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 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 (Munro 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 (Deshai et al., 1988; Mura- kami 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; Zylicz 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 (Sudler 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 (Luke 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 Sec63p 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 Scllp 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 YEpSCJ1 and YEpSCJ1AceoR (SCJ1 truncated after codon 273) were described before (Blumberg and Silver, 1991). Plasmid YCpSCJ1 contains the 1700-bp KpnI/StB1 fragment of YEpSCJ1 (550-bp 5' sequence, 1,134-bp coding sequence, and 15-bp 3' sequence) in the Smal site of the CEN6/LEU2 plasmid pRS315 (Sikorski and Hieter, 1989). Plasmid YCpSCJ2 contains an oligonucleotide-derived insertion (5'-CAGTCGGAACTTCTCG' for a N-linked glycosylation signal) in the unique Ncol site of YCpSCJ1. Plasmid pSfP-SCJ1 contains the PCR-generated coding sequence (starting with the signal sequence) of SCJ1 as a BamHI/XbaI fragment in pSP65 (Promega Corp., Madison, WI). The entire coding region was sequenced by the chain termination method (Sanger et al., 1977) to confirm the absence of mutations. The entire coding sequence of SCJ1 fused to the coding sequence of cysteine inactivates the Psfl/PvuI fragment of Tkt10 (Kuri-

hara and Silver, 1992) was inserted into plasmids YCpSEC63-J-SCJ1, YCpSEC63-J-SISi, and YCpSEC63-J-MDJ1, which were treated with PstI, XhoI, and Klenow polymerase.

The following yeast strains were used: wild-type strain W303 (MATa ura3-52 leu2-3,112 ade2-1 trpl-1), PSY173 (ASCJ1 [Blumberg and Silver, 1991], MATa ura3-52 leu2-3,112 ade2 ade3 SCJ1::URA3, PSI797 (MATa ura3-52 leu2-3,112 sec63-1), PSY76 (MATa ura3-52 leu2-3,112 trpl-1 sec63-100), PSY43 (Sadler et al., 1989; MATa ura3-52 ura3-52-leu2-3,112 ade2-1 trpl DI+ SEC63::URA3), and PSY662 (MATa ura3-52 leu2-3,112 trpl DI SEC63::URA3 + plasmid YCpSEC63-J-SCJ1). YMB12-6D (MATa ura3-52 leu2-3,112 trpl sec63-1), MY176 (MATa ura3-52 kar2-159), M1378 (MATa ura3-52 kar2-133), and M13000 (MATa ura3-52 kar2-1).

Site-directed Mutagenesis

A point mutation in the DNA region coding for the J domain in Sec63-J-Sislp was obtained by a method that relies on homologous recombination in yeast (Ma et al., 1987). Yeast cells were transformed with plasmid YCpSEC63-J-SISi linearized with HindIII (at the 3' border of the J region), and excess amounts of PCR products (200-fold molar excess), which served as substrates for plasmid repair. The mutant J region PCR primer 5'-GCCAACAGGTTACAGAGAAAAGGTITGCGAGTCTCAGAGGCTTTTGAAGATT-3' contains flanking homologous sequence (permitting recombination on the 5' end and priming on the 3' end), contains restriction site BglII, and changes codon 42 (see Fig. 8 B) of the J domain of Sec63-J-Sislp from Lys to Thr. The 3' PCR primer contains SEC63 wild-type sequence. DNA recovered from random yeast colonies was treated as templates for analytical PCR reactions (using an oligonucleotide that anneals to the 5' border of the SISI J region and the 3' SEC63 primer). About 50% of the PCR products contained the new BglII site. Plasmids prepared from E. coli transformants were sequenced in their J region to confirm the mutations and used for transformation of temperature-sensitive sec63 strains. The same strategy was used to mutate Gin 13 to Arg and Lys 17 to Ser. Here the oligonucleotide 5'-GGGATCTCTCAAGGTGCTAATGAGAGAGAAC'IGAAA-TGGAAATT-3' contains the new BglII site. Plasmids YCpSEC63-J-SCJ1, YCpSEC63-J-SIS1, and YCpSEC63-J-MDJI, which were treated with PstI, XhoI, and Klenow polymerase.

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 sephrose followed by a yeast extract from a strain missing SCJ1 (PSY173) 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).

