Mutation of the ATP-Binding Pocket of *SSA1* Indicates That a Functional Interaction Between Ssa1p and Ydj1p Is Required for Post-translational Translocation Into the Yeast Endoplasmic Reticulum

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ABSTRACT

The translocation of proteins across the yeast ER membrane requires ATP hydrolysis and the action of DnaK (hsp70) and DnaJ homologues. In *Saccharomyces cerevisiae* the cytosolic hsp70s that promote post-translational translocation are the products of the Ssa gene family. Ssa1p maintains secretory precursors in a translocation-competent state and interacts with Ydj1p, a DnaJ homologue. Although it has been proposed that Ydj1p stimulates the ATPase activity of Ssa1p to release preproteins and engineer translocation, support for this model is incomplete. To this end, mutations in the ATP-binding pocket of *SSA1* were constructed and examined both *in vivo* and *in vitro*. Expression of the mutant Ssa1p's slows wild-type cell growth, is insufficient to support life in the absence of functional Ssa1p, and results in a dominant effect on post-translational translocation. The ATPase activity of the purified mutant proteins was not enhanced by Ydj1p and the mutant proteins could not bind an unfolded polypeptide substrate. Our data suggest that a productive interaction between Ssa1p and Ydj1p is required to promote protein translocation.

PROTEINS fated to leave the cell, or to ultimately reside in some cellular compartments, must first traverse the secretory pathway. The first committed step in secretory protein biogenesis is the translocation of newly synthesized polypeptides across the membrane of the endoplasmic reticulum (ER; for review, see JOHNSON and VAN WAES 1999). In the yeast *Saccharomyces cerevisiae*, translocation may proceed either cotranslationally or post-translationally. Cotranslational translocation dictates that translocation proceeds concomitant with polypeptide elongation. However, precursor proteins that are fully synthesized and released from the ribosome prior to targeting to the ER membrane undergo post-translational translocation. In either case, translocation requires the actions of 70-kD molecular chaperones (hsp70s; CHIRICO *et al.* 1988; DESHAIES *et al.* 1988; SANDERS *et al.* 1992; BRODSKY *et al.* 1995).

The hsp70 family of molecular chaperones consists of highly conserved members that assist protein folding and intracellular targeting (for reviews see HARTL 1996; RASSOW *et al.* 1997; BUKAU and HORWICH 1998). Hsp70s contain a highly conserved N-terminal ATPase domain and a more variable C-terminal peptide-binding domain. The coupling of these domains allows hsp70s to bind and release polypeptide substrates in a cycle that is dependent upon ATP binding and hydrolysis (SCHMID *et al.* 1994; MCCARTY *et al.* 1995; BANECKI and ZYLICZ 1996). In *S. cerevisiae*, there are at least 10 members of this family, two subclasses of which reside in the cytosol. These are the Ssb and Ssa gene families. The Ssb subclass, which consists of two genes, *SSB1* and *SSB2*, is involved in protein translation (CRAIG and JACOBSEN 1985; NELSON *et al.* 1992; PFUND *et al.* 1998). The absence of functional Ssb results in a slow-growth phenotype (CRAIG and JACOBSEN 1985), whereas the Ssa gene family is essential; the expression of at least one *SSA* gene is necessary for viability (WERNER-WASHBURNE *et al.* 1987). The Ssa subclass consists of four genes, *SSA1–4*. *SSA1* and *SSA2* are constitutively expressed under normal growth conditions while *SSA3* and *SSA4* resemble classical heat-shock proteins in that their expression is heat inducible (WERNER-WASHBURNE *et al.* 1987).

The most extensively studied member of the Ssa subclass is the product of the *SSA1* gene. *In vivo* evidence suggests that Ssa1p functions in a variety of cellular processes under normal growth conditions. These include microtubule assembly (OKA *et al.* 1998) and nuclear transport (SHULGA *et al.* 1996), as well as the translocation of precursor proteins into mitochondria and the ER (DESHAIES *et al.* 1988; CHIRICO *et al.* 1988; BECKER *et al.* 1996). Because urea can substitute for Ssa1p to support *in vitro* translocation, it has been proposed that Ssa1p maintains secretory precursor proteins in an extended, translocation-competent conformation and prevents their aggregation (CHIRICO *et al.* 1988).

