Sec72p Contributes to the Selective Recognition of Signal Peptides by the Secretory Polypeptide Translocation Complex

David Feldheim and Randy Schekman

Department of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California at Berkeley, Berkeley, California 94720

Abstract. *SEC72* encodes the 23-kD subunit of the Sec63p complex, an integral ER membrane protein complex that is required for translocation of presecretory proteins into the ER of *Saccharomyces cerevisiae*. DNA sequence analysis of *SEC72* predicts a 21.6-kD protein with neither a signal peptide nor any transmembrane domains. Antibodies directed against a carboxyl-terminal peptide of Sec72p were used to confirm the membrane location of the protein. *SEC72* is not essential for yeast cell growth, although an *sec72* null mutant accumulates a subset of secretory precursors in vivo. Experiments using signal peptide chimeric proteins demonstrate that the *sec72* translocation defect is associated with the signal peptide rather than with the mature region of the secretory precursor.

THE targeting and translocation of presecretory proteins across the 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).[1] Cytosolic Hsp70s, the products of the *SSA* gene family, are required for the posttranslational translocation of pre-pro-α-factor (ppαf) both in vivo and in vitro (Chirico et al., 1988; Deshaies 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- and 19-kD yeast homologues of mammalian SRP show strong defects in preprotein translocation (Hann et al., 1989; Hann and Walter, 1991; Nunnari and Walter, 1992; Ogg et al., 1992). The ER lumenal Hsp70 homologue BiP, the product of the *KAR2* gene, is also required for translocation (Vogel et al., 1990; Sanders et al., 1992; Brodsky et al., 1993).

The *SEC61* and *SEC63* gene products reside in two different integral ER membrane protein complexes whose subunits have been characterized genetically and biochemically. The Sec61p complex consists of Sec61p, a 54-kD integral membrane protein containing five to nine predicted transmembrane domain segments (Stirling et al., 1992), and Sss1p, a 9-kD peripheral membrane protein (Esnault et al., 1993). A temperature-sensitive allele of *SEC61* was first isolated in a genetic selection designed to isolate mutants that failed to localize properly a signal peptide–bearing cytoplasmic enzyme chimera to the lumen of the ER (Deshaies and Schekman, 1987). Sec61p is intimately associated with preproteins as they are being translocated across the ER membrane; translocation intermediates, synthesized and imported in vitro, can be cross-linked to Sec61p in an ATP-dependent fashion (Müsch et al., 1992; Sanders et al., 1992). *SSS1*, isolated as a multicopy suppressor of the *sec61-1* mutation, is an essential gene that encodes a small polar protein with neither a signal peptide nor any predicted transmembrane domains. Depletion of Sss1p in vivo leads to a dramatic defect in the translocation of both soluble and membrane proteins (Esnault et al., 1993). Antibodies directed against Sss1p coimmunoprecipitate Sec61p from solubilized membrane extracts, suggesting that the two molecules are in a complex (Esnault Y., D. Feldheim, M.-O. Blondel, R. Schekman, and D. Kepes, manuscript submitted for publication).

The Sec63p complex consists of four polypeptides: Sec62p, Sec63p, Sec71p, and a 23-kD protein. Antibodies directed against Sec62p coprecipitate the other three molecules (Deshaies et al., 1991). *SEC62* and *SEC63* were originally defined by the isolation of temperature-sensitive mutations in the same genetic selection that isolated *SEC61* (Deshaies and Schekman, 1987; Rothblatt et al., 1989). *SEC62* encodes a 30-kD protein with two membrane-spanning domains oriented such that the amino and carboxyl termini are exposed to the cytosol (Deshaies and Schekman, 1989; Deshaies and Schekman, 1990). *SEC63* encodes a 73-kD protein that spans the bilayer three times (Feldheim et al.,

Address all correspondence to Randy Schekman, Department of Molecular and Cell Biology, Howard Hughes Medical Institute, 401 Barker Hall, University of California at Berkeley, Berkeley, CA 94720. Phone: 510-642-5686; fax: 510-642-7846.

1. *Abbreviations used in this paper*: CPY, carboxypeptidase Y; ORF, open reading frame; ppαf, pre-pro-α-factor; SRP, signal recognition particle; Tx-100, Triton X-100; YPD medium, 2% bactopeptone, 1% yeast extract, 2% glucose.

1992). Sec63p contains an ER lumenal domain that is 42% identical to the *Escherichia coli* DnaJ protein (Sadler et al., 1989). Yeast BiP (*KAR2* gene product), which is also required for secretory polypeptide translocation, is 50% identical to the *E. coli* hsp70 homologue DnaK (Normington et al., 1989; Vogel et al., 1990; Rose et al., 1989). In *E. coli*, the DnaK and DnaJ proteins 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). The association of BiP and Sec63p is supported by the isolation of a functional complex that includes BiP, Sec63p, Sec71p, and p23 (Brodsky and Schekman, 1993). BiP is not retained in the complex purified from a strain that harbors a mutation in the DnaJ domain of *SEC63* (Brodsky and Schekman, 1993).

SEC70, *SEC71*, and *SEC72* were identified in a genetic selection designed to obtain nonconditional mutations in genes required for translocation (Green et al., 1992). Sec71p was biochemically identified as the 31.5-kD glycoprotein subunit of the Sec63p complex and was also independently cloned as a multicopy suppressor of a *sec63* temperature-sensitive mutation (Feldheim et al., 1993; Kurihara and Silver, 1993). The glycoprotein spans the ER membrane once, and it is predicted to be oriented with the NH_2 terminus in the ER lumen and the COOH terminus in the cytosol. A null mutation in *SEC71* results in a temperature-sensitive growth defect, and it causes the accumulation of a subset of preproteins in vivo (Feldheim et al., 1993; Kurihara and Silver, 1993).

The Sec63p complex is also required for the translocation of yeast α-factor precursor (ppαf) into reconstituted proteoliposomes (Brodsky and Schekman, 1993). When proteoliposomes are derived from wild-type membrane proteins, ppαf is efficiently translocated in an ATP-dependent manner, whereas proteoliposomes derived from *sec63-1*, *sec71*, or p23 mutant membranes are defective in this reaction. If the purified wild-type Sec63p complex, containing Sec63p, Sec71p, and p23 and is supplied during proteoliposome formation, the mutant defects are repaired (Brodsky and Schekman, 1993; Brodsky et al., 1993).

We have now characterized the 23-kD subunit of the Sec63p complex. In this report, we show that p23 is encoded by *SEC72* and has neither a predicted signal peptide nor any transmembrane domains. *sec72* null mutant cells are viable but accumulate a subset of preproteins in vivo. This translocation defect is associated with the composition of the signal peptide rather than the mature region of the secretory protein.

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 trpΔ63 MATα*), RSY925 (*ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ63/trp1Δ63 leu2Δ1/leu2Δ1 sec71::LEU2/SEC71*), RSY1005 (*ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 his3Δ200/his3Δ200 trp1Δ63/trp1Δ63 leu2Δ1/leu2Δ1 sec72::HIS3/SEC72*), *RSY926 (ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 MATα*), RSY1006 (*ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 sec72::HIS3 MATα*), RSY607 (*ura3-52 leu2-3,112 pep4::URA3 MATα*), and RSY587 (*ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 sec63::HIS3 MATα*) containing the *SEC63*-c-*myc* plasmid pDF41 (Feldheim et al., 1993). *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 pDF59 contains a 3.3-kb EcoRI fragment containing *SEC72* inserted into the EcoRI restriction site in pBluescript KS (Stratagene, La Jolla, CA). The gene disruption plasmid pDF68 was constructed as follows: a 145-bp BclI fragment was removed from pDF59. An 1.8-kb BamHI fragment containing the *HIS3* gene of pJJ250 (Jones and Prakash, 1990) was ligated to the cleaved form of pDF59. The carboxypeptidase Y-invertase chimeric plasmid pCYIC50, has been described, as has the random leader peptide, 4H11-invertase (Johnson et al., 1987; Kaiser and Botstein, 1990).

