al.*, 1989; Sadler *et al.*, 1989; Stirling *et al.*, 1992).

Strains Deleted for SEC66 Accumulate a Variety of Secretory Precursors In Vivo

To determine the role of Sec66p in translocation, we tested whether the *sec66* null mutant strain accumulates untranslocated precursor proteins in vivo. Biogenesis of the ER luminal protein, Kar2p, the periplasmic enzyme, invertase, and the mating phermone, ppαf, were examined by immunoprecipitation of radiolabeled precursors from wild-type, *sec66*, and for comparison, *sec63*-1 mutant cells.

Kar2p is translated with a signal peptide that is cleaved upon translocation into the ER lumen. Only mature Kar2p was seen when immunoprecipitation with anti-Kar2p antibodies was performed on lysates of wild-type cells that were grown at 25°C or shifted to 37°C 1 h before labeling (Figure 2A, lanes 1 and 2). In contrast, the *sec66* mutant showed a strong temperature dependent accumulation of pre-Kar2p when cells were preincubated at 37°C for 1 h (Figure 4A, lanes 3 and 4). This defect in Kar2p maturation was also seen in *sec63* mutant cells at permissive and restrictive temperatures (Figure 4A, lanes 5 and 6).

The periplasmic enzyme invertase is translated as a preprotein that undergoes signal peptide cleavage and core glycosylation in the ER lumen. Upon transport to the Golgi apparatus, the core oligosaccharides become heterogeneously outer chain glycosylated. To assay invertase maturation, cells were derepressed for invertase expression in 0.1% glucose for 3 h. Cultures were divided into two portions and either kept at 25°C or shifted to 37°C for 1 h and pulse labeled for 30 min. Aliquots of cell lysates were immunoprecipitated with anti-invertase antiserum. Wild-type cells displayed predominately the Golgi form of invertase at 25 or 37°C (Figure 4B, lanes 1 and 2). When the glycosylation inhibitor tunicamycin was added before pulse labeling, a signal-processed, unglycosylated form of invertase was produced (Figure 4B, lane 3). The *sec66* strain showed a temperature-dependent accumulation of a novel form of invertase with a mobility consistent with that expected for preinvertase (Figure 4B, lanes 4 and 5). This result was in contrast to *sec63* (Figure 4B, lanes 6 and 7) and *sec62* temperature-sensitive strains that show no significant block of invertase at their respective nonpermissive temperatures (Rothblatt *et al.*, 1989).

In contrast to the temperature-dependent accumulation of Kar2p and invertase in *sec66*, we found that untranslocated ppαf was accumulated in mutant cells at all temperatures (Figure 4C). During translocation into the ER, the pheromone preprotein has its signal peptide removed and becomes core glycosylated on three asparagine residues. Wild-type cells did not accumulate ppαf at 25 or 37°C (Figure 4C, lanes 1 and 2). The addition of tunicamycin before pulse labeling led to an accumulation of signal-cleaved pro-α-factor (pαf) (Figure 4C, lane 3). *sec66* accumulated ppαf when radiolabeled at 25°C and to a greater extent at 37°C