As is the case with other hsp70s, the cellular functions of Ssa1p are likely modulated by interaction with a DnaJ-like partner protein. The most well-established cochaperone of Ssa1p is the cytosolic protein Ydj1p. *In vitro*,

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Ydj1p stimulates the ATPase activity of Ssa1p and also catalyzes the release of bound substrate from Ssa1p in an ATP-dependent manner (Cyr *et al.* 1992; Ziegelhoffer *et al.* 1995; Srinivasan *et al.* 1997; McClellan *et al.* 1998). In addition, *SSA* and *YDJ1* mutant alleles exhibit synthetic lethality (Becker *et al.* 1996). Ydj1p also appears to play a role in ER protein translocation because (1) a strain harboring a temperature-sensitive allele of *YDJ1* accumulates an untranslocated preprotein at the nonpermissive temperature (Caplan *et al.* 1992) and (2) translocation defects are observed if *YDJ1* is deleted in an *SSA1 ssa2 ssa3 ssa4* strain (Becker *et al.* 1996). Together, these data suggest that the interaction of Ssa1p and Ydj1p is important for both viability and protein translocation into the ER.

It is assumed that the ability of Ssa1p to couple ATP binding and hydrolysis to secretory precursor protein binding and release and the ability of Ydj1p to modulate these activities are important for Ssa1p to support post-translational protein translocation. Yet, each aspect of this model has not been directly tested in a single study. To this end, we engineered point mutations in the ATP-binding pocket of *SSA1* which, when present in the ER lumenal hsp70, BiP, were lethal and had dominant effects on translocation (McClellan *et al.* 1998). We found that these new *SSA1* mutations are not lethal, but cannot support ER translocation. *In vivo* and *in vitro* evidence suggests that the observed translocation defect arises from the inability of the mutant Ssa1p's to interact productively with Ydj1p and because they fail to associate with unfolded polypeptide substrates.

MATERIALS AND METHODS

Site-directed mutagenesis, cloning, strains, and media: Four mutant alleles of *SSA1*, *ssa1-101* (K69Q), *ssa1-102* (G198D), *ssa1-103* (G199D), and *ssa1-104* (G226D), were created using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). *SSA1* inserted between the *Hin*dIII and *Bam*HI sites of YEp351 (Hill *et al.* 1986; YEp351-*SSA1* plasmid provided by E. Craig, University of Wisconsin, Madison) served as the template. The primer pairs used were as follows: for *ssa1-101*, 5′-CCGTTTTCGACGCT**C**AGCGTTTGATCGG-3′ and 5′-CCGATCAAACGCT**G**AGCGTCGAAAACGG-3′; for *ssa1-102*, 5′-GTCTTGATTTTCGACTTGG**A**TGGTGGTACTTTCG-3′ and 5′-CGAAAGTACCACCA**T**CCAAGTCGAAAATCAA GAC-3′; for *ssa1-103*, 5′-GATTTTCGACTTGGGTG**A**TGG TACTTTC GATGTC-3′ and 5′-GACATCGAAAGTACCA**T**CACCCAAGTC GAAAATC-3′; and for *ssa1-104*, 5′-GGTGACACCCATTTGG**A**T GGTGAAGATTTTGAC-3′ and 5′-GTCAAAATCTTCACCA**T**C CAAATGGGTGTCACC-3′. In each primer the altered nucleotide is boldfaced and underlined. Mutagenesis resulted in plasmids YEp351-*ssa1-101*, YEp351-*ssa1-102*, YEp351-*ssa1-103*, and YEp351-*ssa1-104*. Subsequently, ~400 bp of *SSA1* and *ssa1-101* sequence were amplified by PCR, maintaining a unique *Nco*I site at the 3′ end and introducing a *Hin*dIII site, a new start codon, and six consecutive histidine residues at the 5′ end. The primers used were 5′-CCGTG**AAGCTTATG**CATCA TCATCATCATCATTCAAAAGCTGTCGGTATTGATTTA GG-3′ and 5′-CCTTCATCTTACCCAAGA**CCATGG**-3′. In the first primer, the underlined and boldfaced sequence represents the *Hin*dIII site, the sequence boldfaced indicates the new start codon, and the underlined sequence represents the primer bases that anneal to the template. In the second primer, the boldfaced and underlined sequence shows the *Nco*I recognition site. The $(His)_6$-tagged PCR fragment generated from *SSA1* was subcloned into YEp351-*SSA1*, YEp351-*ssa1-102*, YEp351-*ssa1-103*, and YEp351-*ssa1-104*, and the $(His)_6$-tagged PCR fragment generated from *ssa1-101* was subcloned into YEp351-*ssa1-101* between the *Hin*dIII and *SSA1 Nco*I sites. This created YEp351-$(His)_6$-*SSA1*, YEp351-$(His)_6$-*ssa1-101*, YEp351-$(His)_6$-*ssa1-102*, YEp351-$(His)_6$-*ssa1-103*, and YEp351-$(His)_6$-*ssa1-104*. Next, the wild-type and mutant $(His)_6$-*SSA1* alleles were inserted into the galactose-regulated pYES2 vector (Invitrogen, Carlsbad, CA) between the *Hin*dIII and *Bam*HI sites. The identities of the desired mutations and the absence of mutations elsewhere were confirmed by DNA sequence analysis. These plasmids were used to generate AJMY01–AJMY12 (see Table 1).

