MOLECULAR AND CELLULAR BIOLOGY, Aug. 1996, p. 4378–4386 Vol. 16, No. 8
0270-7306/96/$04.00+0

Functional Interaction of Cytosolic hsp70 and a DnaJ-Related Protein, Ydj1p, in Protein Translocation In Vivo

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Received 5 February 1996/Accepted 2 May 1996

In order to analyze the in vivo role of the *SSA* class of cytosolic 70-kDa heat shock proteins (hsps) of *Saccharomyces cerevisiae*, we isolated a temperature-sensitive mutant of *SSA1*. The effect of a shift of mutant cells (*ssa1^{ts} ssa2 ssa3 ssa4*) from the permissive temperature of 23°C to the nonpermissive temperature of 37°C on the processing of several precursor proteins translocated into the endoplasmic reticulum or mitochondria was assessed. Of three mitochondrial proteins tested, the processing of only one, the β subunit of the F_1F_0 ATPase, was dramatically affected. Of six proteins destined for the endoplasmic reticulum, the translocation of only prepro-α-factor and proteinase A was inhibited. The processing of prepro-α-factor was inhibited within 2 min of the shift to 37°C, suggesting a direct effect of the hsp70 defect on translocation. More than 50% of radiolabeled α-factor accumulated in the precursor form, with the remainder rapidly reaching the mature form. However, the translocation block was complete, as the precursor form could not be chased through the translocation pathway. Since DnaJ-related proteins are known to interact with hsp70s and strains containing conditional mutations in a *dnaJ*-related gene, *YDJ1*, are defective in translocation of prepro-α-factor, we looked for a genetic interaction between *SSA* genes and *YDJ1* in vivo. We found that a deletion mutation of YDJ1 was synthetically lethal in a *ssa1^{ts} ssa2 ssa3 ssa4* background. In addition, a strain containing a single functional *SSA* gene, *SSA1*, and a deletion of *YDJ1* accumulated the precursor form of α-factor. However, no genetic interaction was observed between a *YDJ1* mutation and mutations in the *SSB* genes, which encode a second class of cytosolic hsp70 chaperones. These results are consistent with *SSA* proteins and Ydj1p acting together in the translocation process.

Molecular chaperones of the 70-kDa size class, which were originally identified as heat-inducible proteins (hsp70s), are abundant, highly conserved, essential proteins (reviewed in reference 44). hsp70s bind to a variety of unfolded proteins (reviewed in references 15, 29, 30, and 34). This binding to hydrophobic stretches of nascent or partially denatured proteins is thought to prevent interactions with other proteins which would lead to malfolding or aggregation (52). All known hsp70s have two functionally defined domains. The N-terminal 44-kDa fragment binds adenine nucleotides with high affinity and has a weak ATPase activity (10, 24); the adjacent 18-kDa fragment contains the peptide binding domain, which binds to short stretches of amino acids with a preference for hydrophobic residues (3, 25, 26, 65). Current models of hsp70 action assert that the ATP-bound form of hsp70 binds to substrate polypeptides with high affinity and yet also rapidly releases them and thus can be considered the "fast-binding, fast-release" form. The ADP-bound form is relatively slow in binding to substrates but is also slow in releasing them and can be considered the "slow-binding, slow-release" form of hsp70. Therefore, ATP hydrolysis converts the fast-binding, fast-release form to the slow-binding, slow-release form, thus stabilizing the interaction, with the exchange of ATP for ADP stimulating polypeptide release. Through such cycles of binding and release with substrate polypeptides, hsp70s function in a variety of cellular processes (46, 49, 56).

In the cytosol of the budding yeast *Saccharomyces cerevisiae*, there are two classes of hsp70s, *SSB* and *SSA* proteins (4). *SSB* proteins are encoded by two closely related genes, *SSB1* and *SSB2* (17). The bulk of the *SSB* proteins is associated with translating ribosomes, most likely interacting with the nascent chain since both nascent chains and *SSB* proteins are released by puromycin (48). The *SSA* proteins are encoded by the *SSA1*, *SSA2*, *SSA3*, and *SSA4* genes (67). The *SSA* subfamily is essential; expression of at least one of the four genes at relatively high levels is required for viability. Ssa1p and Ssa2p are constitutively expressed, while Ssa3p and Ssa4p are not expressed under optimal growth conditions but are induced by stress. Evidence exists for the involvement of *SSA* proteins in a number of cellular processes. *SSA* proteins are involved in regulating transcription of heat shock genes (61). Deletion of the two constitutively expressed members, *SSA1* and *SSA2*, results in the increased expression of hsp genes, including *SSA3* and *SSA4*. In addition, the *SSA* proteins appear to play a role in the translocation of at least some proteins into the endoplasmic reticulum (ER) and mitochondria. Addition of *SSA1* and *SSA2* proteins to in vitro translocation assay mixtures increases the rate of translocation of proteins into microsomes and mitochondria (12, 47). In vivo, a possible role of *SSA* proteins in protein translocation was studied by using a strain containing disruptions of the *SSA1*, *SSA2*, and *SSA4* genes with *SSA1* under the control of the repressible *GAL1* promoter. Repression of the *GAL1* promoter by the addition of glucose resulted in a drop in *SSA* protein levels. After several hours of depletion, defects in the translocation of two proteins destined for the ER, α-factor (α-F) and carboxypeptidase Y (CPY), and of a protein destined for mitochondria, the β subunit of the F_1F_0 ATPase ($F_1\beta$), were observed (21).

It is well established that DnaK, an hsp70 of *Escherichia coli*, functions with two other hsps, DnaJ and GrpE. Interaction of DnaJ with DnaK stimulates the ATPase activity of DnaK (43).

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TABLE 1. Strains

Strain	Designation used in text	Genotype
JN55	Wild type	*MAT*α *leu2-3,112 his3-11 ura3-52 trp1*Δ*1 lys2*
JN516	*a2 a3 a4*	*MAT*α *leu2-3,112 his3-11 ura3-52 trp1*Δ*1 lys2 ssa2*::*LEU2 ssa3*::*TRP1 ssa4*::*LYS2*
JB67	*a1-45 a2 a3 a4*	*MAT*α *leu2-3,112 his3-11 ura3-52 trp1*Δ*1 lys2 ssa1-45*:*URA3 ssa2*::*LEU2 ssa3*::*TRP1 ssa4*::*LYS2*
WY1	*ydj1*	*MAT*α *leu2-3,112 his3-11 ura3-52 trp1*Δ*1 lys2 ydj1*::*HIS3*
WY3	*a2 a3 a4 ydj1*	*MAT*α *leu2-3,112 his3-11 ura3-52 trp1*Δ*1 lys2 ssa2*::*LEU2 ssa3*::*TRP1 ssa4*::*LYS2 ydj1*::*HIS3*
WY4	*a1-45 a2 a3 a4 ydj1*	*MAT*α *leu2-3,112 his3-11 ura3-52 trp1*Δ*1 lys2 ssa1-45*:*URA3 ssa2*::*LEU2 ssa3*::*TRP1 ydj1*::*HIS3*
5B6		*MAT*α *leu2-3,112 his3-11 ura3-52 trp1*Δ*1 lys2 ssa1*::*HIS3 ssa2*::*LEU2 ssa4*::*LYS2 pGAL1*:*SSA1*(*URA3*)

In addition, DnaJ is able to interact with substrate proteins on its own, as it has been found associated with the heat shock sigma factor σ^{32} and nascent chains attached to ribosomes (27, 42). GrpE binds to the ATPase domain of DnaK, stimulating the release of bound ADP (5, 43). While no proteins related to GrpE have been identified in the eucaryotic cytosol, two DnaJ-related proteins have been found in the cytosol of *S. cerevisiae*, Ydj1p (Mas5p) (2, 7) and Sis1p (45). *SIS1* mutants show defects in protein synthesis (69), while temperature-sensitive *YDJ1* mutants are defective in the translocation of certain precursors into the mitochondria and the ER (6).

In order to more critically assess the role of *SSA* proteins in protein translocation, we isolated and analyzed a temperature-sensitive allele of *SSA1*. In addition, as the relevance of the interaction between Ydj1p and Ssa1p observed in vitro has not been determined in vivo, we investigated the function of this interaction in *S. cerevisiae*. The results of this analysis suggest that *SSA* proteins are important for the translocation of some, but not all, precursor proteins in vivo and that the functional interaction of Ydj1p and *SSA* proteins in vivo is important for cell viability.

