A Yeast DnaJ Homologue, Scj1p, 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 Scj1p 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 Scj1p 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, Sis1p and Mdj1p, do not function when swapped into Sec63p. However, only three amino acid changes in the Sis1p 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 (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; Zylicz et al., 1989; Alfano and McMacken, 1989) and

similarly for P1 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 Npl1p), 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 lumenal 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 Sis1p, located in the cytoplasm and the nucleus (Luke et al., 1991; Zhong and Arndt, 1993), Ydj1p, located in both the cytoplasm and the outer surface of the ER membrane (Caplan and Douglas, 1991; Atencio and Yaffe, 1992), and Mdj1p, 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 Ydj1p (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 NH_2-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 Scj1p 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 Scj1p 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 Scj1p, Sis1p, and Mdj1p, 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 YEpSCJ1ΔEcoRI (SCJ1 truncated after codon 273) were described before (Blumberg and Silver, 1991). Plasmid YCpSCJ1 contains the 1,700-bp KpnI/SnaBI fragment of YEpSCJ1 (550-bp 5' sequence, 1,134-bp coding sequence, and 15-bp 3' sequence) in the SmaI site of the CEN6/LEU2 plasmid pRS315 (Sikorski and Hieter, 1989). Plasmid YCpg-SCJ1 contains an oligonucleotide-derived insertion (5'-CATGGGAATTCT-TCG-3' coding for an N-linked glycosylation signal) in the unique NcoI site of YCpSCJ1. Plasmid pSP-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. A maltose-binding protein (MBP)[1]–Scj1 fusion protein is encoded by pMAL-SCJ1. The coding sequence of SCJ1 was inserted between the StuI and SalI sites of pMALc (New England Biolabs, Beverly, MA).

Plasmid YCpSEC63 containing the entire SEC63 gene was a kind gift of R. Schekman (University of California, Berkeley, CA). Plasmid YCpSEC63-NC is derived from pRS315 and contains two PCR-generated SEC63 gene fragments: the regions 5' and 3' of the J domain but not the coding sequence of the J domain itself. The 783-bp XbaI/BamHI 5' segment includes the promoter and the NH₂-terminal 124 codons and spans SEC63 positions −409 to +374. The 1,607-bp HindIII/XhoI 3' segment contains codons 200–663 plus 205 bp of 3' sequence. Plasmids YCpSEC63-J-SCJ1, YCpSEC63-J-SIS1, and YCpSEC63-J-MDJ1 contain the J domains of SCJ1, SIS1, and MDJ1, respectively, as 200-bp PCR-derived BamHI/HindIII fragments inserted into YCpSEC63-NC, creating fusions with the SEC63 reading frame. The entire coding region of SEC63 and all the J regions were sequenced to confirm the absence of mutations. YCpSEC63-SUC2 plasmids contain all but the last 14 codons of the SEC63 fused to the coding sequence of cytosolic invertase. The PstI/PvuII fragment of pTK10 (Kuri-

hara and Silver, 1992) was inserted into plasmids YCpSEC63-J-SCJ1, YCpSEC63-J-SIS1, 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 trp1-1*), PSY173 (*ΔSCJ1* [Blumberg and Silver, 1991], *MATa ura3-52 leu2-3,112 trp1-1 ade2 ade3 SCJ1::URA3*), PSY97 (*MATa ura3-52 leu2-3,112 sec63-1*), PSY76 (*MATa ura3-52 leu2-3,112 trp1-1 sec63-101*), PSY43 (Sadler et al., 1989; *MATa/α ura3-52/ura3-52 leu2-3,112/leu2-3,112 ade2-101/+ trp1Δ1/+ SEC63::URA3/+*), PSY662 (*MATα ura3-52 leu2-3,112 trp1Δ1 SEC63::URA3 + plasmid YCpSEC63-J-SCJ1*), MBY12-6D (*MATα ura3-52 leu2-3,112 trp1 sec18-1*), MY176 (*MATα ura3-52 kar2-159*), MS1378 (*MATα ura3-52 kar2-133*), and MS1000 (*MATα ura3-52 kar2-1*).