Plasmids constitutively expressing wild-type or mutant $(His)_6$-*SSA1* alleles were created as follows. The genes were excised from pYES2 and inserted into p426GPD (Mumberg *et al.* 1995) using the *Hin*dIII and *Xho* I sites. Next, *Pvu*II was used to remove the full-length genes, along with the 5′ glyceraldehyde-3-phosphate dehydrogenase promoter and 3′ *CYC1* terminator contained in the p426GPD sequence, and these fragments were blunt-end cloned into pRS317 (Sikorski and Boeke 1991) at the *Sma*I site. These plasmids, along with pYES2-$(His)_6$-*SSA1*, were used to generate AJMY28A1–AJMY28A6 (see Table 1). The insertion of wild-type and mutant GPD-$(His)_6$-*SSA1-CYC1* fragments into pRS426 (Sikorski and Hieter 1989) was performed by blunt-end ligating the *Pvu*II fragments from the p426GPD constructs into pRS426 at the *Pvu*II site. These plasmids were used to create AJMY36–AJMY39 (see Table 1).

The insertion of wild-type and mutant $(His)_6$-*SSA1* alleles into a copper-inducible expression vector (pCu426; Labbé and Thiele 1999) was accomplished by excising the wild-type and mutant $(His)_6$-*SSA1* constructs from pYES2 using *Hin*dIII and *Xho* I and ligating them into pCu426 at the same sites. These plasmids were used to make AJMY48–AJMY51 (see Table 1).

To create a strain in which $(His)_6$-*SSA1* was the only source of Ssa protein (AJMY28), MW332 and JN515 (see Table 1) were mated to obtain the diploid AJMY22. Then, 5-fluoroorotic acid (5-FOA) medium was used to select against cells containing pGAL-*SSA1*. Next, pYES2-$(His)_6$-*SSA1* was introduced by transformation to create AJMY23, and random spore analysis (Ausubel *et al.* 1998) was performed to obtain meiotic products containing pYES2-$(His)_6$-*SSA1* as the only cellular *SSA*.

AJMY28A, a *lys2* derivative of AJMY28, was created by selecting for AJMY28 isolates that grew on α-aminoadipate medium (Adams *et al.* 1997) containing 2% galactose. Mutation of *LYS2* in AJMY28A was confirmed since the transformation of pRS317 (Sikorski and Boeke 1991) into this strain restored growth on medium lacking lysine.

