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 luminal, 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 SSA4 gene family, are required for the posttranslational translocation of pre-pro-α-factor (ppaf) both in vivo and in vitro (Chirico et al., 1988; Deshaies et al., 1988). Ydjlp, a cytosolic DnaJ homologue, is also required for the efficient translocation of ppaf 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 luminal 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 Ssp1p, 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-I 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.,
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 (Norton 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 Dnak 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 (Brody and Schekman, 1993). BiP is not retained in the complex purified from a strain that harbors a mutation in the Dnak domain of SEC63 (Brody and Schekman, 1993).

**Materials and Methods**

### Strains, Materials, Plasmids, and General Methods

The following strains were used in this study: YPH501 (ura3-52 lys2-801 ade2-101 his3A200 trplA63 leu2-112 met15-1), YPH500 (ura3-52 lys2-801 ade2-101 his3A200 trplA63 leu2-112 met15-1), RSY925 (ura3-52 lys2-801 ade2-101 his3A200 trplA63 leu2-112 met15-1), RSY926 (ura3-52 lys2-801 ade2-101 his3A200 trplA63 leu2-112 met15-1), RSY927 (ura3-52 lys2-801 ade2-101 his3A200 trplA63 leu2-112 met15-1), RSY907 (ura3-52 leu2-3,112 pep4::URA3 MATa), and RSY587 (ura3-52 lys2-801 ade2-101 his3A200 trplA63 sec72::HIS3 MATa) containing the SEC63-c-myc plasmid pDF41 (Feldheim et al., 1993). *E. coli* DH5α harboring plasmid pUV5-GS was used to isolate lytic β3, glycanase (Shen et al., 1991). Yeast cells were grown in rich or minimal medium as described previously (Feldheim et al., 1992). Plasmid pDP59 contains a 3.3-kb EcoRI fragment containing SEC72 and a 170-bp deletion in the SEC72 open reading frame (Sherry and Botstein, 1990). The barcoded peptide Y-invertase chimeric plasmid pCYS50, has been described, as has the random leader peptide, 4H1-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 OD600/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 H2O. Cells were resuspended to 100 OD600/ml in lysis buffer (200 mM mannitol, 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 (100 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 10,000 g for 30 min to remove insoluble material. Solubilized proteins were cyclized 10 times over a 5-m median c-myc monoclonal antibody column (Evans et al., 1985) that was made by coupling 10 mg purified human c-myc antibody to 5 ml Swell Agar gel resin according to the manufacturer's instructions (Bioprobe International, Inc., Tuske, 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 M cold acetone, resolubilized in 0.1 ml 8 M urea, 100 mM (NH4)2CO3, 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). Sec72-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 sequenator (2.1 × 150 mm; Applied Biosystems, Inc.). A single sequence, VTELYANNSKLITA (underlined in Fig. 1), was obtained. The degenerate oligonucleotide primers 5' GGGGAATTCGTT-ACNYTNGARTAY 3' and 5' CTTGGGATCCNCNCTDATYYAYTT 3' corresponding to all codon possibilities of VTELY 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 (Stratagene, La Jolla, CA). The gene disruption plasmid pDF68 was constructed as follows: a 145-bp BclI fragment was removed from pDP59. An 1.8-kb BamHI fragment containing the HIS3 gene of pJJ250 (Jones and Prakash, 1990) was ligated to the cleaved fragment of pDP59. The carboxy terminal Y-invertase chimeric plasmid pCYS50 has been described, as has the random leader peptide, 4H1-invertase (Johnson et al., 1987; Kaiser and Botstein, 1990).
**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.

**Radio labeling 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/OD600 cell equivalent, invertase (Schaner et al., 1992). Antibodies raised against CPY (Stevens et al., 1982) and Kar2p (Jeff Brodsky, Department of Molecular and Cell Biology, Howard Hughes Medical Institute, University of California at Berkeley) at 2 µl serum/OD600 cell equivalent were used at 1 µl serum/OD600 cell equivalent, invertase (Schauer et al., 1985) at 2 µl serum/OD600 cell equivalent, α-factor (A. Eun, Department of Molecular and Cellular Biology, Howard Hughes Medical Institute, University of California at Berkeley) 4 µl serum/OD600 cell equivalent, SeC72p at 5 µl serum/OD600 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/OD600 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 OD600 cells were lysed by agitation with glass beads in 2 ml of buffer G (0.1 M sorbitol, 50 mM KAc, 2 mM EDTA, 20 mM Hepes, pH 7.4, 1 mM DTT, 1 mM PMFS). Homogenates were collected at 370 g for 4 min in a microcentrifuge (E34 Beckman Instruments, Inc.). Pellet fractions were resuspended to an equal volume in buffer G, and samples were prepared for SDS-PAGE. Aliquots (0.5 OD600 cell equivalents) were applied to each lane. Transfer of proteins from SDS-PAGE to nitrocellulose was performed as described previously (Harlow and Lane, 1988). Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits. Peptide (200 µg) was injected subcutaneously into rabbits.

**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 NH2-terminal peptide generated from microsequencing of purified Sec72p is underlined. The sequence accession number is L29340.
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 Δsec63::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 (Deshayes et al., 1991), except that no Sec6p 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 NH2-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. Microsequence analysis, we produced a strain in which a BclI fragment between bp 130-274 of the gene encoding 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 proteins required for translocation (Green et al., 1992; Fang et al., 1992, 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 (Deshayes 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).

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). Sec6lp 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 35S-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...
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 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 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 proteins in vivo. The data in Fig. 6 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 pre-CPY. 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 5. sec72 cells are defective in Sec63p complex formation. [35S]-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.

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 [35S]-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 OD600 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 [35S]-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.
peptide sequence according to Kaiser et al. (1987) is MNSPKK-

and 5) cells containing the 4H11-invertase signal peptide chimera

glass bead extracts were immunoprecipitated with antiinvertase

For comparison, the leader peptide of preproCRY is MKAFTS-

ER. Upon transport to the Golgi apparatus, the core oligosac-

invertase. Invertase is translocated as a preprotein that un-

to 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

immunoprecipitated with antiinvertase antiserum and pro-

Analysis of Invertase Chimeric Proteins

Because the sec72 null strain affected the maturation of secretory proteins discriminatingly, we wished to determine

Discussion

Four integral membrane proteins required for secretory pro-

Figure 7. Signal peptide-invertase chimeras accumulate in sec72

cells. (A) 4H11-invertase: wild-type (lanes 1–3) or sec72 (lanes 4

Discussion

Four integral membrane proteins required for secretory pro-

to the mature region of invertase. Fig. 7 A shows that 4H11-

Taken together, these data indicate that a null mutation in

Four integral membrane proteins required for secretory pro-

invades ER-modified forms of 4H11-invertase. The leader

Because the sec72 null strain affected the maturation of secretory proteins discriminatingly, we wished to determine

Four integral membrane proteins required for secretory pro-

The ER and Golgi forms of the CPY-invertase chimera pro-

To assay invertase maturation, we derepressed cells for in-

invertebrate. Invertase is translocated as a preprotein that un-

to ensure signal peptide cleavage and core glycosylation in the

Four integral membrane proteins required for secretory pro-

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 propro into translocation-competent proteoliposomes, although it is not required for propro 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 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 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.

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