Site-directed Mutagenesis

A point mutation in the DNA region coding for the J domain in Sec63-J-Sis1p was obtained by a method that relies on homologous recombination in yeast (Ma et al., 1987). Yeast cells were transformed with plasmid YCpSEC63-J-SIS1 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 mutagenic 5' PCR primer 5'-GCC-AACAGGTGACACAGAAAAGTTTGTCGAGATCTCAGAGGCCTT-TGAAATT-3' contains flanking homologous sequence (permitting recombination on the 5' end and priming on the 3' end), the silent restriction site BglII, and changes codon 42 (see Fig. 8 *B*) of the J domain of Sec63-J-Sis1p from Lys to *Val*. The 3' PCR primer contains *SEC63* wild-type sequence. DNA recovered from random yeast colonies served as templates for analytical PCR reactions (using an oligonucleotide that anneales to the 5' border of the *SIS1* 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 Gln 13 to *Arg* and Lys 17 to *Ser*. Here the oligonucleotide 5'-GGAGTATCTCCAAGTGCTAATGAGAGAGAACTGAAA-TCCGGATATAGAAAAGCAGCTCTA-3', which also introduces a BspEI site, was used as 5' PCR primer.

Cell Fractionation

Mitochondria were prepared from wild-type strain D273-10B grown overnight in lactate medium and harvested at 1.5 A₆₀₀nm 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).

Production of Antibodies

Antibodies specific to Scj1p were prepared as follows. MBP fused to Scj1p (MBP-Scj1p) 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* (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 immunoblots using the ECL detection kit (Amersham Corp., Arlington Heights, IL) were performed according to standard methods (Sambrook et al., 1989). Immunofluorescence was performed as described (Bossie et al., 1992) with anti-Scj1p 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 vitro transcription using SP6 polymerase (Promega Corp., Madison, WI) and cell-free translation in rabbit reticulocyte lysates

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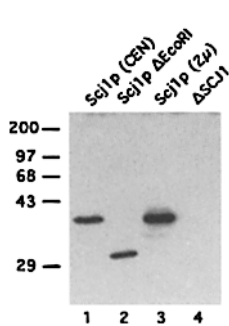1. *Abbreviation used in this paper*: MBP, maltose binding protein.

Figure 1. Characterization of anti-Scjlp antibodies. Yeast cell extracts from PSY173 (Δ*SCJ1*) 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. 3 *A*). The protein was enriched in the ER fraction similar to a marker protein for this compartment (Kar2p), whereas cytochrome c_i heme lyase (CC$_i$HL) 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
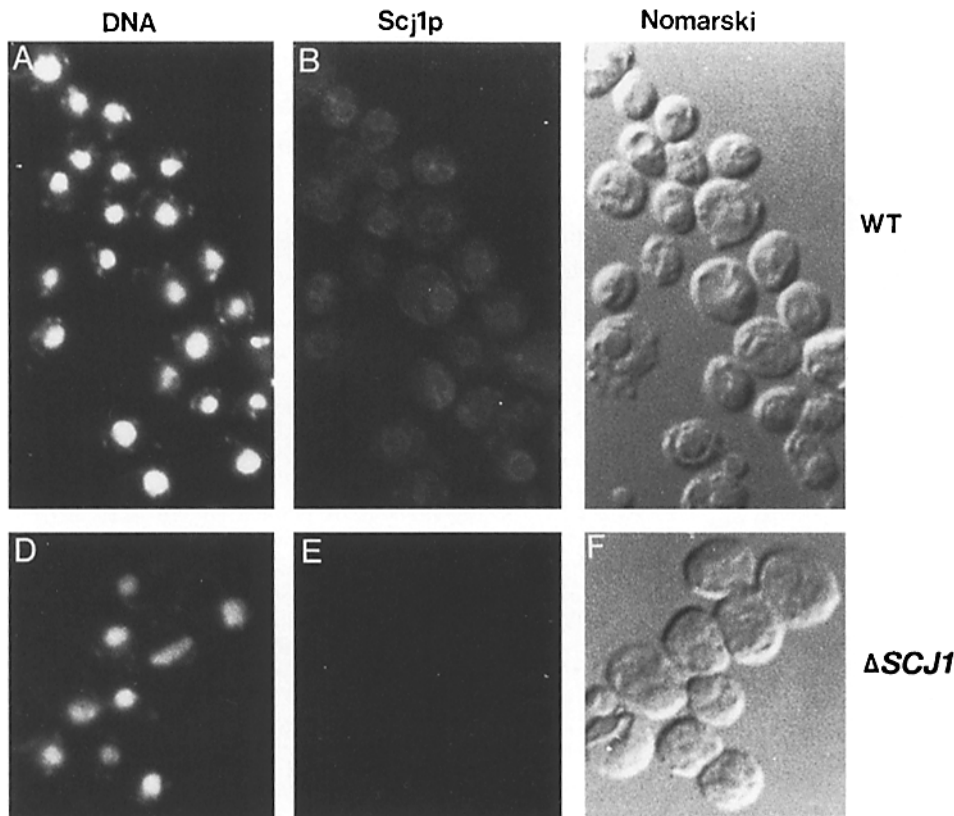
DNA   Scjlp   Nomarski

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′-diamiolino-2-phenylindole), and *C* and *F* show cells viewed by Nomarski optics.

