A Yeast DnaJ Homologue, Scjlp, Can Function in the Endoplasmic Reticulum with BiP/Kar2p Via a Conserved Domain That Specifies Interactions with Hsp70s

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Abstract. Eukaryotic cells contain multiple Hsp70 proteins and DnaJ homologues. The partnership between a given Hsp70 and its interacting DnaJ could, in principle, be determined by their cellular colocalization or by specific protein–protein interactions. The yeast SCJ1 gene encodes one of several homologues of the bacterial chaperone DnaJ. We show that Scjlp is located in the lumen of the endoplasmic reticulum (ER), where it can function with Kar2p (the ER-lumenal BiP/Hsp70 of yeast). The region common to all DnaJ homologues (termed the J domain) from Scjlp can be swapped for a similar region in Sec63p, which is known to interact with Kar2p in the ER lumen, to form a functional transmembrane protein component of the secretory machinery. Thus, Kar2p can interact with two different DnaJ proteins. On the other hand, J domains from two other non-ER DnaJs, Sislp and Mdjlp, do not function when swapped into Sec63p. However, only three amino acid changes in the Sislp J domain render the Sec63 fusion protein fully functional in the ER lumen. These results indicate that the choice of an Hsp70 partner by a given DnaJ homologue is specified by the J domain.

One major class of constitutive and stress-induced proteins in all cell types consists of ATP-binding proteins of 70 kD termed Hsp70s. Members of the Hsp70 family are found in the bacterial cytoplasm as well as in subcompartments of the eukaryotic cell. One function of Hsp70s is to mediate the folding and assembly of protein complexes (Pelham, 1986). BiP, the Hsp70 of the ER lumen (Murphy and Pelham, 1986), binds to newly synthesized proteins as they pass across the ER membrane from their site of synthesis in the cytoplasm (Sanders et al., 1992). Cytosolic Hsp70s stimulate protein translocation across ER and mitochondrial membranes (Deshaies et al., 1988; Murakami et al., 1988; Chirico et al., 1988). Heat shock proteins in the mitochondrial matrix are involved in import, proper folding, and assembly of proteins once they have crossed the inner membrane (Kang et al., 1990).

In Escherichia coli, the activity of DnaK, the cytoplasmic Hsp70, is modulated by DnaJ and GrpE. Together, DnaK, DnaJ, and GrpE are necessary for proper assembly of proteins at the origin of λ DNA replication (Liberek et al., 1989; Zylarcz et al., 1989; Alfano and McMacken, 1989) and similarly for PI plasmid replication (Wickner et al., 1991; Wickner et al., 1992). DnaK and DnaJ together are also important for protein export (Wild et al., 1992) and folding of nascent polypeptides (Langer et al., 1992; Hendrick et al., 1993). One proposal is that DnaJ is necessary to stimulate the ATPase activity of DnaK, which in turn drives protein assembly/folding reactions (Liberek et al., 1991). A second nonexclusive possibility is that DnaJ helps to target DnaK to its substrate.

Only within the past 5 y have eukaryotic DnaJ homologues been identified, thus generalizing the DnaK–DnaJ interaction of eukaryotes. The first DnaJ of yeast Saccharomyces cerevisiae to be described with Sec63p (also termed NpUp), a membrane protein component of the ER translocation machinery (Sadler et al., 1989; Rothblatt et al., 1989; Feldheim et al., 1992; Kurihara and Silver, 1992). Sec63p contains only 70 amino acids that are similar to the NH2 terminus of E. coli DnaJ. These amino acids are embedded between two membrane-spanning regions and exposed on the luminal side of the ER membrane where they interact with Kar2p, the yeast BiP homologue (Normington et al., 1989; Rose et al., 1989; Brodsky and Schekman, 1993; Scidmore et al., 1993). These findings led to the prediction that this region forms the J domain, which is sufficient for interaction of DnaJs with Hsp70s. Sequence comparisons of additional DnaJs have supported this proposal (Silver and Way, 1993). Moreover, the NH2-terminal 108 amino acids of E. coli
DnaJ were recently shown to be sufficient to stimulate DnaK ATPase activity (Wall et al., 1994).