All procedures were performed and all media were prepared using standard protocols (Adams *et al.* 1997). Except where stated, yeasts were grown at 26° to prevent the induction of endogenous chaperones that might interfere with the effects of mutant protein expression.

Purification of wild-type and mutant hexahistidine-tagged Ssa1p's: Hexahistidine-tagged Ssa1p was purified from either AJMY02 or AJMY28A2. For purification from AJMY02, cells were grown to an optical density measured at 600 nm (OD_{600}) of 0.7–0.8 in 2 liters of synthetic complete medium lacking uracil and supplemented with raffinose to a final concentration of 2% (SC −ura raf), harvested, washed once with sterile water, and resuspended in 4 liters of YPGal (1% Bacto yeast

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
JN516	*MAT*α *his3-11, 15 leu2-3, 112 ura3-52 trp1-Δ1lys2 SSA1 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*	Becker *et al.* (1996)
AJMY01	JN516 with pYES2(*URA3*)	This study
AJMY02	JN516 with pYES2-$(His)_6$-*SSA1*	This study
AJMY03	JN516 with pYES2-$(His)_6$-*ssa1-101* (K69Q)	This study
AJMY04	JN516 with pYES2-$(His)_6$-*ssa1-102* (G198D)	This study
AJMY05	JN516 with pYES2-$(His)_6$-*ssa1-103* (G199D)	This study
AJMY06	JN516 with pYES2-$(His)_6$-*ssa1-104* (G226D)	This study
JB67	*MAT*α *his3-11, 15 leu2-3, 112 ura3-52 trp1-Δ1lys2 ssa1::ssa1-45 ssa2::LEU2 ssa3::TRP1ssa4::LYS2*	Becker *et al.* (1996)
AJMY07	JB67 with pYES2(*URA3*)	This study
AJMY08	JB67 with pYES2-$(His)_6$-*SSA1*	This study
AJMY09	JB67 with pYES2-$(His)_6$-*ssa1-101* (K69Q)	This study
AJMY10	JB67 with pYES2-$(His)_6$-*ssa1-102* (G198D)	This study
AJMY11	JB67 with pYES2-$(His)_6$-*ssa1-103* (G199D)	This study
AJMY12	JB67 with pYES2-$(His)_6$-*ssa1-104* (G226D)	This study
MW332	*MAT*α *his3-11, 15 leu2-3, 112 lys2 ura3-52 trp1-Δ1 ssa1::HIS3 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2* pGAL-*SSA1* (*URA3*)	E. Craig
JN515	*MAT***a** *his3-11, 15, leu2-3, 112 ura3-52 trp1-Δ1lys2 SSA1 ssa2-1 ssa3-1 ssa4-2*	E. Craig
AJMY22	*MAT***a**/*MAT*α *his3-11, 15/his3 leu2-3, 112/leu2 lys2/lys2 ura3-52/ura3 trp1-Δ1/Δtrp1SSA1/ssa1::HIS3 ssa2-1/ssa2::LEU2ssa3-1/ssa3::TRP1* pGAL-*SSA1* (*URA3*)	This study
AJMY23	AJMY22 with pYES2-*SSA1* (*URA3*) instead of pGAL-*SSA1* (*URA3*)	This study
AJMY28	*MAT*α *his3 leu2 lys2 Δtrp1 ura3 ssa1::HIS3ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*pYES2-$(His)_6$-*SSA1* (*URA3*)	This study
AJMY28A	AJMY28, but *lys2*	This study
AJMY28A1	AJMY28A with pRS317 (*LYS2*)	This study
AJMY28A2	AJMY28A with pRS317-GPD-$(His)_6$-*SSA1*	This study
AJMY28A3	AJMY28A with pRS317-GPD-$(His)_6$-*ssa1-101* (K69Q)	This study
AJMY28A4	AJMY28A with pRS317-GPD-$(His)_6$-*ssa1-102* (G198D)	This study
AJMY28A5	AJMY28A with pRS317-GPD-$(His)_6$-*ssa1-103* (G199D)	This study
AJMY28A6	AJMY28A with pRS317-GPD-$(His)_6$-*ssa1-104* (G226D)	This study
MW141	*MAT*α *his3-11, 15 leu2-3, 112 lys2 ura3-52 trp1-Δ1ssa1::HIS3 ssa2::LEU2 ssa4::URA3* pGAL-*SSA1* (*TRP1*)	Deshaies *et al.* (1988)
AJMY36	JN516 with pRS426 (*URA3*)	This study
AJMY37	JN516 with pRS426-GPD-$(His)_6$-*SSA1*	This study
AJMY38	JN516 with pRS426-GPD-$(His)_6$-*ssa1-101* (K69Q)	This study
AJMY39	JN516 with pRS317-GPD-$(His)_6$-*ssa1-103* (G199D)	This study
AJMY48	JB67 with pCu426 (*URA3*)	This study
AJMY49	JB67 with pCu426-$(His)_6$-*SSA1*	This study
AJMY50	JB67 with pCu426-$(His)_6$-*ssa1-101* (K69Q)	This study
AJMY51	JB67 with pCu426-$(His)_6$-*ssa1-103* (G199D)	This study