Protein Purification, Protein Fragmentation, and Peptide Sequence Analysis

Purification of the Sec63p complex: RSY587 was grown to midlog phase (between 2 and 10 OD_{600}/ml) in YPD medium (2% bactopeptone, 1% yeast extract, 2% glucose; Difco Laboratories, Detroit, MI). Cells were harvested at 3,000 *g* in a GSA rotor and were 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 PMSF, 10 μM leupeptin, and 10 μM pepstatin) and were 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 centrifugation for 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. Membranes in the pellet were solubilized by the addition of Triton X-100 (Tx-100) 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. Solubilized proteins were cycled 10 times through a 5-ml human c-*myc* monoclonal antibody column (Evan et al., 1985) that was made by coupling 10 mg purified human c-*myc* antibody to 5 ml swollen Avid gel resin according to the manufacturer's instructions (Bioprobe International, Inc., 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 it was eluted with 0.2 M glycine, pH 2, 0.2% Tx-100. Peak fractions, as judged by SDS-PAGE and silver staining, were concentrated by TCA precipitation, washed with 0.5 ml cold acetone, resolubilized in 0.1 ml 8 M urea, 100 mM $(NH_4)_2CO_3$, reduced with 10 mM β-mercaptoethanol, and separated by reverse phase HPLC on an RP-300 C8 column (ProBlott; Applied Biosystems, Inc., Foster City, CA). Sec72p-containing fractions were transferred to membranes (ProBlott; Applied Biosystems, Inc.). Amino terminal protein sequencing was done by standard chemistry using fast cycles on a protein peptide 477A sequencer (2.1 × 150 mm; Applied Biosystems, Inc.). A single sequence, VTLEYNANSKLITA (underlined in Fig. 1), was obtained. The degenerate oligonucleotide primers 5′ GGGGAATTCGTNACNYTNGARTAY 3′ and 5′ GGGGGATCCNGCNCTDTAYAAYTT 3′ corresponding to all codon possibilities of VTLEY and the inverse codons of KLITA were used to amplify yeast genomic DNA using the polymerase chain reaction. The resultant 60-bp PCR fragment was cloned into pBluescript KS, and the nucleotide sequence of the product confirmed the internal NANS codons of the NH_2-terminal peptide. A full-length copy of the corresponding gene was cloned by screening a YEp13-based library (10 kb average insert length; T. Yoshihisa, Dept. of Molecular & Cell Biology, Howard Hughes Medical Institute, University of California at Berkeley) using a degenerate oligonucleotide (5′ ACGCTNGAATATAATGCAAACAGTAAGCT 3′ corresponding to the peptide TLEYNANSK) that was end labeled by reaction with polynucleotide kinase and [^{32}P]γ-ATP. Colony hybridization (Ausubel et al., 1987) was performed at 50°C in 6× SSC, 1× Denhardt's solution, 100 μg/ml yeast tRNA, 0.05% sodium pyrophosphate for 16 h, and 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.

DNA Sequencing

To determine the DNA sequence of *SEC72*, we sequenced pDF59 by standard methods using Sequenase (United States Biochemical Corp., Cleveland, OH) following the manufacturer's instructions.

```
  1 attagattatatctttgaacataaaaccaatagccttaaaatcaaagaaacgtctccttcaacttgcgttaaaggcatac 80

 81 caaagcaagcttattcggcc ATG GTT ACC CTT GAA TAC AAT GCA AAC AGT AAA CTG ATC ACT GCG 145
  1                    M   V   T   L   E   Y   N   A   N   S   K   L   I   T   A  15

146 AGT GAT GCT GTT GTT GCA CTA TCT ACC GAA ACT AAT ATC GAT CAA ATA AAT CTT CTC ACT 205
 16 S   D   A   V   V   A   L   S   T   E   T   N   I   D   Q   I   N   L   L   T  35

206 ACA TCT TTG ATT GGA GAA ACC AAC CCA AAT TTT ACA CCA CAA CCG AAT GAA GCT CTA AGC 265
 36 T   S   L   I   G   E   T   N   P   N   F   T   P   Q   P   N   E   A   L   S  55

266 AAA ATG ATC AAG GGT TTA TTT GAA AGT GGT ATG AAG AAT TTA CAA CAA AAA AAA TTG AAT 325
 56 K   M   I   K   G   L   F   E   S   G   M   K   N   L   Q   Q   K   K   L   N  75

326 GAG GCA TTG AAG AAT GTT TCT TTA GCA ATC GAA ATG GCA CAA AGA AAA AGA GCG CCT TGG 385
 76 E   A   L   K   N   V   S   L   A   I   E   M   A   Q   R   K   R   A   P   W  95

386 GAA GCT TTT GCT ATT CAG CTA CCA GAG CTA CAC TTT ATG CTT CGT AGT AAA ATA GAT TTA 445
 96 E   A   F   A   I   Q   L   P   E   L   H   F   M   L   R   S   K   I   D   L  115

446 TGT TTA ATA CTC GGA AAG CAT TTA GAG GCG TTG CAA GAC TTG GAT TTC TTA CTT GGT ACG 505
116 C   L   I   L   G   K   H   L   E   A   L   Q   D   L   D   F   L   L   G   T  135

506 GGA CTT ATC CAA CCA GAC GTA TTT GTC AGG AAG GCG GAC TGT TTG CTA AAA TTG AGA CAG 565
136 G   L   I   Q   P   D   V   F   V   R   K   A   D   C   L   L   K   L   R   Q  155

566 TGG GAA GAG GCT AGG GCA ACA TGC GAG AGA GGT TTA GCT TTA GCC CCA GAG GAT ATG AAA 625
156 W   E   E   A   R   A   T   C   E   R   G   L   A   L   A   P   E   D   M   K  175

626 CTT AGA GCT CTT TTA ATA GAA ACT GCA AGA AAT ATG GCC GAA TAT AAC GGT GAA TAA ggtg 686
176 L   R   A   L   L   I   E   T   A   R   N   M   A   E   Y   N   G   E   *      194

687 cataaccgaagatattttagacctttttcttgatatgtctctatgctacacacgactacatagcaagaaattttagataga 766

767 ttcaaaaacacatagcaagcccattatcatatatactaaggaatagcgtttatcaaaggctttgaactatacagcttgag 846

847 tatatgatacctctgcttcccaaccgcaagctttttttcttcgtgatgctgaaaaaaaaaaatggacatagaaaaaacct 926

927 tcataaatgaaacagaccattcattactcgtgcggaaccagtaaacacaacac                              979
```

Figure 1. Nucleotide sequence of the *SEC72* gene and the predicted amino acid sequence of Sec72p. Sequences potentially involved in the initiation (TATA boxes) or termination of transcription are underlined. The NH_2-terminal peptide generated from microsequence of purified Sec72p is underlined. The sequence accession number is L29340.