Other DnaJs from yeast include Sislp, located in the cytoplasm and the nucleus (Lutke et al., 1991; Zhong and Arndt, 1993), Ydjlp, located in both the cytoplasm and the outer surface of the ER membrane (Caplan and Douglas, 1991; Atencio and Yaffe, 1992), and Mdjlp, located in the mitochondrial matrix (Rowley et al., 1994). Each of these can be predicted to interact with particular Hsp70s at each location and has been shown directly for Ydjlp (Cyr et al., 1992; Cyr and Douglas, 1994) and Sec63p (Brodsky and Schekman, 1993; Scidmore et al., 1993). It may be that each DnaJ interacts with a certain Hsp70 and/or that specificity is achieved by colocalization.

The SCJ1 gene of the yeast S. cerevisiae encodes a protein with 40% identity to bacterial DnaJ over its entirety (Blumberg and Silver, 1991). SCJ1 could encode 27 NH2-terminal amino acids that were suggested to act as a mitochondrial targeting sequence. These are followed by a second in-frame methionine and ~20 additional amino acids that could function as a signal sequence for translocation across the ER. Furthermore, the ER retention sequence, KDEL (Munro and Pelham, 1987), occurs at the Scjlp COOH terminus, although HDEL is the preferred signal in yeast (Semenza et al., 1990).

We now present data indicating that most, if not all, of Scjlp is located in the lumen of the ER, where it interacts with Kar2p/Bip. Furthermore, by replacing the J region of Sec63p with that from Scjlp, Sislp, and Mdjlp, we show that the J domain contains the information necessary for specific interaction with its Hsp70 partner.

Materials and Methods

Plasmids and Yeast Strains

Plasmids YEpSCJI and YEpSCJILacEOORI (SCJ1 truncated after codon 273) were described before (Blumberg and Silver, 1991). Plasmid YCpSCJI contains the 1700-bp KpnI/SnaBI fragment of YEpSCJI (550-bp 5' sequence, 1134-bp coding sequence, and 15-bp 3' sequence) in the SmaI site of the CEN6/LEU2 plasmid pRS315 (Sikorski and Hieter, 1989). Plasmid YCpgSCJI contains an oligonucleotide-derived insertion (5'-CA~T'I~T-CA~GTACGAATTCG-3') into the unique NcoI site of YCpSEC63-NC. Plasmid pSP-SCJI contains the PCR-generated coding sequence of SCJI was sequenced by the chain termination method (Sanger et al., 1977) to confirm the absence of mutations. A maltose-binding protein (MBP)-Scjlp fusion protein is encoded by pMAL-SCJ1. The coding sequence of SCJI was inserted into YCpSEC63-NC, creating fusions with the SEC63 read-frame. The entire coding region of SEC63 and all the J segments inserted into YCpSEC63-NC, creating fusions with the SEC63 reading frame. The entire coding region of SEC63 and the J region of SCJ1, Sislp, and Mdjlp was sequenced in their J region to confirm the mutations and used for transformation of temperature-sensitive sec63 strains.

Production of Antibodies

Antibodies specific to Scjlp were prepared as follows. MBP fused to Scjlp (MBP-Scjlp) was purified from an E. coli overproducing strain by amylose affinity chromatography according to the instructions of the manufacturer (New England Biolabs, Beverly, MA) and injected into New Zealand white rabbits. Antibodies were purified from sera by ammonium sulfate precipitation, dialysis into PBS, and absorption against an E. coli extract coupled to CNBr-activated sepharose followed by a yeast extract from a strain missing SCJ1 (PSY713) similarly coupled to CNBr-activated Sepharose. Finally, the antibodies were affinity purified by chromatography on a column containing MBP-SCJ1 coupled to CNBr-activated Sepharose. The generation of other antibodies is described in Rowley et al. (1994) and Zollner et al. (1992).