extract, 2% Bacto peptone, 2% galactose). After 16 hr, cells were harvested, washed, and converted to spheroplasts (Brodsky *et al.* 1993). For purification from AJMY28A2, cells were grown to an OD_{600} of 1–2 in 4 liters of SC −ura medium lacking lysine and supplemented with galactose to a final concentration of 2% (SC −ura −lys gal), harvested, washed, and converted to spheroplasts. In subsequent steps, the following protease inhibitors were included in all buffers at the concentrations recommended by the manufacturer: phenylmethylsulfonyl fluoride, pepstatin-A, leupeptin, and ρ-amino benzamidine (Sigma Chemical, St. Louis). Spheroplasts were resuspended in 30 ml of buffer B [40 mM HEPES, pH 6.8, 5 mM MgOAc, 75 mM KCl, and 1 mM dithiothreitol (DTT)], and a one-half volume of glass beads was added. The cells were agitated on a Vortex mixer six times for 1 min, with 2 min on ice between each disruption. Unbroken cells were removed by centrifugation at 3000 × *g* for 5 min at 4°, and then the resulting supernatant was spun at 22,000 × *g* for 10 min at 4°. This supernatant was loaded onto a 5-ml ATP-agarose (Sigma Chemical) column equilibrated in buffer C (20 mM HEPES, pH 6.8, 2 mM MgOAc, and 25 mM KCl). The column was washed sequentially with 30 ml of buffer C, 30 ml of buffer C containing 1 M KCl, and 20 ml of buffer C. Ssa1p was eluted with 20 ml of buffer C containing 7 mM ATP. Peak fractions, as determined by SDS-PAGE and immunoblot analysis with antipentahistidine antibody (QIAGEN, Hilden, Germany), were pooled and loaded onto a 5-ml Q-sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ) pre-equilibrated in buffer C. The column was washed with 30 ml of buffer C, and then Ssa1p was eluted with a 15-ml × 15-ml gradient of buffer C to buffer C containing 800 mM KCl. Fractions enriched for Ssa1p were pooled, diluted 1:2 in buffer S

(50 mM HEPES, pH 7.4, 300 mM NaCl, 10 mM imidazole, and 5 mM β-mercaptoethanol), and loaded onto a ~2-ml Ni^{2+}-NTA (QIAGEN) or Talon (CLONTECH, Palo Alto, CA) metal affinity resin column pre-equilibrated in buffer S. The column was washed sequentially with 20 ml of buffer S containing 2% Triton X-100 and 5% glycerol, 20 ml of buffer S containing 1 M NaCl and 5% glycerol, and 20 ml of buffer S containing 50 mM NaCl and 5% glycerol. Hexahistidine-tagged Ssa1p [$(His)_6$-Ssa1p] was eluted with 7 ml of buffer S containing 50 mM NaCl, 250 mM imidazole, and 5% glycerol. Peak fractions were dialyzed for 14 hr at 4° in dialysis buffer (50 mM Tris, pH 7.4, 50 mM NaCl, 0.8 mM DTT, 2 mM $MgCl_2$, and 5% glycerol) and then snap-frozen in liquid nitrogen and stored at −70°.