Gene Disruptions

A null allele of *SEC72* was generated in vitro by digesting pDF68 with EcoRI, giving a plasmid DNA fragment terminating with ~1 kb of 5′ *SEC72* sequence and 2 kb 3′ of the *HIS3* gene. The fragment was gel purified using Geneclean (Bio 101 Inc., La Jolla, CA) according to the manufacturer's instructions. The diploid yeast strain YPH501 was transformed with the EcoRI fragment using the lithium acetate method (Ausubel et al., 1987), and transformants were selected on minimal medium lacking histidine. Transformants were induced to sporulate on acetate sporulation plates and dissected into tetrads. Genomic DNA from the heterozygous diploid RSY1005 and representative spores were isolated and used as template in PCR using the primers 5′ ACGTCTCCTTCAACTTGCG 3′ and 5′ GCGCTCTTTTTCTTTGAG 3′ (corresponding to nucleotides 51–68 and 382–365 of Fig. 1). The PCR products were separated on 1% agarose gels. Genomic DNA from the parent diploid strain or from the His^- spores gave rise to an expected 315 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 His^+ 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 CPY (Stevens et al., 1982) were used at 1 μl serum/OD_{600} cell equivalent, invertase (Schauer et al., 1985) at 2 μl serum/OD_{600} cell equivalent, α-factor (A. Eun, Department of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California at Berkeley) 4 μl serum/OD_{600} cell equivalent, Sec72p at 5 μl serum/OD_{600} cell equivalent, and Kar2p (Jeff Brodsky, Department of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California at Berkeley) at 2 μl serum/OD_{600} cell equivalent. Tunicamycin (Sigma Immunochemicals, St. Louis, MO) was added at a final concentration of 10 μg/ml 10 min before radiolabeling. Cross-linking of radiolabeled membranes and immunoprecipitation of the Sec63p complex has been described (Deshaies et al., 1991). Endoglycosidase H (Endo H) treatments were carried on in sodium citrate buffer, pH 5.5, using 0.005 U Endo H in samples that were incubated for 8 h at 37°C.

Cell Fractionation and Immunoblotting

RSY607 cells were grown in YPD medium and lysates were prepared as described (Feldheim et al., 1992). To determine the nature of the association of Sec72p with the membrane, 200 OD_{600} cells were lysed by agitation with glass beads in 2 ml of buffer G (0.1 M sorbitol, 50 mM KOAc, 2 mM EDTA, 20 mM Hepes, pH 7.4, 1 mM DTT, 1 mM PMSF). Homogenates were cleared at 370 *g* for 4 min in a rotor (HB4; Beckman Instruments, Inc., Fullerton, 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, Inc.). 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 (Harlow and Lane, 1988). Filters were blocked, and all antibody incubations and washes were conducted with 2% nonfat dry milk in 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 Corp., Arlington Heights, IL). Antibodies to Sec61p, Sec62p, Sec63p, Sec71p, and Sec23p are described elsewhere (Hicke and Schekman, 1989; Deshaies and Schekman, 1990; Feldheim et al., 1992, 1993; Stirling et al., 1992).

Production of Anti-Sec72p Antiserum

Polyclonal antiserum recognizing the COOH terminus of Sec72p was obtained by immunizing rabbits with the peptide CETARNMAEYNGE (corresponding to the ultimate 12 amino acids of SEC72p) conjugated to BSA (Harlow and Lane, 1988). Peptide (200 μg) was injected subcutaneously in an emulsion containing Freund's complete (first injection) and incomplete (subsequent injections) adjuvant. An antiserum titer of 1/1,000, detected by immunoblot of an SDS-PAGE–resolved whole-cell lysate, developed 16 wk after the initial injection.

Results

Cloning of the Gene that Encodes p23

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. 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 2.1, and neutralized with 1 M Tris/HCl to pH 7.5. The protein profile as judged by SDS-PAGE was similar to the components of the complex described previously (Deshaies et al., 1991), except that no Sec61p was detected in the eluate. The eluate was blotted onto polyvinyldifluoride membranes, and the region containing the 23-kD protein was removed and subjected to NH_2 terminal amino acid sequencing. A 14–amino acid sequence derived from p23 (VTLEYNANSKLITA) was used to generate a degenerate oligonucleotide primer corresponding to TLEYNANSK (see Materials and Methods). This oligonucleotide hybridized predominately to one band on a total yeast genomic DNA Southern blot (not shown). A yeast multicopy chromosomal library propagated in *E. coli* was screened by colony hybridization, and positive clones were isolated and further characterized by restriction mapping.

Nucleotide Sequence of p23

The DNA sequence surrounding the hybridizing fragment was determined by the dideoxynucleotide chain termination method (Sanger et al., 1977). Fig. 1 shows that the nucleotide sequence contains a single open reading frame (ORF) of 582 bp found between nucleotides 101-683. A potential transcriptional initiation signal noted at position 7, falls within the usual range of −20 to −200 for TATA boxes in yeast (Struhl, 1985). Putative termination signals are found at positions 719-875. We conclude that this ORF is the gene encoding p23 because the NH_2-terminal peptide generated from the microsequencing of p23 is contained within this reading frame (underlined amino acids in Fig. 1). Sequence analysis of the ORF predicts a polypeptide consisting of 194 amino acids, corresponding to a molecular mass of 21.6-kD with an isoelectric point of 5.48. There is neither a predicted NH_2-terminal signal peptide nor any stretch of amino acids predicted to span a membrane bilayer. A search of the data base using the FASTA program revealed that the gene encoding p23 is identical to a previously uncharacterized ORF located between *GSP1*, a Ras-like GTP binding protein (Belhumeur et al., 1993), and *GCD7*, a gene encoding an essential 43-kD subunit of the guanine nucleotide exchange factor for eIF-2 in yeast (Bushman et al., 1993). This ORF, of unknown function, is on chromosome XII and overlaps the predicted promoter for *GCD7*. There were no other homologies to this gene in the data base.

p23 Is Not Required for Viability

To investigate the role of p23 in vegetatively growing cells, we explored the effects of deletion of the chromosomal copy of the gene. A 134-bp BclI fragment between bp 130-274 of the gene encoding p23 was replaced with the *HIS3* gene of *Saccharomyces cerevisiae*. This deletion removed 48 amino acids of the ORF. An EcoRI fragment containing 1 kb 5′ and 2 kb 3′ of surrounding nucleotide sequence was used to transform the yeast diploid strain YPH501 to histidine prototrophy. PCR analysis, using oligonucleotide primers designed to flank the BclI fragment, was performed using chromosomal DNA as a template to confirm integration of the disruption construct at the locus (see Materials and Methods). Histidine prototrophic heterozygous diploids were sporulated, and asci were dissected into tetrads and germinated at 25°C on rich medium. Twelve tetrads each showed four viable spores. Histidine prototrophy segregated 2 His^+: 2 His^-, 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. Spores containing the *His3* integrant gene were also viable at high (37°) and low temperatures (17°C). The ability of the p23 null mutant to grow at all temperatures was in contrast to the other subunits of the Sec63p complex. *SEC61*, *SEC62*, and *SEC63* are essential for cell viability at all temperatures, and *SEC71* is essential at 37°C (Deshaies and Schekman, 1989; Sadler et al., 1989; Stirling et al., 1992; Feldheim et al., 1993; Kurihara and Silver, 1993).