Cell Fractionation

Mitochondria were prepared from wild-type strain D273-10B grown overnight in lactate medium and harvested at 1.5 A260nm according to the procedure of Daum et al. (1982). Isolated crude mitochondria were stored in buffer A (20 mM Hepes, pH 7.2, 0.6 M sorbitol) containing 10 mg/ml fatty acid-free BSA. Further purification by density gradient centrifugation was according to Lewin et al. (1990). The following published procedures were used: preparation of microsomal membranes and cytosol (Caplan and Douglas, 1991) with modification by Rowley et al. (1994); and digitonin fractionation (Hartl et al., 1986).

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1. Abbreviation used in this paper: MBP, maltose binding protein.
Figure 1. Characterization of anti-Scjlp antibodies. Yeast cell extracts from PSY173 (ΔSCJ1) containing SCJ1 on a CEN plasmid (lane 1); a partial deletion of SCJ1 on a 2μ plasmid (lane 2); SCJ1 on a 2μ-containing plasmid (lane 3); or no SCJ1 (lane 4) were electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with affinity-purified polyclonal antibodies raised against Scjlp. Molecular mass markers are in kilodaltons.

Immunofluorescence with the anti-Scjlp antibodies was used to localize Scjlp. In wild-type cells, the antibodies stained predominantly a region around the rim of the nucleus (Fig. 2, A–C). A similar staining pattern is observed when yeast cells are probed with antibodies against other ER-localized proteins (e.g., Rose et al., 1989; Kurihara and Silver, 1993). No staining was observed when anti-Scjlp antibodies were used to probe cells missing SCJ1 (Fig. 2, D–F).

To further investigate the subcellular localization of Scjlp, fractions enriched in mitochondria, ER, and cytosol were analyzed by immunostaining for the abundance of Scjlp and various marker proteins. Scjlp was found in both mitochondrial and ER fractions, but was not present in the cytosol (Fig. 3 A). The protein was enriched in the ER fraction similar to a marker protein for this compartment (Kar2p), whereas cytochrome c1 heme lyase (CC1HL) of the mitochondrial inner membrane was detectable only within the mitochondrial fraction. This suggested that at least some Scjlp cofractionated with the ER. However, the data left open whether the presence of Scjlp in the mitochondrial fraction was exclusively due to contaminating ER membranes. We therefore asked whether part of Scjlp present in mitochondria can be localized to one of the organelle’s subcompartments. To this end, isolated mitochondria were subfractionated by treatment with digitonin, which preferentially solubilizes the mitochondrial outer membrane (as well as the membrane of the ER), whereas the mitochondrial inner membrane is only solubilized at higher concentrations of digitonin (Hartl et al., 1986). As a result, soluble components are sequentially released from the organelles, while membrane-bound proteins become susceptible to attack by added protease. As shown in Fig. 3 B, Scjlp became accessible to protease at low concentrations of digitonin. Thus, it behaved like proteins located in the intermembrane space.

Results

Localization of Scjlp

To better establish the intracellular distribution of Scjlp, high-titer affinity-purified anti-Scjlp antibodies were generated against a MBP–Scjlp fusion protein purified from an E. coli overproducing strain. These antibodies do not bind to any proteins on immunoblots when used to probe a yeast extract from a strain deleted for SCJ1 (Fig. 1, lane 4). However, when the same strain carried SCJ1 on a single-copy plasmid, a 40-kD protein was recognized that corresponds to Scjlp (Fig. 1, lane 1). Cells containing SCJ1 on a multicopy plasmid produce more Scjlp (Fig. 1, lane 3). Extracts from cells bearing a plasmid encoding a truncated version of Scjlp contain a 31-kD protein (Fig. 1, lane 2).