Other Methods

DNA techniques, SDS-PAGE, and immunoblot using the ECL detection kit (Amersham Corp., Arlington Heights, IL) were performed according to standard methods (Sambrook et al., 1989). Immunodotfluorescence was performed as described (Boxx et al., 1992) with anti-Scjlp affinity-purified antibody (1:100) followed with FITC-conjugated anti-rabbit secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at a dilution of 1:500. In vivo transcription using SP6 polymerase (Promega Corp., Madison, WI) and cell-free translation in rabbit reticulocyte lysates.
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.

(Boehringer Mannheim Corp., Indianapolis, IN) in the absence or presence of dog pancreas microsomes (Boehringer Mannheim Corp.) were performed according to the instructions of the manufacturers and as previously reported by Hansen et al. (1986). Protease protection assays (proteinase K concentration 50 μg/ml) were as described in Schlenstedt et al. (1990).

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).

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. 3A). 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. 3B, Scjlp became accessible to protease at low concentrations of digitonin. Thus, it behaved like proteins located in the intermembrane space.
Data were quantitated by densitometry. and membranes were collected by centrifugation for 5 min at 9,000 g. Analysis for the indicated marker proteins was as in A. min on ice, 1 ml of buffer A containing 1 mM PMSF was added, 0°C was in the presence or absence of 125 #g/ml proteinase K mitochondrial fraction. 200 #g mitochondria in 20 #l of buffer A PK).

The mitochondria fraction was subjected to additional purification by density gradient centrifugation (DGC). Proteins were visualized by immunostaining of nitrocellulose blots with antibodies against CC,HL, Scjlp, Kar2p, and fructose 1,6-bisphosphatase (FBP). (B) Digitonin fractionation of the mitochondrial fraction. 200 #g mitochondria in 20 #l 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 #g/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 for 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.

(CC,HL or cytochrome b2) or the ER (Kar2p), but not like the matrix protein Mpilp (Maarse et al., 1992). Treatment of the mitochondrial fraction in hypotonic solution leads to swelling of the mitochondria, thereby rupturing the outer membrane but leaving the ER membrane intact. Protease treatment after osmotic swelling indicated Scjlp was not in the intermembrane space of the mitochondria, suggesting localization in the microsomal contamination (data not shown). 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 CC,HL 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 Hisl73 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 3. Cell fractionation. (A) Analysis of cell fractions. The indicated amounts of protein corresponding to cytosolic, microsomal, or mitochondrial fractions were applied to SDS-PAGE. 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 CC,HL, 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 for 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.

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 ΔSCJ1 bearing plasmid YCPSCJ1 encoding Scjlp (lane 1) or YCpgSCJ1 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 ΔSCJ1 cells transformed with YCpSCJ1 (lanes 1–4) and YCpgSCJ1 (lanes 5–8). Yeast cultures were grown in the presence of 10 μg/ml tunicamycin. Aliquots were removed after the indicated amount of time, and cell extracts were loaded on a 10% SDS–polyacrylamide gel. Molecular size markers are in kilodaltons.
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.

The 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 F$_{1}$β-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 (ΔSCJ1) and temperature-sensitive alleles of SEC61, SEC62, SEC63, KAR2, SSC1 (encoding the mitochondrial matrix Hsp70), and also a strain deleted for SEC71 (Deshaies et al., 1988; Sadler et al., 1989; Rothblatt et al., 1989; Craig et al., 1989; Kurihara and Silver, 1993). For SEC61, SEC62, SEC63, SEC71, and SSC1, the double mutants were always viable at both 25°C and 30°C. The same was true for double mutants containing ΔSCJ1 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 suggest that Scjlp is located in the ER lumen where it can interact with Kar2p, the luminal 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 (Fig. 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 luminal DnaJ domain of Sec63p (Fig. 8, A and B). That this protein was made in
Table I. Synthetic Lethal Combinations of ΔAscjl and KAR2 Mutations

| Cross                  | Live/dead* | Genotype of spores† |
|------------------------|------------|---------------------|
| ΔAscjl × kar2-1        | 4:0 5      | 2 Ascjl KAR2, 2 SCJ1 kar2-1 |
|                        | 4:0 9      | 1 SCJ1 KAR2, 1 ΔAscjl KAR2, 1 SCJ1 kar2-1, 1 ΔAscjl kar2-1 |
|                        | 4:0 2      | 2 SCJ1 KAR2, 2 ΔAscjl kar2-1 |
| ΔAscjl × kar2-133      | 4:0 1      | 2 ΔAscjl KAR2, 2 SCJ1 kar2-133 |
|                        | 4:0 12     | 1 SCJ1 KAR2, 1 ΔAscjl KAR2, 1 SCJ1 kar2-133, 1 ΔAscjl kar2-133 |
|                        | 4:0 2      | 2 SCJ1 KAR2, 2 ΔAscjl kar2-133 |
| ΔAscjl × kar2-159      | 4:0 5      | 2 ΔAscjl KAR2, 2 SCJ1 kar2-159 |
|                        | 3:1 15     | 1 SCJ1 KAR2, 1 ΔAscjl KAR2, 1 SCJ1 kar2-159, ΔAscjl kar2-159 |
|                        | 2:0 1      | 2 SCJ1 KAR2, 2 ΔAscjl kar2-159 |