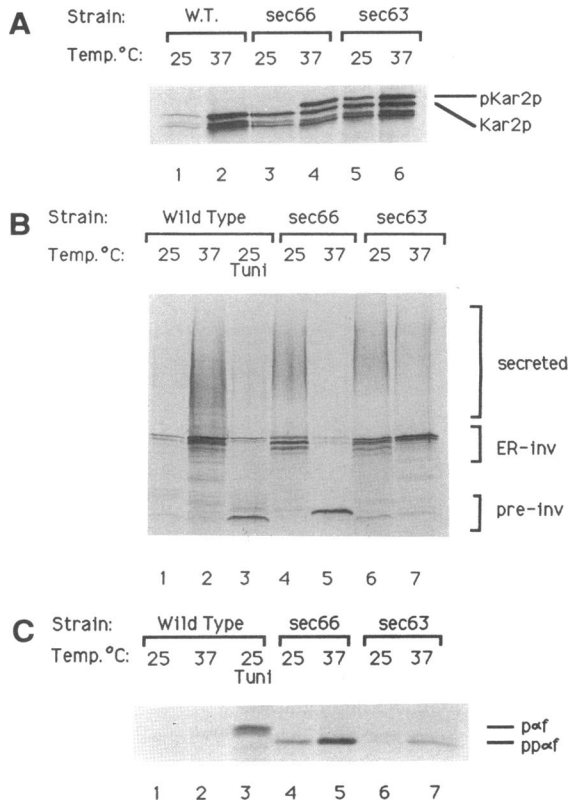

Figure 4. Unmodified secretory precursors accumulate in *sec66* cells. (A) Kar2p: wild-type (lanes 1 and 2), *sec66* (lanes 3 and 4), or *sec63* (lanes 5 and 6) cells were pulse labeled at 25°C (lanes 1, 3, and 5) or 37°C (lanes 2, 4, and 6) with ^{35}S Translabel for 20 min. Glass bead extracts were immunoprecipitated with anti-Kar2p antiserum. Immunoprecipitates were resolved on 7.5% SDS-PAGE and subjected to fluorography. *pKar2p*, precursor form of Kar2p. Lane 1 is underloaded. The bands of higher mobility are unrelated to Kar2p. (B) Invertase: wild-type (lanes 1–4), *sec66* (lanes 5 and 6), and *sec63* (lanes 7 and 8) cells were grown to OD_{600} of 0.5 in minimal medium containing 2% glucose and shifted to minimal medium containing 0.1% glucose for 3 h at 25°C. Cells were then grown at either 25°C (lanes 1, 3, 4, and 6) or 37°C (lanes 2, 5, and 7) for 1 h, and pulse labeled for 20 min with ^{35}S Translabel. Glass bead extracts were immunoprecipitated with anti-invertase serum and resolved on a 7.5% SDS-PAGE followed by fluorography. Tunicamycin (lane 3) was added at a concentration of 10 μg/ml 10 min before labeling. *pre-inv*, preinvertase; *ER-inv*, ER modified form of invertase; *secreted*, secreted and Golgi-modified form of invertase. (C) α-factor precursor: wild-type (lanes 1–3), *sec66* (lanes 4 and 5), and *sec63* (lanes 6 and 7) cells were pulse labeled at either 25 or 37°C for 20 min. Glass bead extracts were immunoprecipitated with anti-α-factor antiserum. Immunoprecipitates were resolved on 12.5% SDS-PAGE gels. Tunicamycin (lane 3) was added (10 μg/ml) 10 min before labeling.

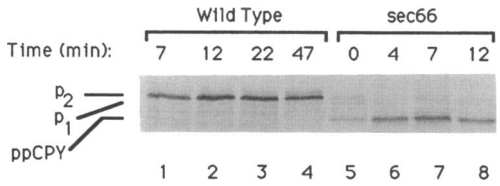

Figure 5. The *sec66* translocation defect is imposed immediately after temperature shift. Wild-type (lanes 1–4) or *sec66* (lanes 5–8) cells were grown to an OD_{600} cell equivalence of 0.25/ml at 25°C in minimal medium and shifted to 37°C. Aliquots were taken out at the times indicated and pulse labeled with ^{35}S Translabel for 2 min at 37°C. Ice-cold sodium azide was added to a final concentration of 10 mM, and samples were lysed by glass beads and immunoprecipitated with anti-CPY antiserum. Immunoprecipitates were resolved on 7.5% SDS-PAGE and fluorography. *ppCPY*, pre-proCPY; *p1*, ER modified form of CPY; *p2*, Golgi-modified form of CPY.

similar to the defect seen in *sec63* (Figure 4C, lanes 4–7). Taken together, these data show that Sec66p is required for the translocation of a variety of presecretory proteins.

sec66 Strains Accumulate Untranslocated Precursor Proteins Immediately After Temperature Shift

To determine how fast the block in translocation occurred after a temperature shift in *sec66* null mutant cells, we followed the biogenesis of the vacuolar protease CPY at various times after shifting the cells to 37°C. CPY is translated as a pre-pro-protein and is signal peptide processed upon translocation into the ER (Stevens *et al.*, 1982). In the ER, CPY is core glycosylated to the p1 form. Subsequent outer chain mannose residues are added in the Golgi complex, converting p1 CPY to the higher molecular weight p2 form. Upon reaching its final destination in the vacuole, p2 CPY is cleaved by a vacuolar protease to a smaller mature form that comigrates with unglycosylated prepro-CPY. The overall maturation of CPY takes about 6 min (Haslilik and Tanner, 1978). In a brief (2 min) pulse labeling, wild-type cells contain predominately the p1 and p2 forms of CPY. Wild-type (Figure 5, lanes 1–4) or *sec66* (Figure 5, lanes 5–8) strains were shifted to 37°C, and at various times cells were pulse labeled with ^{35}S Translabel for 2 min after which the labelings were terminated by the addition of 20 mM sodium azide. Lysed cells were immunoprecipitated with antibody against CPY, and the radioactive proteins were resolved by SDS-PAGE and visualized by fluorography. Figure 5 shows that as with ppα-factor, there was a partial translocation block of CPY at 25°C (Figure 5, lane 5). A complete translocation block was imposed in the *sec66* strain within 4 min after temperature shift (Figure 5, lane 6). Kar2p maturation was also affected in this time frame. The immediacy of the introduction of the translocation block suggests that *sec66* plays a direct role in translocation.