Figure 2. Localization of Scjlp by immunofluorescence. Wild-type (W303) cells (A–C) or cells missing SCJ1 (D–F) were prepared for immunofluorescence and probed with anti-Scjlp rabbit antibodies followed by FITC-conjugated anti-rabbit antibodies (B and E). A and D show cells stained with the DNA-specific dye DAPI (4′,6-diamidino-2-phenylindole), and C and F show cells viewed by Nomarski optics.
The mitochondrial fraction was also analyzed for the indicated marker proteins after further purification by density gradient centrifugation (DGC). Proteins were visualized by immunostaining of nitrocellulose blots with antibodies against CCIHL, Scjlp, Kar2p, and fructose 1,6-bisphosphatase (FBP). (B) Digitonin fractionation of the mitochondrial fraction. 200 μg mitochondria in 20 μl of buffer A were mixed with the indicated concentrations of digitonin (added from a 20-fold stock solution in buffer A). Treatment for 3 min at 0°C was in the presence or absence of 125 μg/ml proteinase K (PK). Samples were diluted by adding 4 vol of buffer A. After 30 min on ice, 1 ml of buffer A containing 1 mM PMSF was added, and membranes were collected by centrifugation for 5 min at 9,000 g. Analysis of the indicated marker proteins was as in A. Data were quantitated by densitometry. Cyt b2, cytochrome b2; Mpilp, a mitochondrial matrix protein; Kar2p, ER lumen.

To further evaluate the distribution of Scjlp, the mitochondrial fraction was subjected to additional purification by density gradient centrifugation (Fig. 3 A; Lewin et al., 1990). This procedure removes most contaminating membranes as well as damaged mitochondria. Purification resulted in at least a 25-fold depletion of both Scjlp and Kar2p, whereas the content in CCIHL was virtually unchanged. Taken together, these results support a localization for Scjlp primarily in the ER. Thus, the previous report of a mitochondrial localization of Scjlp (Blumberg and Silver, 1991) is explained by a high-ER contamination of the mitochondria used in those studies. Support for at least a partial ER location also came from earlier results showing that fusion proteins containing the Scjlp NH2 terminus fused to invertase are Suc*, suggesting that the NH2 terminus could function as a signal for ER translocation (Blumberg and Silver, 1991).

Scjlp is normally not glycosylated, as determined by a lack of potential N-linked glycosylation sites and its behavior during gel electrophoresis, where it migrates at 40 kD, the predicted molecular mass (Fig. 4 B, lane 1). To further test the idea that Scjlp is in fact in the lumen of the ER, a mutated version that contains a consensus site for glycosylation was constructed by inserting the amino acids GNSSH between His173 and Gly174 (Fig. 4 A). When the mutated gene was expressed in yeast that otherwise lacked SCJ1, the mutant Scjlp (gScjlp) migrated slower (at 43 kD) than wild-type Scjlp when extracts were assayed by gel electrophoresis and immunoblotting with anti-Scjlp antibodies (Fig. 4 B, lane 2). When cells were incubated with the glycosylation inhibitor tunicamycin, the mutated Scjlp shifted in molecular mass (Fig. 4 C, lanes 5–8) and migrated like wild-type unglycosylated Scjlp (Fig. 4 C, lanes 1–4). Enzymatic digestion of the mutant Scjlp by EndoH yielded a protein that migrates at 41 kD consistent with removal of glycosyl units (data not shown). These data indicate that most, if not all, Scjlp must gain access to the lumen of the ER, where the mutant protein is glycosylated.

Tunicamycin treatment stimulates the production of Scjlp (Fig. 4 C, lanes 1–4). Wild-type Scjlp levels increase approximately fourfold (as determined by densitometry) after 4 h of tunicamycin treatment. This is consistent with the

Figure 4. Glycosylation of mutant form of Scjlp. (A) The signal sequence, the J domain, the glycine-rich region, and the COOH-terminal ER retention signal Lys-Asp-Glu-Leu are indicated. The exact cleavage site of signal peptidase is not known. The sequence of the glycosylated form of Scjlp contains the insertion Gly-Asn-Ser-His between amino acids 173 and 174. (B) Cell extracts prepared from yeast strain ΔScjlp bearing plasmid YCpSCJ1 encoding Scjlp (lane 1) or YCpSCJ1 encoding the glycosylated derivative gScjlp (lane 2) were analyzed by SDS-PAGE and immunoblotting with anti-Scjlp antibodies. (C) Immunoblot using anti-Scjlp antibodies on ASCJ1 cells transformed with YCpSCJ1 (lanes 5–8) and migrated like wild-type unglycosylated Scjlp (Fig. 4 C, lanes 1–4). Enzymatic digestion of the mutant Scjlp by EndoH yielded a protein that migrates at 41 kD consistent with removal of glycosyl units (data not shown). These data indicate that most, if not all, Scjlp must gain access to the lumen of the ER, where the mutant protein is glycosylated.
Figure 5. Potential UPRE in the promoter region of SCJ1. The previously identified UPRE of KAR2 was aligned to the 5'-region of the SCJ1. Identical nucleotides are boxed, and the numbers refer to the distance from the translation start.