MATERIALS AND METHODS

General methods and materials, strains, and plasmids. Standard techniques for yeast strain propagation and genetic manipulation were used as described by Sherman et al. (59). Yeast transformations were performed as described by Ito et al. (35). Standard techniques for manipulation of DNA were used as described by Sambrook et al. (53).

A 605-bp *YDJ1* fragment was isolated by PCR during a search for unidentified *dnaJ*-related genes and cloned into the vector pUC18 digested with *Bam*HI and *Sal*I (41). A 1.4-kb DNA fragment carrying the *HIS3* gene was inserted into the plasmid carrying the *YDJ1* fragment after digestion with *Eco*RV, deleting the 300 bp of the protein-coding region, from the codons of amino acids 94 to 193. The disrupted *YDJ1* fragment was liberated from vector sequences by digestion with *Bam*HI and *Sal*I and used to establish a chromosomal *ydj1*::*HIS3* disruption in JN55 by homologous recombination. The disruption was verified by Southern analysis and the typical temperature-sensitive phenotype of a *YDJ1* null mutant (7).

Isolation of *SSA1* thermosensitive mutants. A plasmid [pYe(CEN3)30 SSA1] carrying a 4.3-kb *Pvu*II-*Bam*HI fragment containing the entire *SSA1* gene with its own promoter was subjected to mutagenesis with hydroxyl amine (38). 5B6 (Table 1) was transformed with the mutagenized plasmid, and transformants were selected for their ability to grow at 23°C, but not 37°C, on glucose-based medium. Segments of the plasmid containing the candidate $SSA1^{ts}$ genes were cloned into the unmutagenized plasmid [pYe(CEN3)30 SSA1] to determine if a mutation conferring temperature-sensitive growth was in the *SSA1* gene. As described in Results, one *SSA1* gene carrying a mutation within a carboxy-terminal 600-bp *Cla*I-*Sal*I fragment was selected for further analysis and named *ssa1-45*. To replace the chromosomal *SSA1* gene with the *ssa1-45* allele, a 1.2-kb *Hin*dIII fragment with *Sph*I linkers, containing the entire *URA3* gene, was cloned into the *Sph*I site immediately downstream of the termination site of the *SSA1* gene of pYe(CEN3)30 ssa1-45. A 5.5-kb *Pvu*II-*Bam*HI fragment of pYe(CEN3)30 containing *ssa1-45*:*URA3* DNA was integrated into yeast strain JN516, giving rise to yeast strain JB67. Integrations were confirmed by Southern blot analysis.

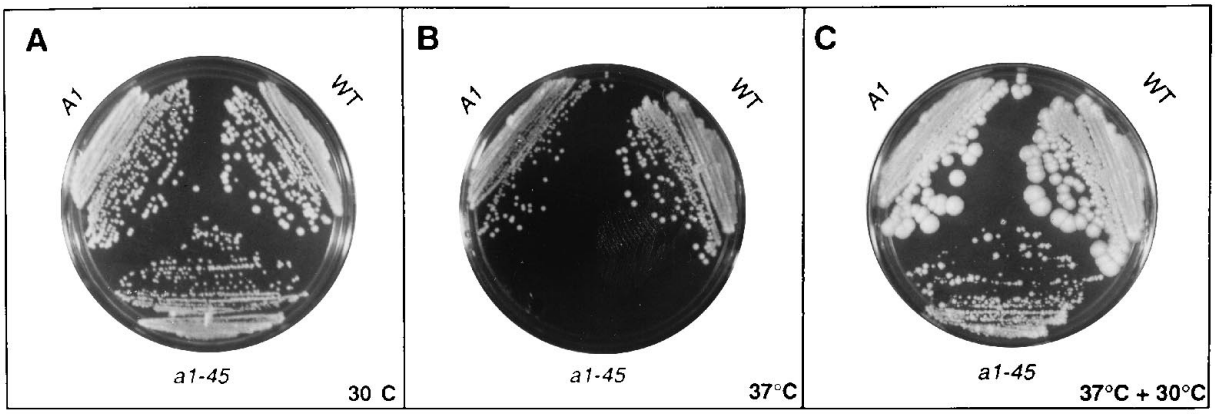
Cell labeling and immunoprecipitation. Cells were grown overnight in yeast minimal medium containing 5% glucose and lacking methionine, cysteine, and other appropriate amino acids for selection purposes. Cells in log phase (optical density at 600 nm [OD_{600}] = 0.5 to 1.5) were resuspended in fresh medium at 3 OD_{600} units per ml and incubated for 30 min at the permissive temperature (25°C) before the labeling protocol was carried out. The cells were labeled with 50 μCi of Translabel (Amersham) per OD_{600} unit. For pulse-chase experiments the chase was initiated by adding either cycloheximide to a final concentration of 0.5 mg/ml or cysteine-methionine to a final concentration of 0.03%. For the experiment investigating the processing of $F_1\beta$ after de- and re-energizing mitochondria, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to a final concentration of 20 μM at the time of labeling and the chase was initiated by adding cycloheximide (0.5 mg) and β-mercaptoethanol (0.05%). The labeling and chase were terminated by adding ice-cold NaN_3 (20 mM) and shifting the cells onto ice.

The cells were washed once with phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4) and resuspended in an equal volume of 1% sodium dodecyl sulfate (SDS)–0.5 mM phenylmethylsulfonyl fluoride. After addition of glass beads (diameter = 0.5 mm) to the fluid meniscus, the cells were broken with a bead beater twice for a duration of 1 min. The lysate was boiled for 5 min before 9 volumes of IP buffer (50 mM Tris Cl [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS) lacking SDS was added. Preimmune serum (0.02%, vol/vol) and 2% (vol/vol) of a protein A-Sepharose suspension (50%, vol/vol) were added, and the lysate was incubated for 2 h at 4°C with gentle agitation. The lysate was cleared by centrifugation at 10,000 × *g* for 5 min at 4°C. Trichloroacetic acid-precipitable counts were determined, and equal amounts of radioactivity (10^7 to 10^8 cpm) were added to a suitable antiserum and to 20 μl of protein A-Sepharose suspension (50%, vol/vol) before overnight incubation at 4°C. The protein A-Sepharose-immune complex was pelleted by a 5-s spin and washed five times with IP buffer. The final pellet was resuspended in 20 μl of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (40) and boiled for 5 min. Following SDS-PAGE, the gels were treated with Amplify (Amersham), dried, and autoradiographed at −70°C. *SEC62* mutants (*SEC2* encodes a protein required for translocation across the ER membrane [22]), were labeled for use as controls in immunoprecipitation experiments.

For the protease protection assay, the spheroplasts were broken by vortexing the spheroplast suspension for 30 s. After centrifugation, the resulting 500 × *g* supernatant was incubated with 500 μg of proteinase K per ml on ice for 5 and 15 min in the presence and absence of detergent. The reactions were stopped by adding 0.5 volume of 10 mM phenylmethylsulfonyl fluoride. These samples were subsequently prepared for immunoprecipitation with antiserum against α-F.

RESULTS

Isolation of temperature-sensitive *SSA1* mutants. To further analyze the function of Ssa1p, we isolated thermosensitive yeast mutants carrying mutated *SSA1* genes. For this purpose, a yeast strain, 5B6, was constructed in which the hsp70 genes, *SSA1*, *SSA2*, and *SSA4*, were inactivated by insertion of selectable markers (Table 1). To sustain the viability of this otherwise inviable triple mutant, 5B6 also contained a centromeric plasmid with the *SSA1* gene under transcriptional control of the galactose-inducible and glucose-repressible *GAL1* promoter element. To isolate conditional alleles, this strain was transformed with a centromeric plasmid carrying an *SSA1* gene under control of its endogenous promoter, which had been mutagenized with hydroxylamine. Transformants were screened for growth at 23 and 37°C on glucose-containing



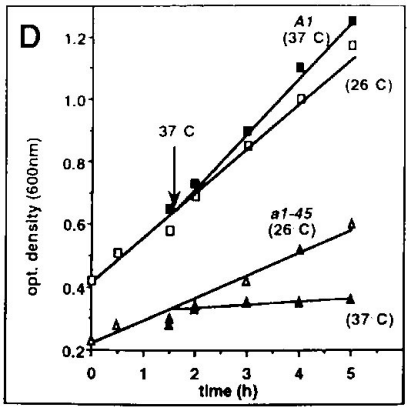


FIG. 1. The thermosensitive growth phenotype of *a1-45 a2 a3 a4* cells sets in quickly but is not lethal. Strains JN55 (wild type [WT]), JN516 (*a2 a3 a4* [*A1*]), and JB67 (*a1-45 a2 a3 a4* [*a1-45*]) were grown on rich medium for 3 days at 30°C (A) or 37°C (B) or for 3 days at 37°C followed by 6 days at 30°C (C). (D) *A1* and *a1-45* cells were grown in rich liquid medium at 25°C; at the time indicated by the arrow, half of the cultures were shifted to 37°C, and growth was measured as OD_{600}.

medium, conditions under which the wild-type gene, under control of its *GAL1* promoter, was not expressed.