The Gene Encoding p23 Corresponds to SEC72

Complementation analysis of the null allele of p23 showed that it corresponded to *SEC72*, a mutant identified in a genetic screen designed to isolate nonconditional alleles of proteins required for translocation (Green et al., 1992; Fang and Green, 1994). We therefore will refer to the gene encoding p23 as *SEC72*.

Detection of Sec72p in Yeast Cell Extracts

To characterize Sec72p further, we raised polyclonal antibodies to a predicted peptide derived from the COOH terminus of the gene product. The peptide was conjugated to BSA and injected into rabbits. Antibodies were affinity purified by binding to and elution from an immobilized form of this peptide, and the resultant antiserum was tested for its specificity by immunoblotting SDS-PAGE–resolved whole-cell extracts made from either wild-type, *sec71*, or *sec72* mutant cells (Fig. 2). Wild-type cell extracts displayed a 23-kD immunoreactive polypeptide that was absent in a *sec72* null strain (Fig. 2, lanes *1* and *3*). Surprisingly, cell extracts made from *sec71* null cells contained little or no Sec72p (Fig. 2, lane *2*). In contrast, cell extracts made from either wild type or *sec72* cells contained Sec71p. The loss of Sec71p or Sec72p had no affect on the steady-state levels of Sec61p, Sec62p or Sec63p (Fig. 2). A reduced level of Sec72p in *sec71* cell extracts is consistent with our previous data that showed no Sec72p present in the Sec63p complex isolated from *sec71* mutant cells (Feldheim et al., 1993). Also consistent with this was the observation that a *sec71*, *sec72* double mutant was no more growth defective than the *sec71* mutant alone.

Sec72p is Tightly Associated with Membranes

Sec72p is associated with Sec62p, Sec63p, and Sec71p, three integral membrane proteins in the ER (Deshaies and Schekman, 1989; Feldheim et al., 1992, 1993; Stirling et al., 1992; Kurihara and Silver, 1993). To test if Sec72p is membrane localized or cytosolic, membrane fractions were prepared from wild-type yeast cells and extracted using condi-

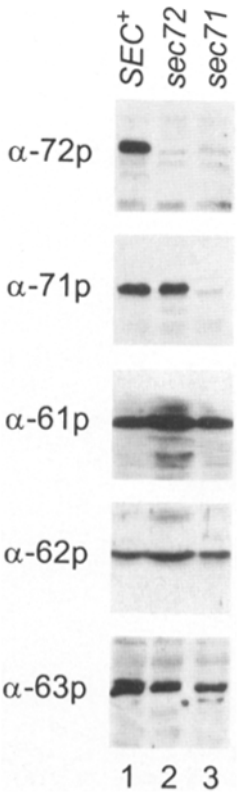

Figure 2. Detection of Sec72p in whole cell extracts. Wild-type (lane *1*, YPH500), *sec71* (lane *2*, RSY925), or *sec72* (lane *3*, RSY1006) cells were grown to midlog phase in rich medium, lysed with glass beads in SDS-PAGE sample buffer, and heated to 65°C for 20 min. One OD_{600} cell equivalent of protein was resolved by 12.5% SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted with antibodies directed against Sec72p, Sec71p, Sec61p, Sec62p, or Sec63p.

tions that either solubilize peripheral membrane proteins or solubilize all membrane proteins. The experiment in Fig. 3 demonstrates that <5% of the Sec72p was 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. 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. This result demonstrated that Sec72p was exclusively associated with membranes, presumably through its association with the other subunits of the Sec63p complex.

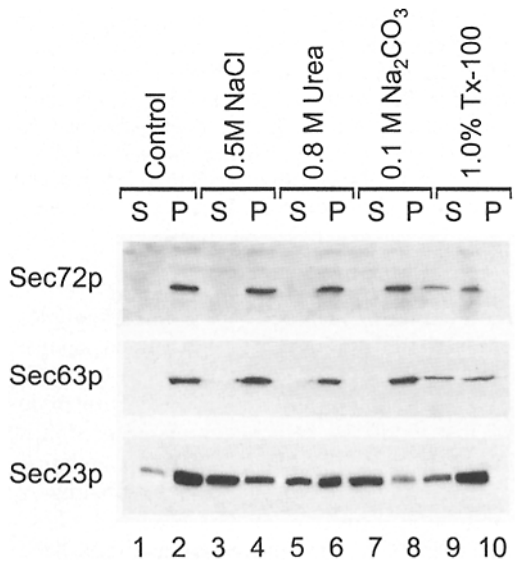

Figure 3. Evidence that Sec72p fractionates with membranes. Low speed supernatant fractions were prepared and treated with either 0.5 M NaCl, 0.1 M Na_2CO_3, pH 11, 0.8 M urea, or 1% Tx-100. After incubation on ice for 20 min, all samples were separated into supernatant (*S*) or pellet (*P*) fractions by centrifugation at 100,000 *g*, subjected to SDS-PAGE, and immunoblotted with anti-Sec72p, anti-Sec63p, or anti-Sec23p antiserum.

Sec72p is Rapidly Degraded in SEC71 Mutant Cells

Sec72p is absent from the Sec63p complex in yeast strains deleted for *SEC71* (Feldheim et al., 1993). We wished to determine whether this defect in the Sec63p complex was caused by the inability of Sec72p to be recruited to the Sec63p complex in the absence of Sec71p, or by the instability of Sec72p in *sec71* null strains. To address the possibility of Sec72p instability, wild-type and *sec71* mutant cells were pulse labeled for 10 min followed by chase with cold amino acids for various times. The labeled cells were lysed with glass beads, and samples were treated with anti-Sec72p antiserum. Immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. The experiment in Fig. 4 showed that Sec72p was stable in wild-type cells (Fig. 4, lanes *1–4*) but was rapidly degraded in *sec71* null cells (Fig. 4, lanes *5–9*). Quantitation showed that the half-life of Sec72p in *sec71* cells was ~12 min. Thus, we propose that Sec71p may have a stabilizing influence on Sec72p, because Sec72p is degraded rapidly in the absence of Sec71p.

Defective Sec63p Complex Formation in the sec72 Strain

Sec72p can be cross-linked to four other proteins (Sec63p, Sec62p, Sec61p, and Sec71p) that are precipitated from detergent-solubilized membranes with antibody against Sec62p or Sec63p (Deshaies et al., 1991; Feldheim et al., 1992). Sec61p is a minor component of this complex and is not always seen under these conditions. To test whether the integrity of this complex is affected in the *sec72* null strain, radiolabeled wild-type (Fig. 5, lane *2*) or *sec72* (Fig. 5, lane *1*) strains were pulse labeled with ^{35}S-Translabel for 30 min. Membranes from lysed cells were solubilized with Tx-100 and treated with the thiol-cleavable cross-linker dithio-bis-(succinimidylpropionate). Samples were quenched, treated with SDS, and then mixed with antibody directed against

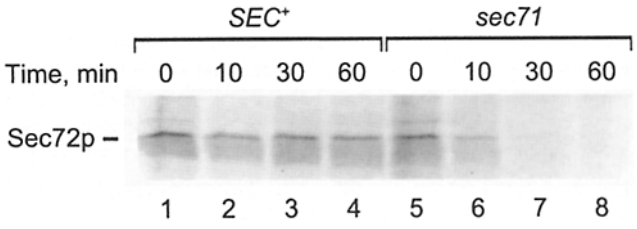

Figure 4. Sec72p is rapidly degraded in *sec71* cell extracts. Wild-type (lanes *1–4*) or *sec71* (lanes *5–8*) cells were pulse labeled at 30°C for 15 min, and incubation was continued in the presence of cold methionine and cysteine (1 mg/ml) for the indicated times. Glass bead extracts were immunoprecipated with anti-Sec72p antiserum. The precipitates were subjected to SDS-PAGE and fluorography. Quantification was performed using a PhosphoImager (Molecular Dynamics, Inc).