A

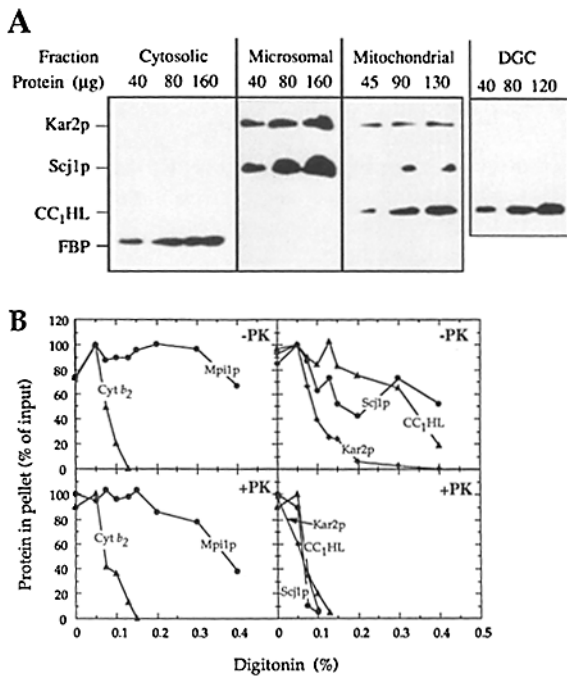| Fraction	Cytosolic			Microsomal			Mitochondrial			DGC		
Protein (µg)	40	80	160	40	80	160	45	90	130	40	80	120
Kar2p —												
Scj1p —												
CC₁HL —												
FBP —												

B

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 CC₁HL, Scj1p, 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; *Mpi1p*, a mitochondrial matrix protein; *Kar2p*, ER lumen.

(CC₁HL or cytochrome b₂) or the ER (Kar2p), but not like the matrix protein Mpi1p (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 Scj1 was not in the intermembrane space of the mitochondria, suggesting localization in the microsomal contamination (data not shown).

To further evaluate the distribution of Scj1p, 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 Scj1p and Kar2p, whereas the content in CC₁HL was virtually unchanged. Taken together, these results support a localization for Scj1p primarily in the ER. Thus, the previous report of a mitochondrial localization of Scj1p (Blumberg and Silver, 1991) is ex-

plained 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 Scj1p NH₂ terminus fused to invertase are Suc⁺, suggesting that the NH₂ terminus could function as a signal for ER translocation (Blumberg and Silver, 1991).

Scj1p 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 Scj1p 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 Scj1p (gScj1p) migrated slower (at 43 kD) than wild-type Scj1p when extracts were assayed by gel electrophoresis and immunoblotting with anti-Scj1p antibodies (Fig. 4 *B*, lane *2*). When cells were incubated with the glycosylation inhibitor tunicamycin, the mutated Scj1p shifted in molecular mass (Fig. 4 *C*, lanes *5-8*) and migrated like wild-type unglycosylated Scj1p (Fig. 4 *C*, lanes *1-4*). Enzymatic digestion of the mutant Scj1p 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, Scj1p must gain access to the lumen of the ER, where the mutant protein is glycosylated.

Tunicamycin treatment stimulates the production of Scj1p (Fig. 4 *C*, lanes *1-4*). Wild-type Scj1p levels increase approximately fourfold (as determined by densitometry) after 4 h of tunicamycin treatment. This is consistent with the
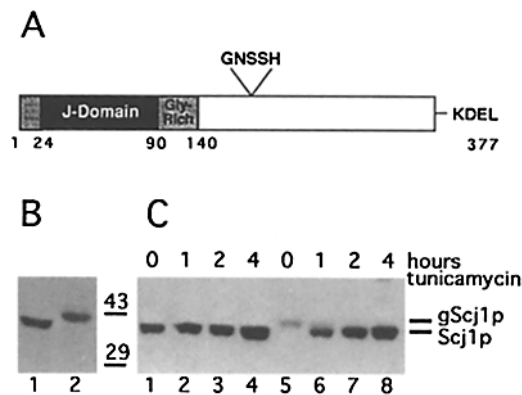
A