To eliminate possible recombination between the mutated episomal *ssa1*^{ts} alleles and their disrupted counterparts on the chromosome, the *ssa1*^{ts} alleles were inserted into the chromosome, replacing the wild-type *SSA1* gene. This replacement was carried out in a strain, JN516, carrying insertion mutations of *SSA2*, *SSA3*, and *SSA4* and henceforth referred to as the *a2 a3 a4* strain. Of eight independent integrated *ssa1*^{ts} mutants, three had reasonable growth rates at 23°C and a pronounced inhibition of growth at 37°C (Fig. 1). Sequence analysis of these three *ssa1*^{ts} mutants revealed that all three mutant genes carried the same point mutation, changing P-417 to L. This residue lies within the peptide binding domain. Further analysis was carried out with the mutation from one of these three isolates, *a1-45*, inserted into the chromosome of JN516 to give the strain JB67. JB67, having the genotype *ssa1-45 ssa2 ssa3 ssa4*, is henceforth referred to as the *a1-45 a2 a3 a4* strain.

The growth of *a1-45 a2 a3 a4* cells ceased almost immediately after a shift to the nonpermissive temperature of 37°C (Fig. 1D). The growth inhibition was cytostatic rather that cytocidal, as prolonged exposure to 37°C over a period of 3 days did not decrease plating efficiency significantly after the cells were shifted back to 30°C. Analysis of these mutant cells at 37°C by light microscopy did not reveal any abnormal morphology or cell cycle-specific arrest.

The temperature-sensitive *SSA1* mutant rapidly accumulates α-F precursor. In *a1 a2 a4* cells containing a plasmid with *SSA1* under the control of the *GAL1* promoter element, precursor proteins of both the ER (α-F and CPY) and mitochondria $F_1\beta$) accumulated several hours after the addition of glucose, which represses expression from the *GAL1* promoter (21). Because of the long period required for depletion of Ssa1p, it is possible that secondary pleiotropic effects caused the observed accumulation of precursor proteins. To assess whether *SSA* proteins were directly involved in protein translocation, we investigated the processing of the yeast secretory protein α-F in *a1-45 a2 a3 a4* mutants. Prepro-α-F (ppα-F) contains a signal sequence specific for the ER which is cleaved upon entry into the ER and is then glycosylated to generate gpα-F. This glycosylation increases its apparent molecular mass to about 27 kDa (36). In *a2 a3 a4* cells which contain a wild-type *SSA1* gene, ppα-F was not detectable in cells labeled for 5 min at either 23 or 37°C; only a highly glycosylated form of pα-F was observed, indicating that ppα-F is readily processed. After the mutant cells were shifted to 37°C, the unprocessed form of α-F, ppα-F, was detectable by immunoprecipitation as soon as 2 min after the shift (Fig. 2). The swift accumulation of precursor in the mutant without any preincu-

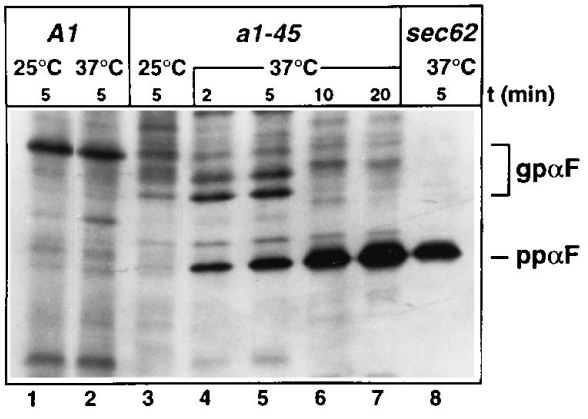


FIG. 2. *a1-45 a2 a3 a4* cells rapidly accumulate prepro-α-F at the nonpermissive temperature. JN516 (*a2 a3 a4* [*A1*]) and JB67 (*a1-45 a2 a3 a4* [*a1-45*]) cells were grown at 25°C and labeled with [^{35}S]methionine-cysteine for 5 min at 25°C (lanes 1 and 3) or for 2 min (lane 4), 5 min (lanes 2 and 5) 10 min (lane 6), or 20 min (lane 7) after a shift to 37°C. Cell extracts were subjected to immunoprecipitation with α-F-specific antibodies. As a migration standard, lane 8 contains ppα-F immunoprecipitated from *sec62* cells harvested 5 min after transfer to 37°C.

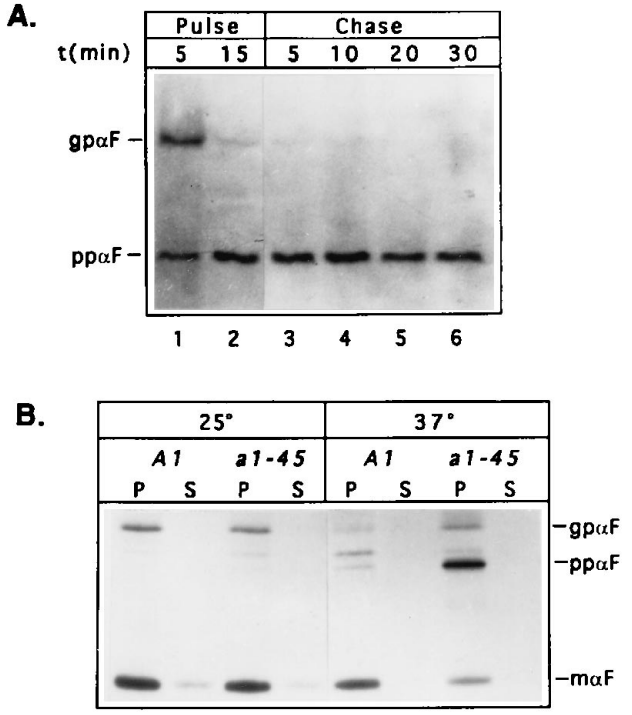


FIG. 3. Stability of prepro-α-F in *a1-45 a2 a3 a4* cells. (A) *a1-45 a2 a3 a4* cells were pulse-labeled with [^{35}S]methionine-cysteine for 5 min (lane 1) and 15 min (lane 2) immediately after a shift to 37°C. After the 15-min labeling period, cycloheximide and unlabeled methionine-cysteine were added, and samples were removed at the times indicated during the chase (lanes 3 to 6). (B) Cultures of *a2 a3 a4* and *a1-45 a2 a3 a4* cells growing at 25°C were split. One aliquot of each culture was pulse-labeled at 25°C for 10 min; the other was shifted to 37°C and labeled 10 to 20 min later. The cells were pelleted; the resuspended cell pellet (P) and the supernatant (S) were subjected to immunoprecipitation with α-F-specific antibodies. mαF, mature α-F.

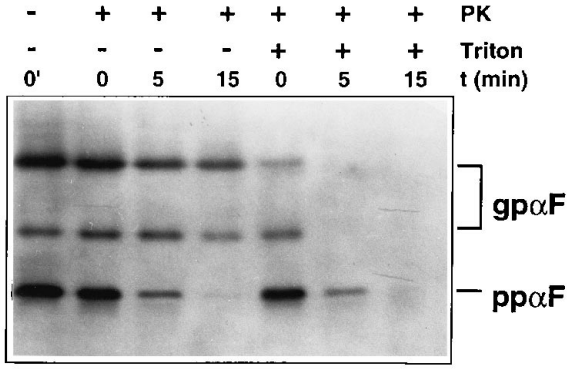


FIG. 4. Accumulated prepro-α-F in *a1-45 a2 a3 a4* cells is exposed to the cytosol. *a1-45 a2 a3 a4* cells labeled with [^{35}S]cysteine-methionine for 10 min immediately after a shift to 37°C were converted to spheroplasts at the nonpermissive temperature and lysed by vortexing. The initial lysate was centrifuged at 500 × *g*, yielding a crude preparation containing all organelles in the supernatant. This lysate was then either mock digested (0′) or treated with 500 μg of proteinase K (PK) per ml in the presence or absence of 0.5% Triton X-100 for 0, 5, and 15 minutes. The digest was terminated by adding PMSF and assessed by immunoprecipitation of α-F, SDS-PAGE, and autoradiography.

bation at the nonpermissive temperature indicates that the failure to translocate α-F was a direct consequence of the malfunction of the altered Ssa1p and strongly suggests a direct involvement of Ssa1p in facilitating protein translocation into the ER.