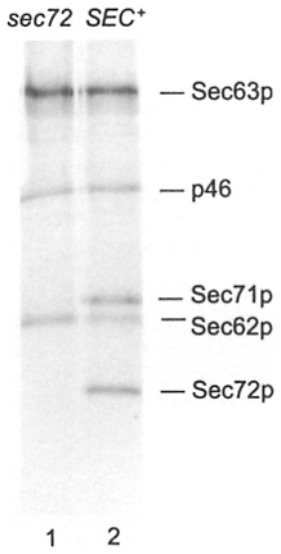

Figure 5. sec72 cells are defective in Sec63p complex formation. ^{35}S-labeled membranes prepared from wild-type (lane *2*) or *sec72* (lane *1*) cells were solubilized with 1% Tx-100 and cross-linked with dithio-bis-(succinimidylpropionate) as previously described (Deshaies et al., 1991). Samples were immunoprecipitated with anti-Sec63p affinity-purified antibodies. The cross-links were cleaved in sample buffer containing 10 mM DTT, and the products were resolved by 12.5% SDS-PAGE followed by fluorography.

Sec63p. The immunoprecipitates were treated with DTT to break the cross-links, resolved by SDS-PAGE, and subjected to fluorography. The immunoprecipitation from wild-type cells produced a protein profile consisting of Sec63p, a 46-kD band, Sec62p, Sec71p, and Sec72p (Fig. 5, lane 2). Sec63p immunoprecipitates from *sec72* cells did not contain *sec72*, as expected, but, in addition, Sec71p was not recovered in the complex (Fig. 5, lane *1*). The absence of Sec71p from the Sec63p complex in *sec72* cells is not because of the absence of Sec71p in the cell: Sec71p was approximately equally abundant in wild-type and *sec72* cells (Fig. 2). Therefore, Sec72p is required to attach or retain Sec71p in the Sec63p complex. In the absence of Sec72p, Sec71p and Sec63p may be associated, but not in manner that can be preserved by chemical cross-linking.

sec72 Null Mutant Accumulates a Subset of Secretory Proteins In Vivo

To determine the role of Sec72p in translocation, we tested whether the *sec72* null strain accumulated untranslocated precursor proteins in vivo. Biogenesis of the periplasmic enzyme invertase and the vacuolar protease carboxypeptidase Y (CPY) were examined by immunoprecipitation of radiolabeled precursors from wild-type, *sec72* null, and control strains.

CPY is translated as a pre-pro-protein and undergoes signal peptide processing upon translocation into the ER (Stevens et al., 1982). In the ER, CPY is core glycosylated to the p1 form. Subsequently 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 molecular weight mature form that comigrates with unglycosylated pre-pro-CPY. The overall maturation of CPY takes ~6 min (Hasilik and Tanner, 1978). A brief (4-min) radiolabel pulse of wild-type cells detects predominantly the p2 form of CPY. Wild-type (Fig. 6 *A*, lanes *1* and *2*), *sec72* (Fig. 5 *A*, lane *3*), or *sec63* (Fig. 6 *A*, lane *4*) cells were pulse labeled with ^{35}S-Translabel for 4 min, after which the labelings were terminated with the addition of 20 mM sodium azide. Cells were lysed, samples were immunoprecipitated with antiserum directed against CPY, and the radioactive proteins were resolved on SDS-PAGE and visualized by fluorography. The experiment in Fig. 6 *A* shows that wild-type cells accumulated predominantly p2 CPY (Fig. 6 *A*, lane *1*). When the glycosylation inhibitor tunicamycin was added before pulse labeling, a more rapidly migrating form of CPY was observed whose mobility was consistent with signal-processed, unglycosylated CPY (Fig. 6 *A*, lane *2*). The *sec72* strain showed a partial translocation defect: ~50% of CPY accumulated as a species whose mobility was consistent with untranslocated pro-CPY (Fig. 6 *A*, lane *3*). As a control, the translocation-defective *sec63* strain was shown to accumulate this species exclusively. The data in Fig. 6 *B* shows that *sec72* was not defective in the translocation and secretion of the periplasmic enzyme

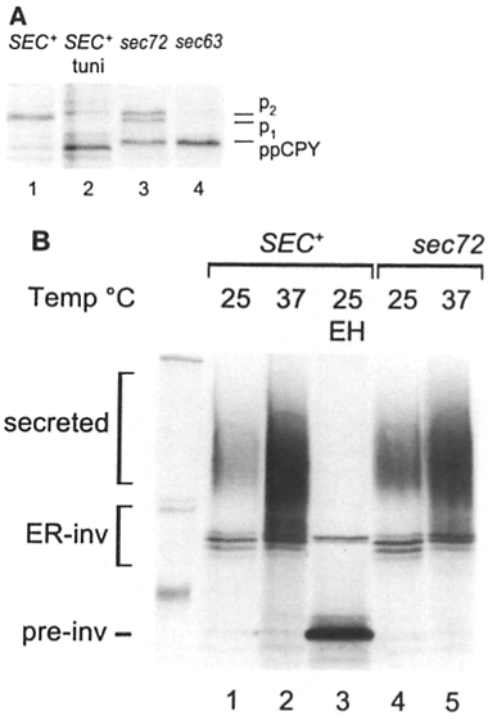

Figure 6. Unmodified secretory precursors accumulate in *sec72* cells. (*A*) CPY: wild-type (lanes *1* and *2*), *sec72* (lane *3*) or *sec63* (lane *4*) cells were pulse labeled at 37°C with ^{35}S-Translabel for 4 min. Glass bead extracts were immunoprecipitated with anti-CPY antiserum. Immunoprecipitates were resolved on 10% SDS-PAGE and subjected to fluorography. Tunicamycin (lane *2*) was added at a final concentration of 10 μg/ml 10 min before labeling. *ppCPY*, Pre-pro-CPY; *p1*, ER-modified form of CPY; *p2*, Golgi-modified form of CPY. (*B*) Invertase: wild-type (lanes *1–3*) or *sec72* (lanes *4* and *5*) cells were grown to OD_{600} of 0.5 in minimal medium containing 2% glucose and were shifted to minimal medium containing 0.1% glucose for 3 h at 25°C. Cells were then grown at 25°C (lanes *1*, *3*, and *4*) or 37°C (lanes *2* and *5*) for 1 h, and pulse labeled for 15 min with ^{35}S-Translabel. Glass bead extracts were immunoprecipitated with antiinvertase antiserum and resolved on a 7.5% SDS-PAGE followed by fluorography. EndoH (lane *3*, 0.005 units) was added to the immunoprecipitates in EndoH buffer and incubated at 37°C for 8 h. *pre-inv*, Unglycosylated forms of invertase; *ER-inv*, ER-modified forms of invertase; *secreted*, secreted and Golgi modified forms of invertase.