Ssa1-K69Qp and Ssa1-G199Dp were purified from strains AJMY03, AJMY05, AJMY50, and AJMY51. AJMY03 and AJMY05 were grown and harvested as described above for AJMY02. For purification from AJMY50 and AJMY51, cells were grown to an OD_{600} of ~0.6–8 in SC −ura medium supplemented with glucose to a final concentration of 2% (SC −ura glu). Then, $CuSO_4$ was added to a final concentration of 100 μM, and the cells were incubated for an additional 2 hr before they were harvested and converted to spheroplasts. The purification procedure after this point was identical to that employed for wild-type $(His)_6$-Ssa1p, with the exception that the ATP-agarose column was omitted as the first step because the mutant proteins bound poorly to this resin (data not shown).

Accumulation of untranslocated ppαf *in vivo*: MW141, AJMY28A1, AJMY28A2, AJMY28A3, and AJMY28A5 (see Table 1) were grown to an OD_{600} of 0.7–1 in SC −ura −lys gal medium. Then, cultures were diluted to an OD_{600} of 0.1 in SC −ura −lys glu medium and grown for 14 hr. Equivalent amounts of cells (~4.5×10^8) were harvested at 0, 10, 12, and 14 hr and washed once with sterile water. Final OD_{600}s were 0.86 (MW141), 1.11 (AJMY28A1), 1.36 (AJMY28A2), 0.8 (AJMY28A3), and 0.84 (AJMY28A5). The cell pellets were resuspended in buffer B containing protease inhibitors and lysed by agitation with glass beads on a Vortex mixer six times for 1 min and kept on ice for 2 min between disruptions. The homogenate was spun for 5 min at 5000 rpm in a microcentrifuge to remove unbroken cells and the amount of protein in the supernatant was quantified using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA) and bovine serum albumin (BSA) as the standard. Equal amounts of protein (~25 μg) were analyzed by SDS-PAGE followed by immunoblot analysis using enhanced chemiluminescence (Pierce, Rockford, IL) with antipentahistidine, Ssa1p, and ppαf antibodies.

***In vitro* translocation assays:** Yeast microsomal membranes, cytosol, and wheat-germ translated ppαf were prepared and *in vitro* translocation reactions were performed as described previously (Brodsky *et al.* 1993).

Assay for Ssa1p association with Ydj1p: This assay was performed essentially as described in Oka *et al.* (1998). AJMY36, AJMY37, AJMY38, and AJMY39 were grown in SC −ura glu medium to an OD_{600} of 0.8–1.0. Then, the cells were harvested, washed once with water, and resuspended to ~20 OD_{600} units/ml in buffer B containing protease inhibitors. A one-half volume of glass beads was added, and the cells were disrupted on a Vortex mixer four times for 1 min, with a 2-min incubation on ice between disruptions. Unbroken cells were removed by centrifugation for 5 min at 5000 rpm in a microcentrifuge. Reactions contained 89 μl of protein extract and an ATP-regenerating system (Brodsky *et al.* 1993) in a total volume of 100 μl. Into each reaction, 100 μl of buffer S, 2 μl of 10% Triton X-100, and 80 μl of a 1:1 slurry of Talon resin in buffer S were introduced. The reactions were then rotated at 4° for 45 min, spun for 1 min at 13,000 rpm in a microcentrifuge to pellet the resin and bound proteins, and the supernatants were removed and ice-cold trichloroacetic acid (TCA) was added to a final concentration of 20%. The TCA-precipitated proteins were washed once with acetone and resuspended in SDS sample buffer (1% β-mercaptoethanol, 2% SDS, 0.05 mg/ml Bromophenol blue, 0.065 M Tris, pH 6.8). The resin was washed three times with buffer S and resuspended in SDS sample buffer. Bound and unbound fractions were analyzed by SDS-PAGE followed by immunoblot analysis using enhanced chemiluminescence with antipentahistidine, Ssa1p, Ydj1p, L3, and Kar2p antibodies.