To determine more directly whether the ppα-F detected in the mutant strain at 37°C was actually blocked in translocation or whether translocation was only slowed, we carried out pulse-chase experiments. *a1-45 a2 a3 a4* cells were labeled for 15 min at 37°C immediately after a shift from 23°C (Fig. 3A). Immunoprecipitations of extracts from cells harvested at various times after initiation of a chase showed a constant amount of labeled ppα-F, indicating that precursor which had not been rapidly translocated remained incompetent for translocation.

To assess the completeness of this block in translocation of ppα-F, the amounts of mature α-F as well as ppα-F were determined. *a2 a3 a4* cells and *a1-45 a2 a3 a4* cells were pulse-labeled between 10 and 20 min after a shift to 37°C, and the protein was immunoprecipitated with a combination of antibodies that reacted with both mature and precursor forms of α-F (Fig. 3B). In *a2 a3 a4* cells, greater than 90% of the precipitable α-F was in the processed glycosylated or mature form, reflecting the rapid processing and maturation of α-F. On the other hand, only 30% of α-F was processed in the mutant, indicating that the block in translocation was not complete but was substantial. We were unable to perform an analysis to determine if the block was complete at later times after the shift to the nonpermissive temperature, because the shutdown of expression of many genes normally found after a temperature upshift (that is, a heat shock) results in such a reduction in synthesis of ppα-F in the mutant strain that quantitation is impossible.

Accumulated ppα-F in *a1-45 a2 a3 a4* cells is protease sensitive and on the cytosolic side of microsomal membranes. To confirm that the observed accumulation of ppα-F was due to a translocation defect, rather than merely a defect in the processing of the precursor by signal sequence peptidase, we tested whether accumulated ppα-F present after a shift of mutant cells to the nonpermissive temperature was on the cytosolic side of the ER membrane. As noted above, both gpα-F and ppα-F were detectable by immunoprecipitations with α-F-specific antibodies if *a1-45 a2 a3 a4* cells were labeled for short periods of time. Extracts prepared from *a1-45 a2 a3 a4* cells labeled 5 min after a shift to 37°C were treated with proteinase K in the presence and absence of detergent (Fig. 4). ppα-F was digested by proteinase K in the presence or absence of detergent, while the glycosylated, ER-localized form of α-F, gpα-F, was degraded only in the presence of detergent. This observation strongly suggested that ppα-F was located outside the ER, since detergent was not required to make it accessible to added protease as was the case for the glycosylated form. Thus, it appears that the *ssa1-45* mutation prevents α-F from entering the ER at the nonpermissive temperature.

***a1-45 a2 a3 a4* cells are defective in PrA processing.** We also analyzed the maturation of another protein which is translocated across the ER membrane, proteinase A (PrA). The signal sequence of prepro-PrA is cleaved upon entrance into the ER lumen, and the resulting pro-PrA is glycosylated at two sites to yield a 47-kDa ER form (39). Upon entry into the vacuole, the propeptide is removed to generate the 42-kDa mature species. About 5 to 20% of the pro-PrA becomes glycosylated at only one site, generating more rapidly migrating pro and mature forms (62). *a2 a3 a4* and *a1-45 a2 a3 a4* cells were pulse-labeled at 25 and 37°C, and extracts were subjected to immunoprecipitation with PrA-specific antibodies. Nearly all of the pulse-labeled PrA in *a2 a3 a4* cells labeled at both temperatures and in *a1-45 a2 a3 a4* cells labeled at 25°C migrated as a 47-kDa protein (Fig. 5A). However, about 40% of the PrA labeled in the mutant at the nonpermissive temperature migrated as the prepro form, suggesting a defect in trans-

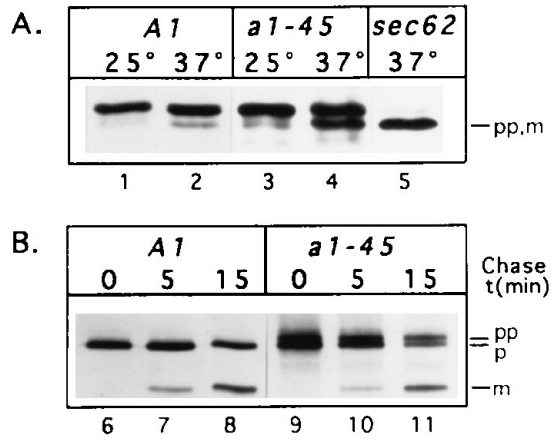


FIG. 5. PrA translocation is defective in *a1-45 a2 a3 a4* cells. *a2 a3 a4* (*A1*) and *a1-45 a2 a3 a4* (*a1-45*) cells were grown at 25°C. The cultures were split, and one aliquot was maintained at 25°C. A second aliquot was shifted to 37°C. (A) [^{35}S]methionine-cysteine was added 10 min after the shift, and the cells were harvested 10 min after the addition of label. The 25°C culture was also pulse-labeled for 10 min. The resulting extracts were subjected to immunoprecipitation by PrA-specific antibodies. A culture of *sec62* cells was pulsed-labeled for 10 min at 90 min after the shift to 37°C. (B) Tunicamycin (20 μg/ml) was added at the time of the temperature shift. [^{35}S]methionine-cysteine was added 15 min after the shift. An aliquot of cells was harvested 5 min later (0); excess unlabeled methionine-cysteine was added to the remainder of the culture, and samples were removed 5 and 15 min later. The aliquot of cells maintained at 25°C was treated in the same manner. m, mature; pp, prepro; p, pro.

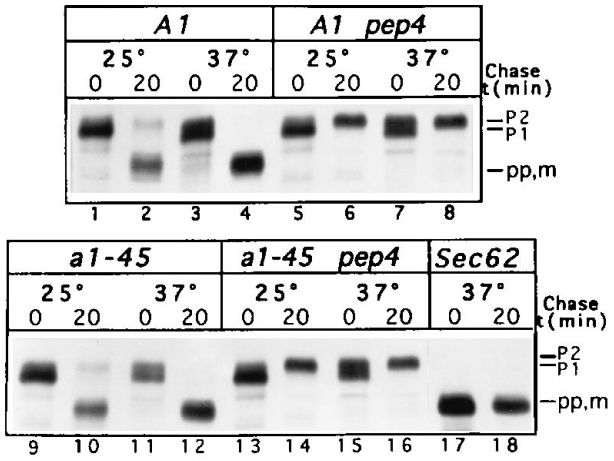


FIG. 6. CPY translocation and transport to the vacuole is not affected in the *SSA1* mutant. *a2 a3 a4* (*A1*), *a2 a3 a4 pep4* (*A1 pep4*), *a1-45 a2 a3 a4* (*a1-45*), and *a1-45 a2 a3 a4 pep4* (*a1-45 pep4*) cells were grown at 25°C. The cultures were split, and one aliquot was maintained at 25°C. A second aliquot was shifted to 37°C; label was added 30 min after the shift. One half of each culture was harvested 10 min after the addition of label. Unlabeled cysteine and methionine were added to the other half, and cells were harvested 20 min later. The aliquot of cells maintained at 25°C was treated in the same manner. m, mature; p, pro; pp, prepro.

location into the ER. However, the prepro form migrates in a position similar to that of the mature form with only a single site of glycosylation. To more definitively test whether processing was defective in the *ssa* mutant, we treated cells with tunicamycin prior to the addition of label to prevent glycosylation. After treatment with tunicamycin, all of the detectable PrA in *a2 a3 a4* cells labeled for 5 min at 37°C was in the pro form, with maturation occurring during the chase period (Fig. 5B). However, about 45% of PrA was in the prepro form in *a1-45 a2 a3 a4* cells after a 5-min labeling at the nonpermissive temperature of 37°C. With time the pro form matured. However, after 15 min after the beginning of the chase, 30% of the PrA was still in the prepro form, indicating that the processing of the prepro form was not slowed but was blocked.