* 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 ΔAscjl to sec63-1 and sec71-1 strains always yielded four viable spores at both 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

Figure 8. Swapping J domains. (A) The topology of Sec63p and Sec63-J-Scjlp in the ER membrane is shown schematically. The J domain of Sec63p is represented by the thin black line and the J domain of Scjlp by the thick line. The small arrow indicates the site of fusion to invertase. (B) Amino acid sequences of the J domains from Sec63p, Scjlp, Mdjlp, and Sislp are shown with regions of identity in black. The italicized amino acids represent nonidentical linker sequences encoded by the introduced restriction sites in the chimeric genes. The numbers refer to the Scjlp amino acid sequence. The arrows indicate the Q13R, K17S, and K42V mutations in the J domain of Sec63p (see text), and the asterisk indicates the alanine that, when mutated in Sec63p (the sec63-1 mutation [Nelson et al., 1993]), renders it unable to interact with Kar2p.
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, test these possibilities, we constructed two additional Sec63p in which the specificity could be 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 (Feldheim 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 sec63-1, sec63-101, 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 sec63-1 or sec63-101 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 interact with Kar2p, can function.

The sequence of the four J domains used in the swap experiments is 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-Scjlp containing both mutations Gln13 to Arg and Lys17 to Ser were not cap able 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-Scjlp hybrid containing the three mutations combined (Gln13 to Arg, Lys17 to Ser and Lys42 to Val) fully rescues the temperature-sensitive growth defect of sec63-1 and sec63-101 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 functional 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 sorting 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 entirety. However, its actual function in the cell has remained an enigma. This is, in part, because a strain deleted for SCJ1 has not obvious growth defect, and uptake of proteins into the nucleus, mitochondria, and ER appears normal (Blumberg, H., and P. A. Silver, unpublished results).
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 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 strain. 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 allele. 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 translocation support this conclusion. Because the in vitro translation product possessed only the second methionine, we conclude that it is the 20 amino acids preceding it that are acting as the Scjlp signal sequence.

Information for the DnaJ-Hsp70 Interaction Is Conferred by a Small Number of Amino Acids

As is the case for the Hsp70 protein family, there is also a family of DnaJ-related proteins in the eukaryotic cell (for review see Kurihara and Silver, 1992). By extension of the prokaryotic DnaK-DnaJ interaction, each DnaJ is predicted to have at least one Hsp70 partner. Since distinct Hsp70s are found in each intracellular compartment, it could have been the case that the interaction with a particular DnaJ would be conferred by colocalization. The other possibility is that it is the sequence of the J domain that confers specificity to the Hsp70 interaction. Since J domains have a high degree of similarity with each other, the question arises as to how much specificity could be achieved.

Previous results have suggested that a particular Hsp70 interacts with a certain DnaJ. For example, a yeast cytosolic Hsp70, Ssalp, cannot substitute for BiP in a reconstituted protein translocation system (Brodsky et al., 1993). In addition, Ydjlp stimulates the ATPase and peptide-binding activities of the cytosolic Ssa but not Ssb Hsp70s (Cyr and Douglas, 1994). We now present data that show that J domains from a mitochondrial and a cytoplasmic DnaJ cannot function in the ER lumen when placed into Sec63p. The result that the Scjlp J domain will, on the other hand, function when inserted into Sec63p allows for the prediction of which amino acids may be important for the specificity of the interaction between Sec63p/Scjlp and Kar2p. This turns out to be only three amino acids in the J domain. Conversion of one of these amino acids in the nonfunctioning Sislp J domain rendered the fusion protein able to partially function, whereas two other mutations had no effect. However, all three mutations combined yielded a fully functional hybrid protein. Based on the structure of the E. coli DnaJ domain (Szyperski et al., 1994), these three residues would lie on the outer face of two predicted a helices. Perhaps this defines a surface through which the J domain directly interacts with a particular Hsp70.

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