Defective Sec63p Complex Formation in the sec66 Strain

Sec66p can be crosslinked to four other proteins (Sec63p, Sec62p, Sec61p, and p23) that are precipitated from detergent solubilized membranes with antibody against Sec62p (Deshaies *et al.*, 1991) or Sec63p (Feldheim *et al.*, 1992). Sec61p is a minor component of this complex and not always seen under these conditions. To test whether the integrity of this complex is affected in the *sec66* null strain, we radiolabeled wild-type (Figure 6, lane 1) or *sec66* (Figure 6, lane 2) strains with ^{35}S Translabel for 30 min. Membranes from lysed cells were solubilized with Tx-100 and treated with the cleavable crosslinker dithio-*bis*-(succinimidylpropionate). Samples were quenched, treated with SDS, and then mixed with antibody directed against Sec63p. The immunoprecipitates were resolved by SDS-PAGE and subjected to fluorography. The immunoprecipitation from wild-type cells produced a protein profile consisting of Sec63p, a 46-kDa band, Sec62p, Sec66p, and p23 (Figure 6, lane 1). Sec63p immunoprecipitates from *sec66* cells did not contain Sec66p, as expected, but, in addition, p23 was also not recovered in the complex (Figure 6, lane 2). Although under steady state conditions we found approximately equal amounts of the 28- and 31.5-kDa forms of Sec66p (Figure 2A), only the 31.5-kDa form of Sec66p is assembled into the Sec63p complex, suggesting that N-linked glycosylation of the protein may be necessary for its biological activity. The 46-kDa protein recovered from the complex is similar in mobility to a species seen when radiolabeled membranes are immunoprecipitated with the anti-Sec62p antibodies directed against a LacZ-Sec62p fusion protein but not a protein A-Sec62p fusion protein (Deshaies *et al.*, 1991), and therefore, may or may not be part of the translocation machinery.

DISCUSSION

We have previously shown that three integral membrane proteins required for preprotein translocation, Sec63p, Sec62p, and Sec61p, exist in a multiprotein complex with two other proteins of 31.5 and 23 kDa. To gain a better understanding of the mechanism of

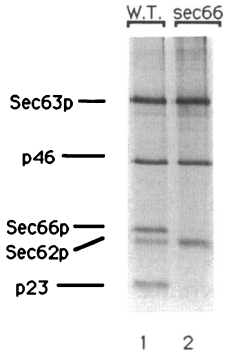

Figure 6. *sec66* cells have defects in Sec63p complex formation. ^{35}S-labeled membranes prepared from wild-type (lane 1) or *sec66* (lane 2) cells were solubilized with 1% Tx-100 and crosslinked with DSP. Samples were immunoprecipitated with anti-Sec63p affinity-purified antibodies. The crosslinks were cleaved in sample buffer containing 10 mM DTT, and the products resolved by SDS-PAGE followed by fluorography.

translocation, we have cloned and characterized the 31.5-kDa glycoprotein (now called Sec66p) of the Sec63p complex. The nucleotide sequence of *SEC66* predicts a 23-kDa protein capable of spanning a lipid bilayer one time. Cell fractionation experiments confirm that Sec66p behaves as an integral membrane protein. Antibodies raised against recombinant Sec66p hybrid proteins recognize two species of Sec66p in whole cell extracts. These species appear to contain one or two N-linked oligosaccharides.

The null mutant of *sec66* is viable at 30°C but inviable at 37°C, a temperature at which the *sec66* mutant accumulates a variety of precursor proteins. *sec66* cells are defective in Sec63p complex assembly, preventing a stable interaction of p23 with the Sec63p complex. This defect may be because of degredation of p23 or failure to recruit or retain p23 in proximity to Sec63p and Sec62p.