Translocation and maturation of CPY occurs normally in *a1-45 a2 a3 a4* cells. We were also interested in analyzing the maturation of CPY through the ER and into the vacuole, since a defect in CPY processing had been observed in earlier studies of *SSA1* protein function (21). CPY is synthesized as a precursor form (ppCPY) of 61 kDa. Upon transport of ppCPY into the ER, cleavage of the signal sequence and addition of core oligosaccharides yields the p1 form, which migrates at 67 kDa. Extension of the oligosaccharides in the Golgi yields a 69-kDa p2 form (60). The p2 form is sorted to the vacuole, where it undergoes proteolytic cleavage by PrA, the product of the *PEP4* gene. *a2 a3 a4* and *a1-45 a2 a3 a4* cells were labeled for 10 min at 25 or 37°C beginning 15 min after a shift to that temperature. The modified p1 and p2 forms of CPY (67 and 69 kDa, respectively) were immunoprecipitated by CPY-specific antibodies from extracts of both types of cells labeled at either temperature. Very little material migrating as ppCPY at 61 kDa was observed (Fig. 6, lanes 1, 3, 9, and 11). Immunoprecipitation of extracts from cells harvested 20 min after the beginning of the chase showed that nearly all of the CPY had been converted to a form that migrated as mature CPY in both strains at both 25 and 37°C (Fig. 6, lanes 2, 4, 10, and 12).

However, the mature form of CPY migrates at 61 kDa, as does ppCPY. Therefore, distinction between the unglycosylated precursor and glycosylated mature forms of CPY was difficult because of their coincident mobility (60). Introduction of a *PEP4* mutation into the *a2 a3 a4* and *a1-45 a2 a3 a4* strains alleviated this problem because the cleavage to the 69-kDa p2 form is blocked in the absence of PrA (33). Analysis of *a2 a3 a4 pep4* and *a1-45 a2 a3 a4 pep4* strains allowed us to unambiguously determine whether the 61-kDa CPY product accumulating in both strains at 25 and 37°C after the chase was the mature form, rather than the precursor that was synthesized after the chase had begun. In the *a2 a3 a4 pep4* and *a1-45 a2 a3 a4 pep4* strains, all of the detectable CPY accumulated in the 69-kDa p2 form after the chase (Fig. 6, lanes 6, 8, 14, and 16). These results indicate that translocation both into the ER lumen and from the ER to the Golgi is normal in the presence of the *ssa1-45* mutation even at the nonpermissive temperature.

***a1-45 a2 a3 a4* and *ydj1* are synthetically lethal.** Several DnaJ-like proteins have been identified in *S. cerevisiae*. It had been shown that one of the cytosolic DnaJ-like proteins in *S. cerevisiae* is needed for efficient translocation of certain proteins into the ER and mitochondria (2, 7). Furthermore, Ydj1p is needed in vitro to dissociate protein complexes of Ssa1p with denatured proteins (20). We attempted to determine whether Ssa1p interacts with Ydj1p in vivo. One way to obtain evidence about possible interactions is to combine thermosensitive alleles of suspected partners in one strain, a strategy which had been used successfully to identify interactions, for example, between genes involved in transport vesicle formation and in transport in the secretory pathway (37). As the disruption of *YDJ1* is not lethal but causes the cell to become thermosensitive for growth at 37°C, we tried to determine the phenotype of an *a1-45 a2 a3 a4 ydj1* strain. Crossing the different *SSA* mutants with the *ydj1* null mutant in a stepwise fashion, we failed to obtain the final pentuple mutant containing the four *ssa* mutations and the *ydj1* insertion allele (Table 2). In all cases either *SSA2* or *SSA4* was needed to ensure germination of

TABLE 2. *SSA* and *YDJ* mutations are synthetically lethal

Cross	Total no. of tetrads[a]	No. of recovered haploids with genotype[b]:				No. with[c]:		
		A1 (or *A2*) *YDJ1*	*a1* (or *a2*) *YDJ1*	A1 (or *A2*) *ydj1*	*a1* (or *a2*) *ydj1*	T	PD	NPD
A1 a2 a3 a4 ydj1 × *a1-45 a2 a3 a4 YDJ1*	22 (1)	23 (0)	20 (1)	19 (2)	0 (23)	17	2	3
a1-45 A2 a3 a4 ydj1 × *a1-45 a2 a3 a4 YDJ1*	15 (2)	13 (0)	16 (1)	14 (3)	0 (13)	9	4	2

[a] Numbers in parentheses indicate the number of tetrads for which the genotype could not be deduced; these were eliminated.
[b] Numbers in parentheses indicate the number of haploids not recovered.
[c] T, tetratype; PD, parental ditype; NPD, nonparental ditype.

spores containing the *ssa1-45* and *ydj1* alleles. On the other hand, when an *ssb1 ssb2* strain was crossed with the *ydj1* mutant, *ssb1 ssb2 ydj1* strains were easily attainable from the subsequent sporulation and dissection. These triple mutants showed no synthetic growth defect; at each temperature tested, the *ssb1 ssb2 ydj1* strains grew at the same rate as either the *ssb1 ssb2* or *ydj1* parental strain, depending on which had the more severe defect at the particular temperature being tested. This result strongly suggests that the combination of *ssa1-45* with the *ydj1* allele in cells with an *ssa2 ssa3 ssa4* background leads to synthetic lethality, indicative of an interaction of Ydj1p and Ssa1p.

***a2 a3 a4 ydj1* mutants accumulate ppα-F constitutively.** While *a1-45 a2 a3 a4 ydj1* cells are inviable, *a2 a3 a4 ydj1* cells which have only the wild-type *SSA1* gene of the *SSA* subfamily are viable. To further elucidate the possible interaction between *YDJ1* and *SSA* proteins and to determine if this interaction is important for translocation, we analyzed the processing of ppα-F in an *a2 a3 a4 ydj1* strain. As reported previously, the translocation of α-F into the ER is impaired neither in a *ydj1* deletion mutant nor in *a2 a3 a4* mutants even after a shift to 37°C (6) (Fig. 2). However, *a2 a3 a4 ydj1* mutants readily accumulate ppα-F at both 23 and 37°C (Fig. 7). Accumulation was also observed in *a1-45 a2 a3 ydj1* cells, which have a functional *SSA4* gene, grown at 23°C. However, at 37°C no accumulation of precursor was observed. The increased expression of *SSA4*, a highly inducible heat shock gene, at 37°C is likely able to compensate for the lack of *YDJ1*/*SSA* function at the higher temperature (Fig. 7, lane 8). These results strongly indicate that Ydj1p functionally interacts with Ssa1p and thus enhances its facilitation of the translocation of proteins into the ER.

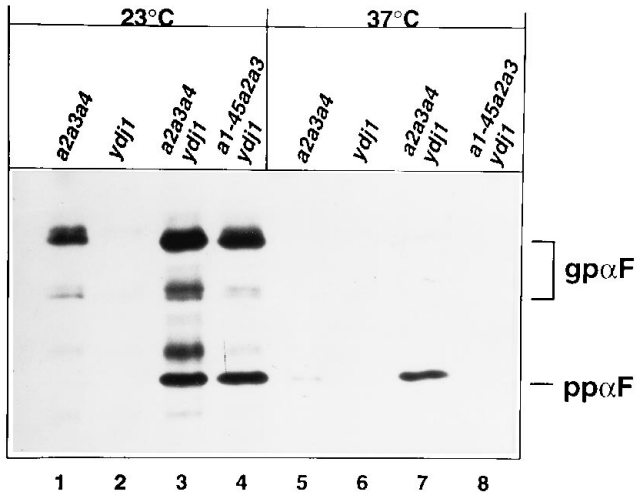


FIG. 7. Accumulation of prepro-α-F in strains containing mutations in *SSA* genes and *YDJ1*. *a2 a3 a4*, *ydj1*, *a2 a3 a4 ydj1*, and *a1-45 a2 a3 a4 ydj1* cells were labeled with [^{35}S]methionine-cysteine for 10 min at 25°C and immediately after the shift to 37°C. Processing of α-F was analyzed by immunoprecipitation, SDS-PAGE, and autoradiography.