B   C

Figure 4. Glycosylation of mutant form of Scj1p. (*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 Scj1p contains the insertion Gly-Asn-Ser-Ser-His between amino acids 173 and 174. (*B*) Cell extracts prepared from yeast strain ΔSCJ1 bearing plasmid YCpSCJ1 encoding Scj1p (lane *1*) or YCpgSCJ1 encoding the glycosylated derivative gScj1p (lane 2) were analyzed by SDS-PAGE and immunoblotting with anti-Scj1p antibodies. (*C*) Immunoblot using anti-Scj1p antibodies on ΔSCJ1 cells transformed with YCpSCJ1 (lanes *1-4*) or 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.

sec18   kar2-159
23 37   23 37   °C

—preKar2p
—Kar2p

—preScj1p
—Scj1p

1   2   3   4

Figure 7. Accumulation of preScjlp in kar2 mutants. Yeast cultures of temperature-sensitive strains sec18 (lanes 1 and 2) and kar2-159 (lanes 3 and 4) were grown at 23°C. The cultures were divided, half was further incubated for 2.5 h at 23°C, and half at 37°C as indicated. Aliquots were removed and analyzed by SDS-PAGE and immunoblotting using anti-Kar2p antibodies (top) or anti-Scjlp antibodies (bottom).

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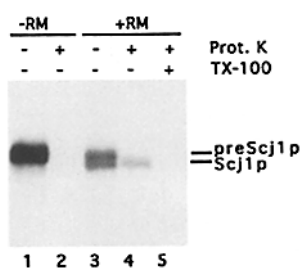
On the other hand, no obvious accumulation of preScjlp or preKar2p was observed in mutants of SEC18 (Fig. 7, lane 2), which acts later in the secretory pathway (Novick et al., 1981).

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 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 (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 lumenal DnaJ domain of Sec63p (Fig. 8, A and B). That this protein was made in

-RM   +RM
- + - + +   Prot. K
- - - - +   TX-100

—preScj1p
—Scj1p

1   2   3   4   5

Figure 6. Processing of preScjlp and assembly of Scjlp in microsomes in reticulocyte lysates. Translation of in vitro–transcribed SCJ1 RNA in the presence of [35S]methionine was performed in the absence (lanes 1 and 2) or presence (lanes 3–5) of dog pancreas microsomes (RM; 1 μl in a 25-μl reaction) for 30 min at 30°C. Reactions were divided into aliquots and further incubated without proteinase K, with proteinase K, or with proteinase K and 0.5% Triton X-100 as indicated. The samples were analyzed by SDS-PAGE and fluorography.
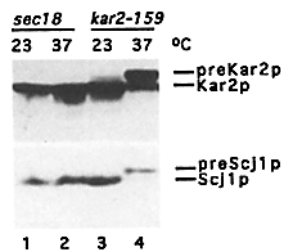
Table I. Synthetic Lethal Combinations of Δscj1 and KAR2 Mutations

Cross	Live/dead*		Genotype of spores‡
Δscj1 × kar2-1	4:0	5	2 Δscj1 KAR2, 2 SCJ1 kar2-1
	4:0	9	1 SCJ1 KAR2, 1 Δscj1 KAR2, 1 SCJ1 kar2-1, 1 Δscj1 kar2-1
	4:0	2	2 SCJ1 KAR2, 2 Δscj1 kar2-1
Δscj1 × kar2-133	4:0	1	2 Δscj1 KAR2, 2 SCJ1 kar2-133
	4:0	12	1 SCJ1 KAR2, 1 Δscj1 KAR2, 1 SCJ1 kar2-138, 1 Δscj1 kar2-13
	4:0	2	2 SCJ1 KAR2, 2 Δscj1 kar2-133
Δscj1 × kar2-159	4:0	5	2 Δscj1 KAR2, 2 SCJ1 kar2-159
	3:1	15	1 SCJ1 KAR2, 1 Δscj1 KAR2, 1 SCJ1 kar2-159, Δscj1 kar2-159
	2:0	1	2 SCJ1 KAR2, 2 Δscj1 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 Δscj1 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-Scj1p antibody (Fig. 9 A, lanes 2 and 4). A protein of the predicted size (75 kD) that reacted with anti-Scj1p was present only in cells containing the chimeric gene.