presence of a sequence in the SCJ1 promoter region with similarity to an unfolded protein response element (UPRE) of the KAR2 promoter (Mori et al., 1992; Kohno et al., 1993; Fig. 5).

Scjlp Is Cleaved upon Import into the ER

Scjlp produced by translation in a reticulocyte lysate migrates slightly slower than wild-type Scjlp (Fig. 6, lane 1) and is fully sensitive to protease treatment (Fig. 6, lane 2).

In the presence of canine pancreas microsomes, the in vitro synthesized Scjlp is processed to a smaller species as determined by relative migration on SDS-polyacrylamide gels (Fig. 6, lane 3). In addition, the lower processed form becomes resistant to added protease (Fig. 6, lane 4) unless detergent is added to disrupt the microsomal membranes (Fig. 6, lane 5). These results are consistent with Scjlp having a cleavable signal sequence that targets it across the microsomal membrane.

A higher molecular weight form of Scjlp accumulates in yeast KAR2 mutants that are thermosensitive for protein translocation across the ER membrane. Extracts were prepared from kar2-159 and sec18 mutants grown at either the permissive temperature of 23°C or for 2.5 h at the nonpermissive temperature of 37°C and examined by immunoblotting for the presence of Kar2p (Fig. 7, top) and Scjlp (Fig. 7, bottom). As has been previously observed (Vogel et al., 1990), pre-Kar2p accumulates in kar2-159 cells that were incubated at the nonpermissive temperature (Fig. 7, lane 4). Similarly, a protein comigrating with the in vitro synthesized precursor was observed in extracts from kar2-159 cells grown at the nonpermissive temperature (Fig. 7, lane 4). This result is consistent with the notion that Scjlp is made as a higher molecular weight precursor in vivo and that its ability to be processed and translocated into the ER is blocked in KAR2 mutants. Mitochondrial precursor proteins, such as pre F1β-ATPase, did not accumulate in kar2-159 cells at the nonpermissive temperature (data not shown). Some preScjlp was also observed to accumulate in sec61 mutants at the nonpermissive temperature (data not shown).

On the other hand, no obvious accumulation of preScjlp or preKar2p was observed in mutants of SEC18 (Fig. 7, lane 2), which acts later in the secretory pathway (Novick et al., 1981).

Scjlp Can Interact with Kar2p Via a Region Conserved in All DnaJs

Because DnaJs are predicted to interact with Hsp70s, Kar2p, the Hsp70 of the ER lumen, is the likely functional partner of Scjlp. No other Hsp70 has been reported to reside within the ER lumen. To test this idea, we attempted to construct double mutants containing a deletion of SCJ1 (∆SCJ1) and temperature-sensitive alleles of SEC61, SEC62, SEC63, KAR2, SSC1, and SEC71, the double mutants were always viable at both 25°C and 30°C. The same was true for double mutants containing ∆SCJ1 and the temperature-sensitive kar2-1 and kar2-133 alleles (Scidmore et al., 1993); they were viable at 25°C and 30°C (Table I). In contrast, double mutants deleted for SCJ1 and containing the kar2-159 thermosensitive allele (Vogel et al., 1990) were not viable, suggesting a synthetic lethal relationship (Table I).