***a1-45 a2 a3 a4* mutants are defective in posttranslational protein translocation of $F_1\beta$ into mitochondria.** We tested the effect of the *ssa1-45* mutation on the processing of three precursor proteins which are translocated into mitochondria, $F_1\beta$, hsp60, and Ssc1p. At 37°C substantial accumulation of $F_1\beta$ was observed (Fig. 8), but hsp60 and Ssc1p precursor accumulated very little, if at all (data not shown). There is disagreement as to whether protein translocation into mitochondria in vivo is cotranslational or posttranslational (63). However, unlike protein translocation into the ER, the import of proteins into mitochondria is dependent on the membrane potential, which is needed to initiate protein translocation (55). By reversibly abolishing the membrane potential of mitochondria, we were able to uncouple protein synthesis and protein translocation into mitochondria and assess the affect of the *ssa1-45* mutation on the posttranslational protein translocation in vivo. Cells were pulse-labeled with [^{35}S]methionine-cysteine in the presence of CCCP, which abolishes the membrane potential and prevents precursors from entering the mitochondria, thus leading to the accumulation of precursor proteins (51). Processing of accumulated pre-$F_1\beta$ was then monitored in *a2 a3 a4* and *a1-45 a2 a3 a4* cells during the chase period in the presence of cycloheximide, which was added to abolish further protein synthesis, and in the presence of the reducing agent β-mercaptoethanol, which was added to reestablish the membrane potential. Over a chase period of 60 min, the reestablishment of the membrane potential led to efficient import of accumulated pre-$F_1\beta$ in *a2 a3 a4* cells at 25 and 37°C and in *a1-45 a2 a3 a4* cells at 25°C. In *a1-45 a2 a3 a4* cells only 60% of $F_1\beta$ accumulated in its precursor form during the labeling in the presence of CCCP (Fig. 8). Whether this reflects a certain characteristic of the *ssa1-45* mutant strain is unclear. However, in *a1-45 a2 a3 a4* cells at 37°C, the import of about 50% of the labeled pre-$F_1\beta$ into reenergized mitochondria remained irreversibly blocked at 37°C (Fig. 8B), indicating a requirement for *SSA* proteins in the posttranslational translocation of $F_1\beta$.

DISCUSSION

We have utilized a temperature-sensitive mutant to assess the role of the cytosolic hsp70, Ssa1p, in protein translocation. Earlier results (21) suggested an in vivo role of *SSA* proteins on the basis of the accumulation of precursor after depletion of *SSA* proteins. This result had been questioned (for example, see reference 63) because of the possibility that the accumulation was due to indirect effects of the depletion of *SSA* proteins which took several hours to occur. However, the rapidity with which the translocation of α-F was inhibited after the shift of the *a1-45 a2 a3 a4* strain to 37°C indicates a direct

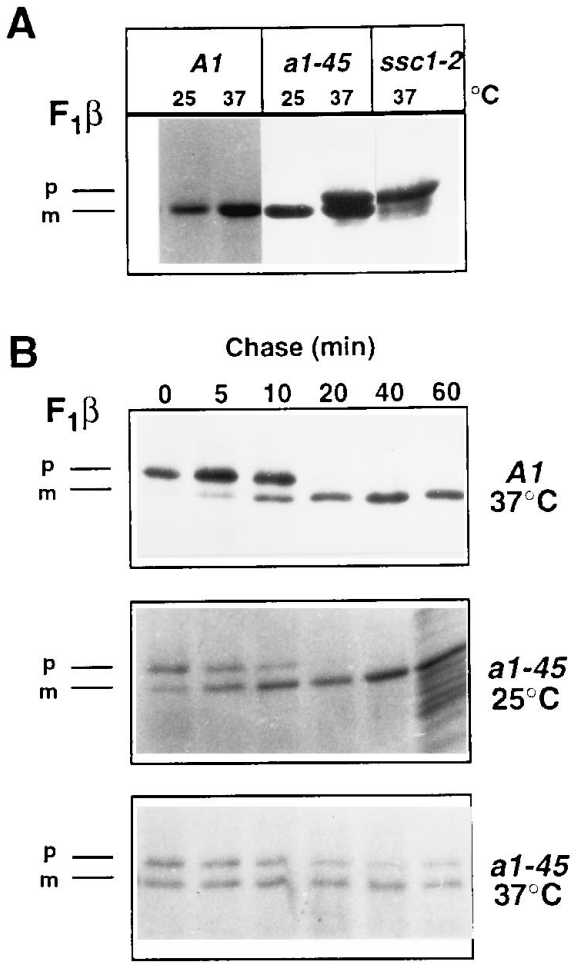


FIG. 8. Accumulation of the $F_1\beta$ precursor in *a1-45 a2 a3 a4* cells at the nonpermissive temperature. (A) Analysis of the processing of $F_1\beta$ by immunoprecipitation. *a2 a3 a4* (*A1*) and *a1-45 a2 a3 a4* (*a1-45*) cells were labeled at 25°C or immediately after a shift to 37°C for 20 min. Extracts were subjected to immunoprecipitation with $F_1\beta$-specific antibodies, SDS-PAGE, and autoradiography. As a migration standard, $F_1\beta$ was immunoprecipitated from *ssc1-2* cells after the shift to the nonpermissive temperature. (B) Pulse-chase and immunoprecipitation analysis of $F_1\beta$ processing after treatment with CCCP and β-mercaptoethanol. Following incubation at 37°C for 30 min, *A1* and *a1-45* cells were labeled for 10 min with [^{35}S]methionine-cysteine in the presence of 20 μM CCCP at 37°C. The cells were chased with β-mercaptoethanol and cycloheximide, and the culture was either maintained at 37°C or shifted to 25°C for periods of time indicated. m, mature; p, pro.

role of *SSA* proteins in the translocation process. Although the block in the translocation process was swift, it was not complete; 70% of the α-F was completely blocked in translocation, while the remaining 30% rapidly matured. A second protein, PrA, was also found to be defective in processing. About 30% of the precursor remained unprocessed even after a chase, while 70% appeared to traverse the pathway to the vacuole at the normal rate.

There are at least two possible explanations for this dichotomy: either complete blockage or normal translocation of affected preproteins. First, the *ssa1-45* mutation could be leaky, with the mutant *SSA* protein having sufficient activity for translocation of only a portion of the α-F and PrA precursors. If this explanation is correct, then Ssap must be required immediately after translation, since preprotein that was not translocated at the normal rate remained translocation incompetent. Since *SSA* proteins are hsp70-type molecular chaperones, it would be consistent with current models of hsp70 activity for Ssap to bind to precursors in the cytosol, preventing aggregation or folding into translocation-incompetent conformations until translocation occurs. In fact, in in vitro translation extracts *SSA* proteins have been found associated with newly synthesized ppα-F (13). However, one would predict that once aggregation occurred, translocation would be impossible.

Alternatively, α-F and PrA could enter the ER via two different pathways, one which is completely blocked in the mutant that is dependent on Ssaps and one that is Ssap independent. Much translocation occurs cotranslationally by utilizing the signal recognition particle (SRP)-dependent pathway. SRP is not essential for growth of *S. cerevisiae*, although deletion of the gene encoding the signal sequence-binding protein (*SRP54*) or the associated small cytosolic RNA, scR1, results in slow growth and impaired translocation into the ER (23, 31). Since translocation in these mutants is not completely abolished, translocation must be able to occur by an SRP-independent pathway. Support for a second pathway comes from in vitro studies in which ppα-F was efficiently translocated into microsomes posttranslationally (32, 66). In addition, Panzer et al. have reconstituted posttranslational protein transport in *S. cerevisiae* with purified components (50). An obvious candidate for an SRP-independent, posttranslational pathway is an hsp70-mediated pathway. It is possible that some precursors partition between the SRP-dependent pathway and the Ssap-mediated pathway. Perhaps more α-F partitions to the Ssap-dependent pathway, while more PrA partitions to the SRP-dependent pathway, as suggested by the percent blockages of translocation of these proteins.

However, a requirement for functional *SSA* proteins in translocation is certainly not universal. No defect in CPY translocation was detected in the *a1-45 a2 a3 a4* strain. A mild defect was seen upon depletion of *SSA* proteins, but only 8 h or longer after shutdown of *SSA* expression. This defect may well have been an indirect effect of the depletion. In addition, three other proteins, protein disulfide isomerase, invertase, and Kar2p, were tested and showed no defect in processing in the *SSA* mutant (18). Many more precursors must be tested before the range of proteins dependent on hsp70 is determined. It is possible that very few proteins are dependent on the hsp70s for translocation into the ER. It is intriguing that the precursor we identified as having a strong dependence on hsp70s for translocation, ppα-F, is the only protein shown to be efficiently translocated posttranslationally in vitro. The rapid cessation of growth of the *ssa-45* mutant after a shift to the nonpermissive temperature may be due to a failure in translocation of these unidentified precursors or to some other function of *SSA* proteins completely unrelated to translocation, such as regulation of expression of the heat shock response (16, 61).