The chimeric gene encoding Sec63-J-Scj1p 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-Scj1p 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-Scj1p 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-Scj1p

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-Scj1p 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
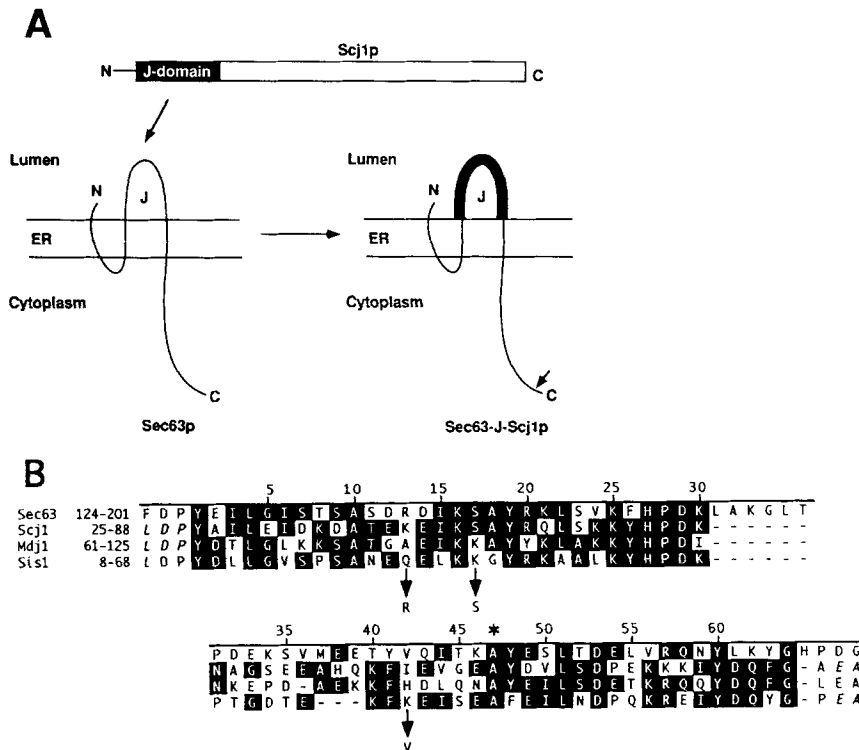

Figure 8. Swapping J domains. (A) The topology of Sec63p and Sec63-J-Scj1p in the ER membrane is shown schematically. The J domain of Sec63p is represented by the thin black line and the J domain of Scj1p by the thick line. The small arrow indicates the site of fusion to invertase. (B) Amino acid sequences of the J domains from Sec63p, Scj1p, Mdj1p, and Sis1p are shown with regions of identity in black. The italicized amino acids represent nonidentical linker sequence encoded by the introduced restriction sites in the chimeric genes. The numbers refer to the Scj1p amino acid sequence. The arrows indicate the Q13R, K17S, and K42V mutations in the J domain of Sec63-J-Sis1p (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.
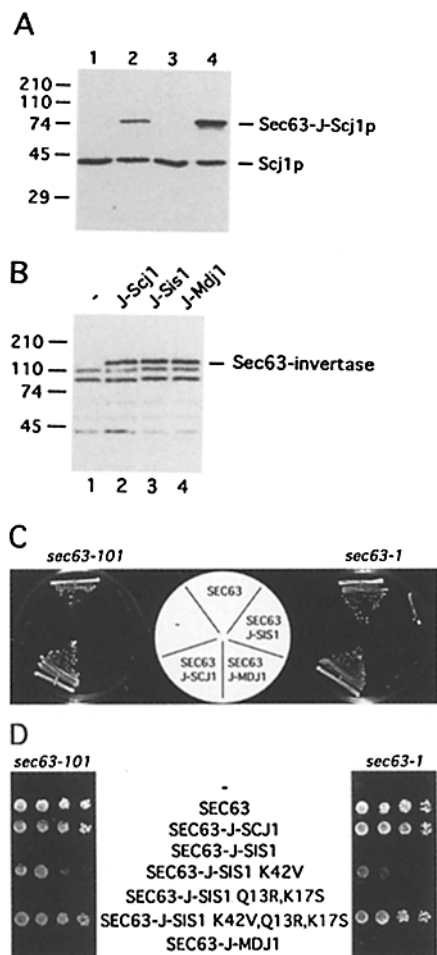
A