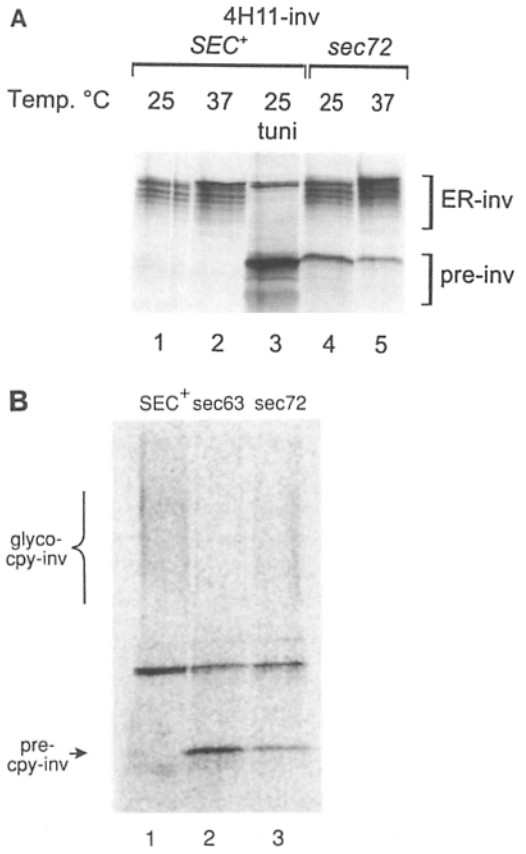

Figure 7. Signal peptide-invertase chimeras accumulate in *sec72* cells. (*A*) 4H11-invertase: wild-type (lanes *1–3*) or *sec72* (lanes *4* and *5*) cells containing the 4H11-invertase signal peptide chimera plasmid were grown to OD_{600} of 0.5 and divided into two cultures. Cells were then grown at 25°C (lanes *1*, *3*, and *4*) or 37°C (lanes *2* and *5*) for 1 h and pulse labeled with ^{35}S-Translabel for 15 min. Glass bead extracts were immunoprecipitated with antiinvertase antiserum, resolved by 7.5% SDS-PAGE, and visualized by fluorography. Tunicamycin (lane *3*) was added to a final concentration of 10 μg/ml 10 min before labeling. *pre-inv*, Pre-4H11 invertase; *ER-inv*, ER-modified forms of 4H11-invertase. The leader peptide sequence according to Kaiser et al. (1987) is MNSPKK-DIHTPPLKYPGGQHRGASCCFVFIMCLLYRICGICGDPRGM. For comparison, the leader peptide of preproCRY is MKAFTS-LLCGLGLSTTLARA (Blanchly-Dyson and Stevens, 1987; Valls et al., 1987). The signal peptide of invertase is MLLQFLFLLAGF-AAKISA (Taussig and Carlson, 1983). (*B*) CPY-invertase: wild-type (lane *1*), *sec63* (lane *2*), or *sec72* (lane *3*) cells containing the CPY-invertase chimera plasmid were pulse labeled for 15 min with ^{35}S-Translabel at 37°C. Glass bead extracts were immunoprecipitated with antiinvertase antiserum and resolved on 7.5% SDS-PAGE followed by fluorography. *pre-cpy-inv*, Untranslocated and signal cleaved unmodified CPY-invertase; *glyco-cpy-inv*, glycosylated forms of CPY-invertase.

invertase. Invertase is translocated as a preprotein that undergoes signal peptide cleavage and core glycosylation in the ER. Upon transport to the Golgi apparatus, the core oligosaccharides become heterogeneously outer chain glycosylated. To assay invertase maturation, we derepressed cells for invertase expression in 0.1% glucose for 3 h. Cultures were divided into two portions, kept at either 25°C or 37°C for 1 h, and pulse labeled for 15 min. Aliquots of cell lysates were immunoprecipitated with antiinvertase antiserum and processed as in Fig. 6 *A*. Wild-type cells and *sec72* cells both accumulated the Golgi and secreted forms of invertase (Fig. 6 *B*, lanes *1, 2, 4 and 5*) at 25°C or 37°C. When the wild-type immunoprecipitates were treated with the enzyme endoglycosidase H, which removes N-linked sugar residues, all forms of invertase collapsed to a mobility consistent with the signal-processed unglycosylated invertase (Fig. 6 *B*, lane *3*). Taken together, these data indicate that a null mutation in *SEC72* affects the translocation of precursors differently. These data are similar to that of Green et al. (1992) who showed that the *sec72-1* allele they isolated accumulated untranslocated CPY, but not invertase or Kar2p.

Analysis of Invertase Chimeric Proteins

Because the *sec72* null strain affected the maturation of secretory proteins discriminantly, we wished to determine whether it was the signal peptide or the mature region of the precursor protein that dictated the requirement for Sec72p for efficient translocation. The maturation of two artificial hybrid proteins was examined: a chimera of an unusual signal peptide fused to the mature region of invertase, and a chimeric protein containing the leader peptide of CPY fused to the mature region of invertase. Fig. 7 *A* shows that 4H11-invertase, a signal peptide invertase fusion described previously by Kaiser and Botstein (1990) required Sec72p for efficient translocation. Wild-type (Fig. 7 *A*, lanes *1–3*) or *sec72* (Fig. 7 *A*, lanes *4* and *5*) cells containing the 4H11-invertase fusion were pulse labeled for 15 min, and cell lysates were immunoprecipitated with antiinvertase antibodies. Wild-type cells displayed predominantly the glycosylated ER form of the 4H11 chimera at both 25°C and 37°C (Fig. 7 *A*, lanes *1* and *2*). When the glycosylation inhibitor tunicamycin was added before pulse labeling, an unglycosylated form of invertase with a mobility consistent with that expected for preinvertase was seen (Fig. 7 *A*, lane *3*). The *sec72* strain showed a partial block of 4H11-invertase maturation at both 25°C and 37°C (Fig. 7 *A*, lanes *4* and *5*). The experiment in Fig. 7 *B* shows that *sec72* cells also accumulated a portion of CPY-invertase. Wild-type (Fig. 7 *B*, lane *1*), *sec63* (Fig. 7 *B*, lane *2*), or *sec72* (Fig. 7 *B*, lane *3*) cells containing the CPY-invertase plasmid were pulse labeled for 15 min with ^{35}S-Translabel. Wild-type cells showed predominantly the ER and Golgi forms of the CPY-invertase chimera protein, while *sec63* and *sec72* cells accumulated a band whose mobility was consistent with pre-CPY-invertase (Fig. 7 *B*, lanes *2–3*). These results show that by changing the signal peptide of invertase, one can alter the specificity of invertase translocation from a non-Sec72p-requiring reaction to a Sec72p-requiring reaction. We suggest that Sec72p discriminates secretory proteins at the level of their signal peptide, rather than at the mature region.

Discussion

Four integral membrane proteins required for secretory protein translocation, Sec61p, Sec62p, Sec63p, and Sec71p, exist in a multiprotein complex with one other protein of 23-kD. To gain a better understanding of the mechanism of translocation, we cloned and characterized the 23-kD protein (now called Sec72p) of the Sec63p complex. The nucleo-

tide sequence of *SEC72* codes for a nonessential 21.6-kD protein with neither a predicted signal peptide nor a stretch of amino acids capable of spanning a lipid bilayer. Cell fractionation experiments show that Sec72p is predominantly localized to the membrane fraction. Sec72p is unstable in *sec71* mutant cells. This may be caused by the inability of Sec72p to assemble into the Sec63p complex in the absence of Sec71p.