The maturation of CPY in the *ssa1-45* mutant is also interesting in light of the suggestions of the involvement of clathrin in movement of proteins from the Golgi to the vacuole and the ability of hsp70s to uncoat clathrin-coated vesicles. The disassembly of cages constructed of bovine clathrin can be facilitated not only by mammalian hsc70 (11) but by other hsp70s as well. *SSA* proteins facilitate the uncoating of clathrin vesicles, while *SSB* proteins do not (28). It has been suggested from analysis of a temperature-sensitive allele of *CHC1*, which encodes clathrin heavy chains, that clathrin vesicles play an important role in ER-to-vacuole translocation of CPY and other vacuolar proteins (14, 58). After a shift of *chc1^{ts}* cells to the nonpermissive temperature, CPY was not sorted correctly to the vacuole; rather, the late Golgi, p2 form was secreted, indicating a defect in Golgi-to-vacuole transport. After extended

periods of time, normal translocation resumed, suggesting the presence of a backup system for the clathrin vesicle system. Our failure to observe a defect in CPY movement from the Golgi to the vacuole in the *ssa1*^{ts} mutants suggests that the *SSA* proteins are not required in *S. cerevisiae* to uncoat clathrin vesicles.

The translocation of four proteins, CPY, dipeptidylaminopeptidase B, α-F, and Kar2p, has been tested in a strain carrying a temperature-sensitive *YDJ1* allele (9). As with the *ssa1-45* mutant, translocation of α-F was affected, but translocation of the other three precursors was not. The genetic interactions reported here support this notion. The *YDJ1* disruption mutation is synthetically lethal with the *a1-45 a2 a3 a4* mutations but not with mutations of the *SSB1* and *SSB2* genes. *SSA* and *SSB* proteins appear to have distinct functions in the cytosol (17, 19). *SSB* proteins are associated with translating ribosomes. *ssb1 ssb2* mutants are hypersensitive to inhibitors of protein synthesis but show no defect in translocation of proteins across the ER membrane. The differences in the phenotypes of *SSA* and *SSB* mutants are consistent with the specificity of the genetic interaction of the *SSA* class of cytosolic hsp70s with *YDJ1*.

A genetic interaction between *SSA* genes and *YDJ1* was also observed with regard to protein translocation. An *a2 a3 a4 ydj1* strain containing a single *SSA* gene, *SSA1*, is defective in translocation of α-F under conditions in which neither the *ydj1* strain nor the *a2 a3 a4* strain shows accumulation of ppα-F. The involvement of both Ydj1p and Ssa1p in protein translocation and the operation of redundant pathways of translocation may be more common than originally envisioned. In *E. coli* both DnaK and DnaJ are involved in an alternative, but overlapping, export pathway to the SecB-dependent pathway (1, 68).

While these genetic interactions suggest that *SSA* proteins and Ydj1p cooperate in a translocation pathway, whether they act together or in parallel in the translocation process can not be ascertained at this time. *YDJ1* may be a chaperone in its own right, as the *E. coli* DnaJ protein has been shown to bind directly to unfolded proteins (42, 57). However, Ydj1p is able to stimulate the ATPase activity of Ssa1p (20, 70), which is indicative of a direct interaction of the two proteins. Ydj1p is farnesylated (8); this modification is necessary for normal function of Ydj1p in protein translocation (6). It is intriguing to consider that localization to the membrane caused by this modification may be important for the translocation process. Ydj1p may act to target either Ssaps or the Ssap-precursor complex to a site on the ER membrane. However, in considering the translocation process, it is necessary to take into account Kar2p/BiP, the hsp70 on the luminal side of the ER membrane. Binding of Kar2p seems to provide the directionality of the translocation process (54, 64). If hsp70s are binding to the precursor on both sides of the membrane, the binding on the cytosolic side must be relatively less stable in order for a net movement into the lumen to occur. Ydj1p allows release of at least some proteins from Ssa1p (8). It could be advantageous for *SSA1* proteins to be more tightly associated with the precursor while in the cytosol, but the binding must be readily reversible to allow translocation once the precursor is associated with the membrane. Ydj1p may serve as such a release factor.

ACKNOWLEDGMENTS

We thank T. Stevens (PrA), R. Schekman (α-F), S. Emr (CPY), P. Silver (invertase), M. Rose (Kar2p), and M. Yaffe ($F_1\beta$) for generously providing antisera.

This work was supported by Public Health Service grant R01 GM 31107 to E.A.C. J.B. was supported by a fellowship from the Deutsche Forschungdgemeinschaft.

REFERENCES

1. **Altman, E., C. A. Kumamoto, and S. D. Emr.** 1991. Heat-shock proteins can substitute for SecB function during protein export in *Escherichia coli*. EMBO J. **10:**239–245.
2. **Atencio, D., and M. Yaffe.** 1992. *MAS5*, a yeast homolog of DnaJ involved in mitochondrial import. Mol. Cell. Biol. **12:**283–291.
3. **Blond-Elguindi, S., S. Cwirla, W. Dower, R. Lipshutz, S. Sprang, J. Sambrook, and M.-J. Gething.** 1993. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. Cell **75:**717–728.
4. **Boorstein, W. R., T. Ziegelhoffer, and E. A. Craig.** 1994. Molecular evolution of the HSP70 multigene family. J. Mol. Evol. **38:**1–17.
5. **Buchberger, A., H. Schroder, M. Buttner, A. Valencia, and B. Bukau.** 1994. A conserved loop in the ATPase domain of the DnaK chaperone is essential for stable binding of GrpE. Struct. Biol. **1:**95–101.
6. **Caplan, A., D. Cyr, and M. Douglas.** 1992. YDJ1p facilitates polypeptide translocation across different intracellular membranes by a conserved mechanism. Cell **71:**1143–1155.
7. **Caplan, A., and M. Douglas.** 1991. Characterization of *YDJ1*: a yeast homologue of the bacterial *dnaJ* protein. J. Cell Biol. **114:**609–621.
8. **Caplan, A., J. Tsai, P. Casey, and M. Douglas.** 1992. Farnesylation of YDJ1p is required for function at elevated growth temperatures in *S. cerevisiae*. J. Biol. Chem. **267:**18890–18895.
9. **Caplan, A. J., D. M. Cyr, and M. G. Douglas.** 1993. Eukaryotic homologues of *Escherichia coli dnaJ*: a diverse protein family that functions with HSP70 stress proteins. Mol. Biol. Cell **4:**555–563.
10. **Chappell, T. G., B. B. Konforti, S. L. Schmid, and J. E. Rothman.** 1987. The ATPase core of a clathrin uncoating protein. J. Biol. Chem. **262:**746–751.
11. **Chappell, T. G., W. J. Welch, D. M. Schlossman, K. B. Palter, M. J. Schlesinger, and J. E. Rothman.** 1986. Uncoating ATPase is a member of the 70 kilodalton family of stress proteins. Cell **45:**3–13.
12. **Chirico, W., M. G. Waters, and G. Blobel.** 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature (London) **332:**805–810.
13. **Chirico, W. J.** 1992. Dissociation of complexes between 70 kDa stress proteins and presecretory proteins is faciliated by a cytosolic factor. Biochem. Biophys. Res. Commun. **189:**1150–1156.
14. **Conibear, E., and T. Stevens.** 1995. Vacuolar biogenesis in yeast: sorting out the sorting proteins. Cell **83:**513–516.
15. **Craig, E. A., B. D. Gambill, and R. J. Nelson.** 1993. Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol. Rev. **57:**402–414.
16. **Craig, E. A., and C. A. Gross.** 1991. Is hsp70 the cellular thermometer? Trends Biochem. Sci. **16:**135–140.
17. **Craig, E. A., and K. Jacobsen.** 1985. Mutations in cognate gene of *Saccharomyces cerevisiae* HSP70 result in reduced growth rates at low temperatures. Mol. Cell. Biol. **5:**3517–3524.
18. **Craig, E. A., and W. Walter.** Unpublished data.
19. **Craig, E. A., T. Ziegelhoffer, J. Nelson, S. Laloraya, and J. Halladay.** 1995. Complex multigene family of functionally distinct Hsp70s of yeast. Cold Spring Harbor Symp. Quant. Biol. **60:**441–449.
20. **Cyr, D., X. Lu, and M. Douglas.** 1992. Regulation of Hsp70 function by a eukaryotic DnaJ homolog. J. Biol. Chem. **267:**20927–20931.
21. **Deshaies, R., B. Koch, M. Werner-Washburne, E. Craig, and R. Schekman.** 1988. A subfamily of stress proteins faciliates translocation of secretory and mitochondrial precursor polypeptides. Nature (London) **332:**800–805.
22. **Deshaies, R. J., and R. Schekman.** 1989. *SEC62* encodes a putative membrane protein required for protein translocation into the yeast endoplasmic reticulum. J. Cell Biol. **109:**2653–2664.
23. **Felici, F., G. Cesareni, and J. Hughes.** 1989. The most abundant small cytoplasmic RNA of *Saccharomyces cerevisiae* has an important function required for normal cell growth. Mol. Cell. Biol. **9:**3260–3268.
24. **Flaherty, K. M., C. DeLuca-Flaherty, and D. B. McKay.** 1990. Three dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. Nature (London) **346:**623–628.
25. **Flynn, G., J. Pohl, M. Flocco, and J. Rothman.** 1991. Peptide-binding specificity of the molecular chaperone BiP. Nature (London) **353:**726–730.
26. **Freeman, B., M. Myers, R. Schumacher, and R. Morimoto.** 1995. Identification of a regulatory motif in Hsp70 that affects ATPasae activity, substrate binding and interaction with HDJ-1. EMBO J. **14:**2281–2292.
27. **Gamer, J., H. Bujard, and B. Bukau.** 1992. Physical interaction between heat shock proteins DnaK, DnaJ and GrpE and the bacterial heat shock transcription factor sigma32. Cell **69:**833–842.
28. **Gao, B., J. Biosca, E. A. Craig, L. E. Greene, and E. Eisenberg.** 1991. Uncoating of coated vesicles by yeast hsp70 proteins. J. Biol. Chem. **266:** 19565–19571.
29. **Georgopoulos, C., and W. Welch.** 1993. Roles of the major heat shock proteins as molecular chaperones. Annu. Rev. Cell Biol. **9:**601–634.
30. **Gething, M.-J., and J. Sambrook.** 1992. Protein folding in the cell. Nature (London) **355:**33–45.