Taken together, these data suggests that Scjlp is located in the ER lumen where it can interact with Kar2p, the lumenal Hsp70 cognate of yeast. Sec63p is an ER membrane protein that has been shown to interact with Kar2p by both genetic and biochemical means (Brodsky and Schekman, 1993; Scidmore et al., 1993). Sec63p spans the ER membrane three times with the region of DnaJ homology located in the ER lumen, but essentially all of the remainder of the protein is located in the cytoplasm (Figs. 8 A; Feldheim et al., 1992; Kurihara and Silver, 1992). This predicts that the J domain mediates the interaction of Sec63p with Kar2p. Moreover, a single amino acid change in a conserved alanine in this region (Fig. 8 B; Nelson et al., 1993) eliminates the ability of Sec63p to interact with Kar2p (Brodsky and Schekman, 1993). Since this also corresponds to the region common to all DnaJs, we predicted that this domain will mediate interaction between DnaJs and their respective Hsp70 partners (Silver and Way, 1993). The following experiments were designed to test this hypothesis.

A chimeric protein was constructed that contains amino acids 25–89 of Scjlp in place of the lumenal DnaJ domain of Sec63p (Fig. 8, A and B). That this protein was made in...
Table 1. Synthetic Lethal Combinations of Δscj1 and KAR2 Mutations

<table>
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<tr>
<th>Cross</th>
<th>Live/dead*</th>
<th>Genotype of spores†</th>
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<tr>
<td>Δscj1 × kar2-1</td>
<td>4:0 5</td>
<td>2 Δscj1 KAR2, 2 SCJ1 kar2-1</td>
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<tr>
<td></td>
<td>4:0 9</td>
<td>1 SCJ1 KAR2, 1 Δscj1 KAR2, 1 SCJ1 kar2-1, 1 Δscj1 kar2-1</td>
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<td>4:0 2</td>
<td>2 SCJ1 KAR2, 2 Δscj1 kar2-1</td>
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<tr>
<td>Δscj1 × kar2-133</td>
<td>4:0 1</td>
<td>2 Δscj1 KAR2, 2 SCJ1 kar2-133</td>
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<tr>
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<td></td>
<td>3:1 15</td>
<td>1 SCJ1 KAR2, 1 Δscj1 KAR2, 1 SCJ1 kar2-159, Δscj1 kar2-159</td>
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<td>2:0 1</td>
<td>2 SCJ1 KAR2, 2 Δscj1 kar2-159</td>
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* Number of tetrads picked with corresponding (Live/dead) spore segregation at 30°C. All spores were allowed to germinate at 25°C, and then checked for growth at 30°C.
† Genotypes were determined by segregation of URA3 and temperature-sensitive markers. For each double-mutant cross, three genotype combinations are shown. Top to bottom, they represent parental ditype, tetratype, and nonparental ditype.

Crosses of Δscj1 to sec63-1 and sec71-1 strains always yielded four viable spores at room temperature and 30°C, indicating a lack of synthetic lethality.

Yeast cells was confirmed by immunoblots of whole-cell extracts with the anti-Scjlp antibody (Fig. 9 A, lanes 2 and 4). A protein of the predicted size (75 kD) that reacted with anti-Scjlp was present only in cells containing the chimeric gene.

The chimeric gene encoding Sec63-J-Scjlp was introduced into sec63-1 and sec63-101 temperature-sensitive alleles. Each mutant strain cannot grow at nonpermissive temperature of 36°C unless it contains a functional SEC63 gene (Fig. 9, C and D). Mutant strains harboring plasmids expressing the Sec63-J-Scjlp chimeric protein also grew at the nonpermissive temperature, indicating that this protein could replace Sec63p in these strain backgrounds (Fig. 9, C and D). Moreover, Sec63-J-Scjlp could function as the only version of Sec63p in a haploid cell (Fig. 9 A). Cells deleted for SEC63 are inviable. The plasmid encoding Sec63-J-Scjlp was introduced into a heterozygous diploid strain containing only one copy of SEC63. The other copy has been deleted from the chromosome by insertion of the URA3 gene (Sadler et al., 1989). Normally, only the Ura- viable spores are recovered when this strain is sporulated. However, when the diploids containing Sec63-J-Scjlp were sporulated, tetrads containing three and four viable spores were obtained. The Ura+ spores always contained the plasmid bearing the chimeric gene (data not shown).