In contrast to the other proteins in the Sec63p complex, *SEC72* is not essential for viability. This is consistent with the lack of a strong translocation defect in the *sec72* deletion strain. In spite of this, we believe that Sec72p is involved selectively in translocation. Cells deleted for Sec72p affect the translocation of at least one precursor, the vacuolar protease CPY. In addition, *sec72-1* was isolated as a nonconditional mutant in a selection designed to identify translocation mutants. The *sec72-1* allele fails to translocate an arginine permease-invertase-HIS4C fusion protein, as well as a Sec63p-invertase chimera protein, both of which use internal hydrophobic segments as signal peptides (Green et al., 1992).

Sec72p is required for the posttranslational translocation of ppαf into translocation-competent proteoliposomes, although it is not required for ppαf translocation in vivo. When microsomes used in the proteoliposome reconstitution are made from a *sec72* null mutant, translocation is decreased by 70%. When purified Sec63p complex containing Sec72p is added to a detergent soluble fraction prepared from *sec72*, followed by dialysis to create proteoliposomes, the mutant defect is repaired (Brodsky and Schekman, 1993; Brodsky et al., 1993). Successful reconstitution may require the proteins of the translocation apparatus either to remain stably associated during the solubilization procedure or to reassociate during the dialysis step of proteoliposome formation.

How is Sec72p involved in translocation? We believe the phenotype of the *sec72* mutant to be consistent with at least two potentially overlapping roles in translocation. Sec72p may be involved in signal peptide recognition for a defined subset of leader peptides, or Sec72p may increase the efficiency of transfer of unusual or "difficult" secretory precursors to the translocation pore. The involvement of Sec72p in signal peptide recognition is suggested by the observation that *sec72* mutants fail to translocate only a defined subset of precursors, and that the defect can be traced to the signal peptide rather than the mature region of a polypeptide. For example, invertase is translocated efficiently in *sec72* cells, while CPY is not. A chimeric protein where the CPY leader peptide is fused to the mature region of invertase encounters a translocation defect in *sec72* cells that is not seen in wild-type cells.

No significant primary amino acid sequence identity or length conservation for leader peptides from the same organism has been found; however statistical analysis suggests that signal peptides from both prokaryotes and eukaryotes have several common features (VonHeijne, 1981, 1990). A typical signal peptide has three distinct regions: a positively charged amino terminus, a central hydrophobic domain, and a more polar carboxyl terminus that helps define the signal peptide cleavage site. The most critical region of the signal peptide is the hydrophobic core because a number of studies have shown that a mutation in this domain abolishes the function of a signal peptide (VonHeijne 1981, 1990).

Yeast cells recognize an unusually diverse spectrum of signal peptide sequences. Kaiser et al. took an empirical approach to define the signal peptide in yeast by replacing the normal signal of invertase with random DNA fragments from a human genomic library (Kaiser et al., 1987; Kaiser and Botstein, 1990). Because invertase is a secreted enzyme responsible for the cleavage of sucrose to glucose, successful secretion directed by the random sequences was selected by recovery of transformants that grew on sucrose as a sole carbon source. Analysis of the successful signal sequences indicated that hydrophobic amino acids were represented more than would be expected by chance and that charged residues were represented less than would be expected by chance, consistent with the previously statistical analysis of naturally occuring signal peptides. However, substantial variations, including unusually long or short signals were recovered. Several signals typified by 4H11-invertase did not reveal the usual stretch of hydrophobic residues (see Fig. 7 *A*).

Among natural yeast signal peptides, pre-pro-CPY does not have a typical hydrophobic core of amino acids. Unlike many yeast and bacterial secreted proteins, preproCPY cannot be translocated efficiently in mammalian cells. Bird and Gething showed that CPY is translocated in mammalian cells both in vivo and in vitro only if the CPY signal peptide is replaced with a mammalian signal peptide (Bird et al., 1987). However, if the sequence of a specific pair of glycines in the hydrophobic central region is changed to code for leucines, altered pre-pro-CPY is translocated efficiently in mammalian cells (Bird et al., 1987). It may be that yeast has developed a means to handle difficult or intractable signal peptides. Because Sec72p is required for efficient translocation of both CPY and the 4H11-invertase chimera, it may be that Sec72p binds charged leader peptides to the membrane until they engage the translocation apparatus. A systematic analysis of leader peptide composition and requirements for Sec72p will test this hypothesis.

We are most grateful to Dr. Neil Green for sharing data before publication, Ari Admon for microsequence analysis, David King for peptide synthesis, Koji Yoshimura for help in protein purification, Tohru Yoshiishisha for expertise with molecular biology, Katayoun Chamany for help in designing PCR oligos, as well as Jeff Brodsky, Karin Romisch, Susie Lyman, Ann Corsi, and Russell Jones for critical reading of the manuscript.

This work is supported in part by National Institutes of Health (GM26755) and Howard Hughes Medical Institute funds.

Received for publication 18 March 1994 and in revised form 25 May 1994.

References

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. *Current Protocols in Molecular Biology*. Harvard Medical School, Boston, MA. p. 13.7.1.

Belhumeur, P., A. Lee, R. Tam, T. DiPaolo, N. Fortin, and M. W. Clark. 1993. *GSP1* and *GSP2*, genetic suppressors of the *prp20-1* mutant in *Saccharomyces cerevisiae*: GTP-binding proteins involved in the maintenance of nuclear organization. *Mol. Cell Biol.* 13:2152–2161.

Bird, P., M.-J. Gething, and J. Sambrook. 1987. Translocation in yeast and mammalian cells: not all signal sequences are functionally equivalent. *J. Cell Biol.* 105:2905–2914.

Blanchly-Dyson, E., and T. H. Stevens. 1987. Yeast carboxypeptidase Y can be translocated and glycosylated without its amino-terminal signal sequence. *J. Cell. Biol.* 77:1183–1191.

Brodsky, J., and R. Schekman. 1993. A Sec63p-BiP complex from yeast is required for protein translocation in a reconstituted proteoliposome. *J. Cell Biol.* 123:1355–1363.

Brodsky, J. L., S. Hamamoto, D. A. Feldheim, and R. Schekman. 1993. Reconstitution of protein translocation from solubilized yeast membranes reveals topologically distinct roles for BiP and cytosolic Hsc70. *J. Cell Biol.* 120:95–102.

Bushman, J., A. I. Asura, R. L. Matts, and A. G. Hinnebusch. 1993. Evidence that *GCD6* and *GCD7*, translational regulators of GCN4, are subunits of the guanine nucleotide exchange factor for eIF-2 in *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 13:1920–1932.

Caplan, A. J., D. M. Cyr, and M. G. Douglas. 1992. YDJ1p facilitates polypeptide translocation across different intracellular membranes by a conserved mechanism. *Cell.* 71:1143–1155.

Chirico, W. J., M. G. Waters, and G. Blobel. 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature (Lond.).* 332:805–810.

Deshaies, R. J., and R. Schekman. 1987. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell. Biol.* 105:633–645.

Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman. 1988. A subfamily of stress protein facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature (Lond.).* 332:800–805.

Deshaies, R. J., and R. Schekman. 1989. SEC62 encodes a putative membrane protein required for protein translocation into the yeast endoplasmic reticulum. *J. Cell Biol.* 109:2653–2664.

Deshaies, R. J., and R. Schekman. 1990. Structural and functional dissection of Sec62p, a membrane-bound component of the yeast endoplasmic reticulum protein import machinery. *Mol. Cell Biol.* 10:6024–6035.

