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

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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 S. 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 (ppof) both in vivo and in vitro (Chirico et al., 1988; Deshaies et al., 1988). Yjlp, a cytosolic DnaJ homologue, is also required for the efficient translocation of ppof 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 genes 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 Ssslp, 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 Ssslp in vivo leads to a dramatic defect in the translocation of both soluble and membrane proteins (Esnault et al., 1993). Antibodies directed against Ssslp 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.,...
1992). Sec63p contains an ER luminal 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 NH2 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 (ppof) into reconstituted proteoliposomes (Brodsky and Schekman, 1993). When proteoliposomes are derived from wild-type membrane proteins, ppof 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 is 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 (ura3-32/ura3-32 lys2-801/lys2-801 ade2-101/ade2-101 his3A200/his3A200 trplA63/trplA63 leu2A12/leu2A12), YS151 (leu2-3-112/leu2-3-112 pep4-3/sec63-1 MATa), YPH500 (ura3-32 lys2-801 ade2-101 his3A200 leu2A12 trplA63 MATa), YPH525 (ura3-32 lys2-801 ade2-101 his3A200 trplA63 MATa), YPH620 (ura3-32 lys2-801 ade2-101 his3A200 leu2A12 trplA63 MATa), YPH621 (ura3-32 lys2-801 ade2-101 his3A200 leu2A12 trplA63 SEC72), YPH622 (ura3-32 lys2-801 ade2-101 his3A200 leu2A12 trplA63 SEC72), YPH623 (ura3-32 lys2-801 ade2-101 his3A200 leu2A12 trplA63 SEC72), YPH624 (ura3-32 lys2-801 ade2-101 his3A200 leu2A12 trplA63 SEC72)

**Protein Purification, Protein Fragmentation, and Peptide Sequence Analysis**

Purification of the Sec63p complex: RSY587 was grown to midlog phase (between 2 and 10 OD600/mI) in YPF medium (2% bacteropetone, 1% yeast extract, 2% glucose; Difco Laboratories, Detroit, MI). Cells were harvested at 3,000 g in a QSA rotor and were washed one time in H2O. Cells were resuspended to 100 OD600/ml in lysis buffer (200 mM mnnitol, 100 mM NaCl, 25 mM sodium phosphate, pH 7.4, 1 mM MgCl2, 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 3,000 g to remove unbroken cells, followed by centrifugation for 15 min at 12,000 g. The medium speed pellet fraction was used for a 50 OD600 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 cyclized 10 times through a 5-ml human c-myc monoclonal antibody column (Evan et al., 1985) that was made by coupling 10 μg 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 0.5 ml 8 M urea, 100 mM (NH4)2CO3, reduced with 10 mL 0.2-mercaptoethanol, and separated by reverse phase HPLC on an RP-300 C8 column (ProBobb; Applied Biosystems, Inc., Foster City, CA). Sec72-containing fractions were transferred to membranes (ProBobb; 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′ GGGAATTCGTGT-ACNYTNGARTAY 3′ (where N is any nucleotide and Y is C or T) together with the reverse complementary sequence of the peptide TLEYNANS (underlined in Fig. 1) were used to screen a plasmid library containing the NANS codons of the NH2-terminal peptide. A full-length copy of the corresponding gene was cloned by screening a YEpl3-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′ ACCTNGGAATATAAATGCAAA-ACATGAATC 3′ (corresponding to the peptide TLEYNANS) that was end labeled by reaction with polynucleotide kinase and [32P]dATP. Colony hybridization (Ausubel et al., 1987) was performed at 50°C in 6× SSC, 1× Denhardt’s solution, 0.1 μg/ml yeast tRNA, 0.02% 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, 0.02% sodium pyrophosphate. Filters were exposed to film at -70°C for 24–48 h, and positive colonies were rescued 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.
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. This 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 representative spores were isolated and used as template in PCR using the primers 5' GCGCTCTTTTCTTTTGAG 3' (corresponding to nucleotides 51-68 and 382-365 of Fig. 1). The PCR products were separated on 1% agarose gels.

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 CYP (Stevens et al., 1982) were used at 1 μl serum/OD 600 cell equivalent. Inverse (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 were used at 1 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 (HIM; Beckman Instruments, Inc., Fullerton, CA) and the low speed supernatant fraction was diluted with 1/10 of one of the following: 5 M NaCl; 8 M urea; 1 M Na 2 CO 3, pH 10.5; 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 \(\Delta sec63::HIS3\) 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., 1993), except that no Sec61p was detected in the eluate. The eluate was blotted onto polyvinylidifluoride 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. NH\(_2\) terminal amino acid sequencing. A 14-amino acid sequence derived from p23 was 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 \(S. cerevisiae\). 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 ascii 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°C) 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~0 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.