31. **Hann, B. C., and P. Walter.** 1991. The signal recognition particle in *S. cerevisiae*. Cell **67:**131–144.
32. **Hansen, W., P. D. Garcia, and P. Walter.** 1986. In vitro protein translocation across the yeast endoplasmic reticulum: ATP-dependent posttranslational translocation of prepro-a-factor. Cell **45:**397–406.
33. **Hemmings, B. A., G. S. Zubenko, A. Hasilik, and E. W. Jones.** 1981. Mutant defective in processing of an enzyme located in the lysosome-like vacuole of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA **78:**435–439.
34. **Hendrick, J., and F.-U. Hartl.** 1993. Molecular chaperone function of heat-shock proteins. Annu. Rev. Biochem. **62:**349–384.
35. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
36. **Julius, D., R. Schekman, and J. Thorner.** 1984. Glycosylation and processing of prepro-alpha factor through the yeast secretory pathway. Cell **36:**309–318.
37. **Kaiser, C., and R. Schekman.** 1990. Distinct sets of SEC genes govern transport vesicle formation and fusion early in the secretory pathway. Cell **61:**723–733.
38. **Kang, P. J., J. Ostermann, J. Shilling, W. Neupert, E. A. Craig, and N. Pfanner.** 1990. Hsp70 in the mitochondrial matrix is required for translocation and folding of precursor proteins. Nature (London) **348:**137–143.
39. **Klionsky, D. J., L. M. Banta, and S. D. Emr.** 1988. Intracellular sorting and processing of a yeast vacuolar hydrolase: proteinase A propeptide contains vacuolar targeting information. Mol. Cell. Biol. **8:**2105–2116.
40. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
41. **Laloraya, S., and E. A. Craig.** Unpublished results.
42. **Langer, T., C. Lu, H. Echols, J. Flanagan, M. K. Hayer, and F. U. Hartl.** 1992. Successive action of DnaK, DnaJ, and GroEL along the pathway of chaperone-mediated protein folding. Nature (London) **356:**683–689.
43. **Liberek, K., J. Marszalek, D. Ang, and C. Georgopoulos.** 1991. Escherichia coli DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. Proc. Natl. Acad. Sci. USA **88:**2874–2878.
44. **Lindquist, S., and E. A. Craig.** 1988. The heat-shock proteins. Annu. Rev. Genet. **22:**631–677.
45. **Luke, M., A. Suttin, and K. Arndt.** 1991. Characterization of *SIS1*, a *Saccharomyces cerevisiae* homologue of bacterial *dnaJ* proteins. J. Cell Biol. **114:**623–638.
46. **McCarty, J., A. Buchberger, J. Reinstein, and B. Bukau.** 1995. The role of ATP in the functional cycle of the DnaK chaperone system. J. Mol. Biol. **249:**126–137.
47. **Murakami, H., D. Pain, and G. Blobel.** 1988. 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. J. Cell Biol. **107:**2051–2057.
48. **Nelson, R. J., T. Ziegelhoffer, C. Nicolet, M. Werner-Washburne, and E. A. Craig.** 1992. The translation machinery and seventy kilodalton heat shock protein cooperate in protein synthesis. Cell **71:**97–105.
49. **Palleros, D. R., K. L. Reid, L. Shi, W. Welch, and A. L. Fink.** 1993. ATP-induced protein-hsp70 complex dissociation requires K^+ but not ATP hydrolysis. Nature (London) **365:**664–666.
50. **Panzer, S., L. Dreier, E. Hartmann, S. Kostka, and T. Rapoport.** 1995. Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. Cell **81:**561–570.
51. **Reid, G. A., T. Yonetani, and G. Schatz.** 1982. Import of proteins into mitochondria: import and maturation of the mitochondrial intermembrane space enzymes cytochrome b2 and cytochrome c peroxidase in intact yeast cells. J. Biol. Chem. **257:**13068–13074.
52. **Rothman, J.** 1989. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell **59:**591–601.
53. **Sambrook, J. E., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
54. **Sanders, S., K. Whitfield, J. Vogel, M. Rose, and R. Schekman.** 1992. Sec61p and BiP directly facilitate polypeptide translocation into the ER. Cell **69:** 353–366.
55. **Schleyer, M., B. Schmidt, and W. Neupert.** 1982. Requirement of a membrane potential for the posttranslational transfer of protein into mitochondria. Eur. J. Biochem. **125:**109–116.
56. **Schmid, D., A. Baici, H. Gehring, and P. Christen.** 1994. Kinetics of molecular chaperone action. Science **263:**971–973.
57. **Schroder, H., T. Langer, F.-U. Hartl, and B. Bukau.** 1993. DnaK, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. EMBO J. **12:**4137–4144.
58. **Seeger, M., and G. Payne.** 1992. Golgi membrane protein retention in *Saccharomyces cerevisiae*. J. Cell Biol. **118:**531–540.
59. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Laboratory course manual for methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
60. **Stevens, T., B. Esmon, and R. Schekman.** 1982. Early stages in the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. Cell **30:**439–448.
61. **Stone, D. E., and E. A. Craig.** 1990. Self regulation of 70-kilodalton heat shock proteins in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**1622–1632.
62. **van den Hazel, H. B., M. C. Kielland-Brandt, and J. R. Winther.** 1992. The propeptide is required for in vivo formation of stable active yeast proteinase A and can function even when not covalently linked to the mature region. J. Biol. Chem. **268:**18002–18007.
63. **Verner, K.** 1993. Co-translational import into mitochondria: an alternative view. Trends Biochem. Sci. **18:**366–371.
64. **Vogel, J. P., L. M. Misra, and M. D. Rose.** 1990. Loss of BiP/grp78 function blocks translocation of secretory proteins in yeast. J. Cell Biol. **110:**1885–1895.
65. **Wang, T.-F., J.-H. Chang, and C. Wang.** 1993. Identification of the peptide binding domain of hsc70. J. Biol. Chem. **268:**26049–26051.
66. **Waters, M. G., and G. Blobel.** 1986. Secretory protein translocation in a yeast cell-free system can occur post-translationally and requires ATP hydrolysis. J. Cell Biol. **102:**1543–1550.
67. **Werner-Washburne, M., D. E. Stone, and E. A. Craig.** 1987. Complex interactions among members of an essential subfamily of hsp70 genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**2568–2577.
68. **Wild, J., E. Altman, T. Yura, and C. A. Gross.** 1992. The DnaK and DnaJ heat shock proteins participate in protein export in *E. coli*. Genes Dev. **6:**1165–1172.
69. **Zhong, T., and K. T. Arndt.** 1993. The yeast *SIS1* protein, a DnaJ homolog, is required for initiation of translation. Cell **73:**1175–1186.
70. **Ziegelhoffer, T., P. Lopez-Buesa, and E. A. Craig.** 1995. The dissociation of ATP from hsp70 of *Saccharomyces cerevisiae* is stimulated by both Ydj1p and peptide substrates. J. Biol. Chem. **270:**10412–10419.