Deshaies, R. J., S. L. Sanders, D. A. Feldheim, and R. Schekman. 1991. Assembly of yeast Sec proteins involved in translocation into the endoplasmic reticulum into a membrane-bound multisubunit complex. *Nature (Lond.).* 349:806–807.

Esnault, Y., M.-O. Blondel, R. Deshaies, R. Schekman, and F. Kepes. 1993. The yeast *SSS1* gene is essential for secretory protein translocation and encodes a conserved protein of the endoplasmic reticulum. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4083–4093.

Evan, G. I., G. K. Lewis, G. Ramsay, and J. M. Bishop. 1985. Isolation of monoclonal antibodies specific for human-c-*myc* proto-oncogene product. *Mol. Cell Biol.* 5:3610–3616.

Feldheim, D. A., J. Rothblatt, and R. Schekman. 1992. Topology and functional domains of Sec63p, an ER membrane protein required for secretory protein translocation. *Mol. Cell Biol.* 12:3288–3296.

Feldheim, D., K. Yoshimura, A. Admon, and R. Schekman. 1993. Structural and functional characterization of Sec66p, a new subunit of the polypeptide translocation apparatus in the yeast endoplasmic reticulum. *Mol. Biol. Cell.* 4:931–939.

Green, N., H. Fang, and P. Walter. 1992. Mutants in three novel complementation groups inhibit membrane protein insertion into and soluble protein translocation across the endoplasmic reticulum membrane of *Saccharomyces cerevisiae*. *J. Cell Biol.* 116:597–604.

Fang, H., and N. Green. 1994. Nonlethal *sec71-1* and *sec72-1* mutations eliminate proteins associated with the Sec63p-BiP complex from *S. cerevisiae*. *Mol. Biol. Cell.* In press.

Hann, B. F., M. A. Poritz, and P. Walter. 1989. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* contain a homologue to the 54-kD subunit of the signal recognition particle that in *S. cerevisiae* is essential for growth. *J. Cell Biol.* 109:3223–3230.

Hann, B. F., and P. Walter. 1991. The signal recognition particle in *S. cerevisiae*. *Cell.* 67:131–143.

Harlow, E., and D. Lane. 1988. Immunizations. *In* Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp. 82–83.

Hasilik, A., and W. Tanner. 1978. Biosynthesis of the vacuolar glycoprotein carboxypeptidase Y: conversion of the precursor into the enzyme. *Eur. J. Biochem.* 85:599–608.

Hicke, L., and R. Schekman. 1989. Yeast Sec23p acts in the cytoplasm to promote protein transport from the endoplasmic reticulum to the Golgi complex in vivo and in vitro. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1677–1684.

Johnson, L. M., V. A. Bankaitis, and S. D. Emr. 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. *Cell.* 48:875–885.

Jones, J. S., and L. Prakash. 1990. Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. *Yeast.* 6:363–366.

Kaiser, C., and D. Botstein. 1990. Efficiency and diversity of protein localization by random signal sequences. *Mol. Cell. Biol.* 10:3163–3173.

Kaiser, C. A., D. Preuss, P. Grisafi, and D. Botstein. 1987. Many random sequences functionally replace the secretion signal sequence of yeast invertase. *Science (Wash. DC).* 235:312–317.

Kurihara, T., and P. Silver. 1993. Suppression of a sec63 mutation identifies a novel component of the yeast endoplasmic reticulum translocation apparatus. *Mol. Biol. Cell.* 4:919–930.

Müsch, A., M. Wiedmann, and T. A. Rapoport. 1992. Yeast Sec-proteins interact with polypeptides traversing the endoplasmic reticulum membrane. *Cell.* 69:343–362.

Normington, K., K. Kohno, Y. Kozutsumi, M. J. Gething, and J. Sambrook. 1989. *S. cerevisiae* encodes an essential protein homologous in sequence and function to mammalian BiP. *Cell.* 57:1223–1236.

Nunnari, J., and P. Walter. 1992. Protein targeting to and translocation across the membrane of the endoplasmic reticulum. *Curr. Opin. Cell Biol.* 4: 573–580.

Ogg, S., M. A. Poritz, and P. Walter. 1992. The signal recognition particle receptor is important for growth and protein secretion in *Saccharomyces cerevisiae*. *Mol. Biol. Cell.* 3:895–911.

Rose, M., L. M. Misra, and J. P. Vogel. 1989. *KAR2*, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell.* 57:1211–1221.

Rothblatt, J., R. J. Deshaies, S. L. Sanders, G. Daum, and R. Schekman. 1989. Multiple genes are required for proper insertion of secretory proteins into the endoplasmic reticulum in yeast. *J. Cell. Biol.* 109:2641–2652.

Sadler, I., A. Chiang, T. Kurihara, J. Rothblatt, J. Way, and P. Silver. 1989. A yeast gene important for protein assembly into the endoplasmic reticulum and the nucleus has homology to DnaJ, an *Escherichia coli* heat shock protein. *J. Cell. Biol.* 109:2665–2675.

Sanders, S. L., and R. Schekman. 1992. Polypeptide translocation across the endoplasmic reticulum membrane. *J. Biol. Chem.* 267:13791–13794.

Sanders, S. L., K. M. Whitfield, J. P. Vogel, M. D. Rose, and R. Schekman. 1992. Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell.* 69:371–380.

Sanger, F. S., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Schauer, I., S. Emr, C. Gross, and R. Schekman. 1985. Invertase signal and mature sequence substitutions that delay intercompartmental transport of active enzyme. *J. Cell Biol.* 100:1664–1675.

Shen, A., P. Chretien, L. Bastien, and S. Slilaty. 1991. Primary sequence of the glucanase gene from *Oerskovia xanthineolytica*. *J. Biol Chem.* 266: 1058–1063.

Stevens, T. B., B. Esmon, and R. Schekman. 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. *Cell.* 30:439–448.

Stirling, C. J., J. Rothblatt, M. Hosobuchi, R. Deshaies, and R. Schekman. 1992. Protein translocation mutants defective in the insertion of integral membrane proteins into the endoplasmic reticulum. *Mol. Biol. Cell.* 3:129–142.

Struhl, K. 1985. Nucleotide sequence and transcriptional mapping of the yeast *pet56-his3-ded1* gene region. *Nucleic Acids Res.* 13:8587–8601.

Taussig, R., and M. Carlson. 1983. Nucleotide sequence for the yeast *SUC2* gene for invertase. *Nucleic Acids Res.* 11:1943–1954.

Valls, L. A., C. P. Hunter, J. H. Rothman, and T. H. Stevens. 1987. Protein sorting in yeast: the localization determinant of yeast vacuolar carboxypeptidase Y resides in the propeptide. *Cell.* 48:887–897.

Vogel, J. L., L. M. Misra and M. D. Rose. 1990. Loss of BiP/GRP78 function blocks translocation of secretory proteins in yeast. *J. Cell. Biol.* 110: 1885–1895.

VonHeijne, G. 1981. Signal sequences. The limits of variation. *J. Mol. Biol.* 184:99–105.

VonHeijne, G. 1990. The Signal Peptide. *J. Membr. Biol.* 115:195–201.

Yamamoto, T. J., J. McIntyre, S. M. Sell, and C. Georgopoulos. 1987. Enzymology of the pre-priming steps in λdv DNA replication in vitro. *J. Biol. Chem.* 262:7996–7999.