Swapping of J Domains Defines the Specificity of Their Interaction with Hsp70s

One question with regard to how DnaJs function is how they interact with certain Hsp70s. It could be that each DnaJ can only interact with a certain Hsp70, and this specificity is...
expression of the chimeric proteins was tested by gene fusions and the other containing the J region from Sislp. Since anti-chimeric proteins, one containing the J region from Mdjlp bodies to the J domains of Mdjlp and Sislp do not exist, ex- test these possibilities, we constructed two additional Sec63p in which the conferred by unique amino acid sequences. Alternatively, specificity could be conferred by intracellular location. To test these possibilities, we constructed two additional Sec63p chimeric proteins, one containing the J region from Mdjlp and the other containing the J region from Sislp. Since antibodies to the J domains of Mdjlp and Sislp do not exist, expression of the chimeric proteins was tested by gene fusions in which the SUC2 gene was fused to the SEC63 chimeric genes (Kurihara and Silver, 1992). If expressed, 128-kD proteins should be produced that have the proper membrane orientation and, in the case of Sec63p, can function (Feld-heim et al., 1992). For Sec63-J-Scjlp (Fig. 9 B, lane 2), Sec63-J-Sislp (Fig. 9 B, lane 3), and Sec63-J-Mdjlp (Fig. 9 B, lane 4), all were produced as chimeric proteins of the correct size that reacted with anti-invertase antibodies. This confirmed that the chimeric proteins could be made in yeast.

Each chimeric gene was introduced into sec63-I, sec63-I01, and SEC63/ASEC63 cells. When tested for growth at the nonpermissive temperature, neither the Sec63-J-Mdjlp or the Sec63-J-Sislp chimeric protein could restore growth to sec63-I or sec63-I01 at the nonpermissive temperature (Fig. 9, C and D). Moreover, no viable spores were obtained that were missing the chromosomal copy of SEC63. Taken together, these results indicate that two J domains from non-ER-resident proteins cannot function when placed into Sec63p, but the domain from Scjlp, which is predicted to inter- act with Kar2p, can function.

The sequence of the four J domains used in the swap experiments are presented in Fig. 8 B. Sequence comparison of Sec63p and Scjlp, which both can function with Kar2p, with Mdjlp and Sislp, which do not function, reveals that amino acids at positions 3, 6, 13, 17, 23, and 42 are conserved between Sec63p and Scjlp but differ in Mdjlp and Sislp. To further define the requirements for the J domain to function, we mutated the three most nonconserved amino acids in Sec63-J-Sislp by site-directed mutagenesis to encode the corresponding Sec63 J domain amino acids (Fig. 8 B). The hybrid protein Sec63-J-Sislp containing both mutations Ginl3 to Arg and Lysl7 to Ser were not capable of restoring growth to sec63 temperature-sensitive mutants (Fig. 9 D). The mutated protein where Lys42 was converted to Val is partially functional (Fig. 9 D). However, the Sec63-J-Sislp hybrid containing the three mutations combined (Glnl3 to Arg, Lysl7 to Ser and Lys42 to Val) fully rescues the tem- perature-sensitive growth defect of sec63-I and sec63-I01 strains (Fig. 9 D).

Discussion

We have presented results from several different experiments that show that Scjlp is located in the ER lumen. These data make Scjlp the second DnaJ homologue to be present in the ER lumen along with Sec63p. The J domain of Sec63p can be successfully replaced with that of Scjlp to make a func- tional protein that can interact with Kar2p. Substitution of other J domain into Sec63p results in chimeric proteins that are not functional and allow for the prediction of which amino acids confer specificity to the Kar2p interaction.