ATPase assays: ATPase assays were performed as described (McClellan *et al.* 1998), with the exception that 3 μl of the reaction was sampled and quenched with 1 μl of stop solution (2 M LiCl, 4 M formic acid, and 36 mM ATP; Miao *et al.* 1997) every 6 min for 30 min.

Binding of ^{125}I-CMLA to Ssa1p: The binding of radiolabeled, permanently unfolded carboxymethylated α-lactalbumin (CMLA; Sigma Chemical) to wild-type and mutant Ssa1p's was performed as previously described (McClellan *et al.* 1998) except that 2 μg of protein was used.

Confirmation of protein expression and quantitative immunoblotting: The expression of wild-type and mutant $(His)_6$-Ssa1p's was confirmed by immunoblot analysis using antipentahistidine primary antibody and enhanced chemiluminescence. In all cases, equal amounts of protein, as determined using the Bio-Rad Protein Assay reagent and BSA as the standard, were analyzed (~15 μg). For direct comparison of expression levels, anti-Sec61p antibody was used as a loading control. Protein levels were assessed by quantitative immunoblot analysis using ^{125}I-labeled protein A (Amersham Pharmacia Biotech) as the secondary antibody for the anti-Ssa1p and anti-Ydj1p primary antibodies and using ^{125}I-labeled anti-mouse whole antibody (Amersham Pharmacia Biotech) as the secondary antibody for the antipentahistidine primary antibody.

RESULTS

Mutations in the ATP-binding pocket of *SSA1* confer a dominant slow-growth phenotype and are null with regard to cell viability: In a previous study we characterized a collection of dominant lethal mutations of the ER lumenal chaperone BiP (McClellan *et al.* 1998). The corresponding mutant proteins, which contained single amino acid substitutions of conserved residues in the ATP-binding pocket, were ATPase-defective, failed to interact productively with their DnaJ-like partner protein, Sec63p, and were unable to support post-translational translocation *in vitro*. To define whether the coupling of ATP hydrolysis, peptide binding, and Ydj1p interaction is critical for Ssa1p to support protein translocation, we created the identical changes in *SSA1* (K69Q, G198D, G199D, and G226D, see materials and methods). In addition, a hexahistidine [$(His)_6$] tag was engineered at the N terminus of the proteins so that their expression could be monitored *in vivo* and to aid in their purification. The genes encoding wild-type and mutant $(His)_6$-*SSA1* were inserted into the galactose-inducible expression vector, pYES2.

We first tested whether expression of the mutant Ssa1p's was lethal. The wild-type and mutant pYES2-$(His)_6$-*SSA1* constructs were transformed into a strain