Unlike other proteins in the Sec63p complex (Sec63p, Sec62p, and Sec61p), Sec66p is not essential for yeast cell growth. At least two possibilities may be offered to explain this phenomenon: Sec66p function may be redundant at 25°C but not at high temperature, or Sec66p may not be involved in translocation directly but may be required for some important function of thermal stress regulation or membrane fluidity that affects translocation indirectly. Several arguments favor a direct role for Sec66p in translocation. First, Sec66p is physically associated with other proteins that are required for translocation. Second, Sec66p is absolutely required for the translocation of a some precursors at 37°C, and a subset of these precursors shows a partial defect at 25°C. Third, *sec66* accumulates untranslocated precursors within 4 min after transfer to 37°C. Moreover, membranes isolated from *sec66* and reconstituted into proteoliposomes (Brodsky *et al.*, 1993) fail to translocate ppα-factor. However, translocation can be restored when reconstitutions are supplemented with Sec63p complex purified from wild-type cells but not by Sec63p complex isolated from *sec66* cells (Jeff Brodsky, unpublished data). This defect is not because of the absence of the 23-kDa protein (p23) in the Sec63p complex isolated from *sec66*, because a null mutation in the gene corresponding to p23 has no affect on the translocation of most precursors (D. Feldheim, unpublished data). Thus, Sec66p function may be directly involved in the translocation process.

Genetic data suggest that Sec66p may serve a redundant function with Sec63p and Sec62p at low temperature. Double mutant combinations of *sec66* and *sec63* or *sec62* are inviable at 25°C, whereas all single mutants are viable at 25°C (D. Feldheim, unpublished data; T. Kurihara, personnel communication). Thus, although Sec66p is dispensable at 25°C and may only be essential when cells are stressed at 37°C, the additional presence of a crippled Sec62p or Sec63p renders the cell incapable of proper secretion even at 25°C. Such synthetic lethal combinations occur with mutations in proteins that are known to interact, such as α- and β-tubulins of yeast (Huffaker *et al.*, 1987). Because Sec66p physically interacts with Sec63p and Sec62p, we interpret the synthetic lethality to mean that these proteins are involved in the same process, namely translocation of preproteins into the ER.

How might Sec66p be involved in translocation? We propose that Sec66p is a subunit of a signal recognition complex that interacts with the signal peptide of a secretory precursor and transfers it to the translocation pore. Consistent with this, Sec63p and Sec62p are required upstream of the putative pore protein, Sec61p (Nunnari and Walter, 1992; Sanders and Schekman, 1992), and the Sec66p null mutant blocks only a subset of precursors at 25°C as do point mutations in Sec63p and Sec62p (Rothblatt *et al.*, 1989). This is conceptually similar to the conclusions drawn from studies on the effect of SRP54 and SRα depletion in yeast cells. Walter and coworkers found that upon depletion of these proteins some, but not all, secretory and membrane proteins accumulate in the cytosol. CPY maturation is unaffected under these conditions, whereas the translocation of Kar2p and dipeptidyl aminopeptidase B, a vacuolar membrane protein, is severely blocked (Hann and Walter, 1991; Nunnari and Walter, 1992; Ogg *et al.*, 1992). It may be that the Sec63p complex and yeast SRP serve complimentary functions in targeting presecretory proteins to the translocation pore. In mammalian cells the 54-kDa subunit of the SRP binds and transfers the signal peptide of a presecretory protein to Tram, a 34-kDa membrane glycoprotein that interacts with a translocating chain early in the process of membrane insertion (Görlich *et al.*, 1992). The Sec63p complex may play a similar role in yeast cells.

We propose that signal peptides interact with one or more members of the Sec63p complex with an intrinsic specificity dictated in the signal. Signal peptides typically contain a hydrophobic core but vary in length and abundance of charged residues. It is possible that some signals that contain an unusual polarity or charge density may require Sec66p for efficient function at 25°C. Less stable interactions at 37°C may render the remaining Sec63p complex less capable of coping without Sec66p. This model predicts that the signal peptide rather than the mature portion of the secretory polypeptide sequence will dictate the stringency of the requirement for Sec66p. Detailed analysis of this model requires the development of a signal peptide dependent precursor binding assay. The isolation of a functional Sec63p complex will facilitate the development of such an assay.

Note added in proof. Dr. Neil Green (Department of Microbiology and Immunology, Vanderbilt University School of Medicine) has found that our temperature sensitive *sec66* null strain fails to complement a nonconditional mutant, *sec71*, published previously (Green, N., Fang, H., and Walter, P. (1992). J. Cell Biol. *116*, 597–604). Thus it is probable that *SEC66* and *SEC71* represent the same complementation group. If this conclusion is corroborated by genetic linkage analysis, we propose to call the gene *SEC71*.

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