Localization of Scjlp in the ER

Scjlp was originally identified by its ability to alter the sort- ing of a normally nuclear targeted protein (Blumberg and Silver, 1991). It was the first eukaryotic DnaJ homologue found to contain similarity to bacterial DnaJs over its entire- ity. However, its actual function in the cell has remained an enigma. This is, in part, because a strain deleted for SCJII has not obvious growth defect, and uptake of proteins into the nucleus, mitochondria, and ER appears normal (Blum- berg, H., and P. A. Silver, unpublished results).
Compared with other DnaJ homologues, Scjlp contains an extra 20 or 45 amino acids at its NH₂ terminus depending on where translation begins. If the first methionine were used, then the first 25 amino acids would have a high degree of similarity to sequences for targeting proteins to the mitochondria. However, initiation at the second methionine would produce a protein with ~20 amino acids that could function as a signal sequence for translocation into the ER. Results presented here show that Scjlp is localized in the ER lumen. Like most proteins that are translocated across the ER membrane, Scjlp is made as a higher molecular weight precursor that is processed to its mature form. Data from both in vitro translation experiments and accumulation of a higher molecular weight precursor in a mutant blocked in ER translocation support this conclusion. Because the in vitro translation product possessed only the second methionine, we conclude that it is the 20 amino acids succeeding it that are acting as the Scjlp signal sequence.

Two DnaJs May Function with Kar2p

Several lines of evidence support an interaction between Sec63p and Kar2p in the ER lumen. Mutant forms of Kar2p can be isolated that restore the ability of sec63-1 J mutants to grow and temperature-sensitive mutations in KAR2 and SEC63 form synthetic lethal combinations (Scidmore et al., 1993). Complexes containing Sec63p and Kar2p can be isolated that can restore ER protein translocation activity to microsomes prepared from a sec63-1 J strain (Brodsky and Schekman, 1993). The association of Kar2p with Sec63p depends on the nucleotide-bound state of Kar2p and is disrupted by a single amino acid change in the J domain present in the sec63-1 J allele (Brodsky and Schekman, 1993; Nelson et al., 1993). These data have led to a model where the association of Kar2p with the translocation machinery is dependent on its ADP-bound state. The hydrolysis of ATP by Kar2p may be stimulated by its interaction with the J domain of Sec63p. On the other hand, it may be that the interaction of Sec63p with Kar2p is simply to position BiP at the site of protein translocation, where it can interact with the nascent chain. Brodsky and Schekman (1993) speculate that, in such a model, a second DnaJ-like activity might exist to stimulate the ATPase activity of BiP in the ER lumen.

Our results suggest that Scjlp is the second DnaJ in the ER lumen that interacts with Kar2p. First, we show that there is a synthetic-lethal relationship between cells missing SCJ1 and kar2-159 mutants. Kar2-159 mutants are blocked in translocation of proteins across the ER membrane (Vogel et al., 1990). On the other hand, cells missing SCJ1 and containing either of two other temperature-sensitive alleles of KAR2, kar2-1 and kar2-133, are viable. These alleles of KAR2 do not affect ER translocation (Vogel, 1993). These results suggest either a direct interaction between Kar2p and Scjlp or that they both participate in similar reactions, as suggested by Brodsky and Schekman (1993).

The second line of evidence that Scjlp can interact with Kar2p comes from results of the J swap experiment. We show that the Scjlp J domain can function in place of the J domain of Sec63p. Since this is the region of Sec63p that must interact with Kar2p, the results of the swap experiment indicate that the Scjlp J domain can do the same, that is, recruit Kar2p to the site of protein translocation. It remains a formal possibility that there is a second Kar2p-like Hsp70 in the ER lumen.

Taken together, we propose a novel situation where two different DnaJs interact with the same Hsp70 through their respective J domains. One model for the role of Scjlp is an extension of that originally proposed by Brodsky and Schekman (1993). Sec63p would bind Kar2p (BiP) and place it at the site of translocation. As nascent proteins emerge from the translocation machinery, BiP is released, converted to its ATP form, and interacts with the emerging nascent chain. Scjlp would then catalyze repeated rounds of ATP hydrolysis that culminate in BiP being converted back to its ADP-bound form and reassociating with Sec63p. The viability of the SCJ1 null mutant could be due to the intrinsic ATPase activity of Kar2p or a third luminal DnaJ homologue. Recently, the mitochondrial Hsp70 has been shown to bind to Mim44p at the inner membrane. It is suggested that Mim44p may act like Sec63p to localize Hsp70 at the site of protein translocation across the mitochondrial membrane (Rassow et al., 1994).
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