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 Na2CO3, 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.

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 immunoprecipitated with anti-Sec72p antiserum. The precipitates were subjected to SDS-PAGE and fluorography. Quantification was performed using a PhosphoImager (Molecular Dynamics, Inc).
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 see72 cells did not contain see72, 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 see72 cells is not because of the absence of Sec71p in the cell: Sec71p was approximately equally abundant in wild-type and see72 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 a 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 see72 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 proteins in vivo. The see72 strain was shown to accumulate this species exclusively. The data in Fig. 6 B shows that see72 was not defective in the translocation and secretion of the periplasmic enzyme...
peptide sequence according to Kaiser et al. (1987) is MNSPKK-

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 OD600 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 35S-Translabel for 15 min.
Glass bead extracts were immunoprecipitated with antiinvertase
antiserum and resolved by 15% SDS-PAGE, and visualized by
fluorography. Tunicamycin (lane 3) was added to a final concentra-
tion of 10 µg/ml 10 min before labeling, pre-inv, Pre-4H11 inver-
tase; ER-inv, ER-modified forms of 4H11-invertase. The leader
peptide sequence according to Kaiser et al. (1987) is MNSPKK-
DIHTTPLKYGGHQRGASCFFIMCLLYRICGICGDPRGM.

For comparison, the leader peptide of preproCPY is MKAFTS-
AIAKISA (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
35S-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, glycosyl-
ated forms of CPY-invertase.

invertase. Invertase is translocated as a preprotein that un-
dergoes signal peptide cleavage and core glycosylation in the
ER. Upon transport to the Golgi apparatus, the core oligosac-
charides become heterogeneously outer chain glycosylated.
To assay invertase maturation, we derepressed cells for in-
vertase expression in 0.1% glucose for 3 h. Cultures were di-
vided 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 pro-
cessed as in Fig. 6 A. Wild-type cells and sec72 cells both
accumulated the Golgi and secreted forms of invertase (Fig.
6B, lanes 1, 2, 4 and 5) at 25°C or 37°C. When the wild-type
immunoprecipitates were treated with the enzyme endogly-
cosidase H, which removes N-linked sugar residues, all
forms of invertase collapsed to a mobility consistent with the
signal-processed unglycosylated invertase (Fig. 6B, 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-I allele they isolated accumulated un-
translocated CPY, but not invertase or Kar2p.

Analysis of Invertase Chimeric Proteins
Because the sec72 null strain affected the maturation of secre-
try 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 sig-
nal 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 pre-
viously 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 lys-
ates 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 tunica-
mycin was added before pulse labeling, an unglycosylated
form of invertase with a mobility consistent with that ex-
pected 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 ex-
periment 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
35S-Translabel. Wild-type cells showed predominantly
the ER and Golgi forms of the CPY-invertase chimera pro-
tein, 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 discrimi-
nates secretory proteins at the level of their signal peptide,
rather than at the mature region.

Discussion
Four integral membrane proteins required for secretory pro-
translocation, Sec61p, Sec62p, Sec63p, and Sec71p, ex-
ist 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 pro-
tein (now called Sec72p) of the Sec63p complex. The nucleo-

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CPY-invertase chimera plasmid were pulse labeled for 15 min with
35S-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, glycosyl-
ated forms of CPY-invertase.
length conservation for leader peptides from the same organ-
amino terminus, a central hydrophobic domain, and a more
ism has been found; however statistical analysis suggests that
shown that a mutation in this domain abolishes the function
of a signal peptide (VonHeijne 1981, 1990).

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 pro-
tease CPY. In addition, sec72-1 was isolated as a noncondition-
tal mutant in a selection designed to identify translo-
cation 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 in-
ternal hydrophobic segments as signal peptides (Green et al.,
1992).

Sec72p is required for the posttranslational translocation of
ppaf into translocation-competent proteoliposomes, al-
though it is not required for ppaf 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 pro-
teins of the translocation apparatus either to remain stably
associated during the solubilization procedure or to reassoc-
iate 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 ef-
iciency of transfer of unusual or "difficult" secretory precurs-
ors 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 pre-
cursors, 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 translo-
cation 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 organ-
ism 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 sig-
nal peptide sequences. Kaiser et al. took an empirical ap-
proach 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 car-
bon source. Analysis of the successful signal sequences indi-
cated 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
occurring 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 leu-
cines, 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 transloca-
tion 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.

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