210 —
110 —
74 —   — Sec63-J-Scj1p
45 —   — Scj1p
29 —

B

210 —
110 —   — Sec63-invertase
74 —
45 —

        1 2 3 4

C

sec63-101   sec63-1

D

sec63-101   sec63-1

SEC63
SEC63-J-SCJ1
SEC63-J-SIS1
SEC63-J-SIS1 K42V
SEC63-J-SIS1 Q13R,K17S
SEC63-J-SIS1 K42V,Q13R,K17S
SEC63-J-MDJ1

Figure 9. Expression and function of chimeric Sec63-J proteins. (*A*) Immunoblot of cell extracts with anti-Scj1p antibodies. Wild-type cells (W303) bearing plasmids pRS315 (lane *1*) YCpSEC63-J-SCJ1 (lane *2*), or plasmid YCpSEC63 (lane *3*). Lane *4* contains extracts from strain PSY662, a haploid yeast strain missing SEC63 but bearing plasmid YCpSEC63-J-SCJ1. (*B*) Immunoblot of cell extracts with anti-invertase antibodies. Cells were transformed with the vector pRS315 (lane *1*) YCpSEC63-J-SCJ1-SUC2 (lane 2), YCpSEC63-J-SIS1-SUC2 (lane *3*), or YCpSEC63-J-MDJ1-SUC2 (lane *4*). Molecular mass markers are in kilodaltons. (*C*) Cells from temperature-sensitive alleles *sec63-1* and *sec63-101* bearing plasmids pRS315, YCpSEC63, YCpSEC63-J-SCJ1, YCpSEC63-J-SIS1, or YCpSEC63-J-MDJ1 were streaked on plates and incubated at 37°C for 4 d. (Control plates incubated at 23°C or wild-type cells containing the same plasmids at 37°C showed no difference in the growth rate of all strains.) (*D*) Cells as described in *C* plus *sec63-1* and *sec63-101* mutants transformed with the plasmids encoding the K42V and the Q13R+K17S and the triple mutations in Sec63-J-Sis1p were applied on plates in fivefold dilution series and incubated at 37°C for 4 d.

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 Mdj1p and the other containing the J region from Sis1p. Since antibodies to the J domains of Mdj1p and Sis1p 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-Scj1p (Fig. 9 *B*, lane 2), Sec63-J-Sis1p (Fig. 9 *B*, lane *3*), and Sec63-J-Mdj1p (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/ΔSEC63* cells. When tested for growth at the nonpermissive temperature, neither the Sec63-J-Mdj1p or the Sec63-J-Sis1p 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 Scj1p, which is predicted to interact 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 Scj1p, which both can function with Kar2p, with Mdj1p and Sis1p, which do not function, reveals that amino acids at positions 3, 6, 13, 17, 23, and 42 are conserved between Sec63p and Scj1p but differ in Mdj1p and Sis1p. To further define the requirements for the J domain to function, we mutated the three most nonconserved amino acids in Sec63-J-Sis1p by site-directed mutagenesis to encode the corresponding Sec63 J domain amino acids (Fig. 8 *B*). The hybrid protein Sec63-J-Sis1p containing both mutations Gln13 to Arg and Lys17 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-Sis1p 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 Scj1p is located in the ER lumen. These data make Scj1p 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 Scj1p to make a functional protein that can interact with Kar2p. Substitution of other J domain into Scc63p 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 Scj1p in the ER

Scj1p 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).

Compared with other DnaJ homologues, Scj1p contains an extra 20 or 45 amino acids at its NH_2 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 Scj1p is localized in the ER lumen. Like most proteins that are translocated across the ER membrane, Scj1p 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 Scj1p 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 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 (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 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 Scj1p 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 directly affect ER translocation (Vogel, 1993). These results suggest either a direct interaction between Kar2p and Scj1p or that they both participate in similar reactions, as suggested by Brodsky and Schekman (1993).

The second line of evidence that Scj1p can interact with Kar2p comes from results of the J swap experiment. We show that the Scj1p 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 Scj1p 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 Scj1p 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. Scj1p 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 lumenal 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).

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, Ssa1p, cannot substitute for BiP in a reconstituted protein translocation system (Brodsky et al., 1993). In addition, Ydj1p 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 Scj1p 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/Scj1p 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 Sis1p 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 J domain (Szpyerski et al., 1994), these three residues would lie on the outer face of two predicted α helices. Perhaps this defines a surface through which the J domain directly interacts with a particular Hsp70.

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