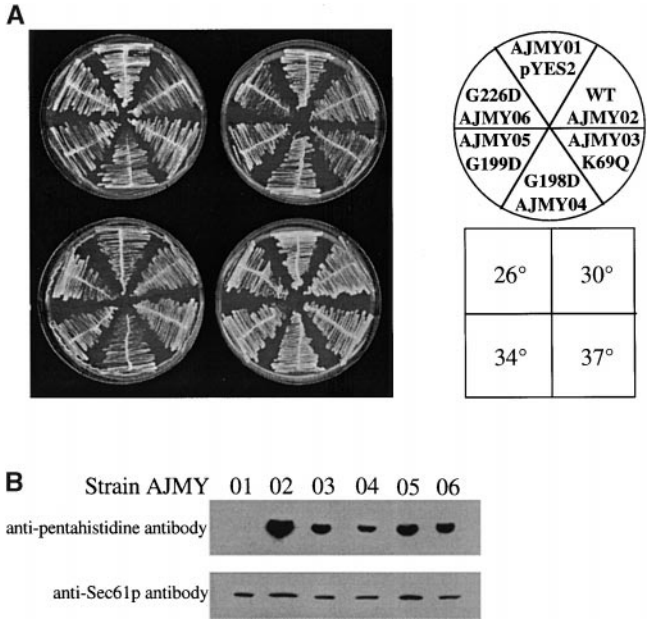

FIGURE 1.—Expression of mutant Ssa1p's is not lethal. (A) Strain JN516 was transformed with the galactose-inducible expression vector, pYES2, lacking insert (AJMY01) or containing $(His)_6$-*SSA1* (AJMY02), $(His)_6$-*ssa1-K69Q* (AJMY03), $(His)_6$-*ssa1-G198D* (AJMY04), $(His)_6$-*ssa1-G199D* (AJMY05), or $(His)_6$-*ssa1-G226D* (AJMY06) (see Table 1). Growth on galactose-containing medium was examined after 3 days at the indicated temperatures. Strains were intentionally overgrown to uncover strong phenotypes, in contrast to the analysis shown in Table 2. (B) Protein expression was confirmed by immunoblot analysis with antipentahistidine antibody. Anti-Sec61p antibody, used to detect an integral membrane protein in the ER, was used as a loading control.

harboring wild-type *SSA1* but with insertion mutations in *SSA2*, *SSA3*, and *SSA4* (JN516, see Table 1). The effect of mutant Ssa1p expression was observed by examining growth on galactose-containing medium at 26°, 30°, 34°, and 37°. As shown in Figure 1A, strains expressing the mutant Ssa1p's were viable at all temperatures tested. Expression of the wild-type and mutant *SSA1* constructs was confirmed by Western blot analysis with antipentahistidine antibody (Figure 1B). Immunoblot analysis using ^{125}I-conjugated secondary antibody revealed that the levels of mutant proteins were 20% (Ssa1-G198Dp) to 46% (Ssa1-G199Dp) of wild-type $(His)_6$-Ssa1p (see Table 2). The growth of these strains at 26° in liquid medium was also examined. As shown in Table 2, the expression of mutant Ssa1p's resulted in a slow-growth phenotype. Although the growth of strain AJMY02, expressing wild-type $(His)_6$-*SSA1*, is somewhat slower (~60%) than the strain containing vector lacking insert (AJMY01), expression of the mutants decreases the doubling times from 107 to 133%. These results demonstrate that this level of mutant *SSA1* expression does not result in lethality, but compromises growth in the presence of wild-type Ssa1p.

To determine whether the mutant $(His)_6$-Ssa1p's are active, two experiments were performed. First, strains AJMY07–AJMY12 were created by transforming the wild-type and mutant *SSA1* constructs into strain JB67, which contains a temperature-sensitive allele of *SSA1*, *ssa1-45* (see Table 1; BECKER *et al.* 1996). Figure 2A shows that only one strain, AJMY08 [containing wild-type $(His)_6$-*SSA1*], grew at 34° and 37°. Ssa1p expression was confirmed in these strains (Figure 2B), as above, and quantification revealed that the mutant protein levels were 27% (Ssa1-G198D) to 47% (Ssa1-K69Qp) that of wild-type $(His)_6$-Ssa1p. Growth in liquid medium at 26° was also examined, and a slow-growth phenotype was again observed for strains expressing the mutant Ssa1p's (see Table 2). Second, strain AJMY28A, in which pYES2-$(His)_6$-*SSA1* is the only source of Ssap, was created (see MATERIALS AND METHODS, Table 1). As such, the viability of this strain depends upon the galactose-induced expression of Ssa1p. Multicopy plasmids lacking an insert or containing wild-type or the four mutant *SSA1* alleles under the control of a strong constitutive promoter [glyceraldehyde-3-phosphate dehydrogenase (GPD); see MATERIALS AND METHODS] were transformed into AJMY28A. Viability was then assessed under conditions of glucose repression. The data in Figure 2C show that only the strain transformed with the wild-type GPD-$(His)_6$-Ssa1p construct grew on glucose-containing medium. This result was confirmed by the demonstration that only this strain was able to grow on medium containing 5′-FOA, indicating that the strains constitutively expressing the *SSA1* mutants cannot lose the pYES2-$(His)_6$-*SSA1* plasmid (data not shown). Expression of the wild-type and mutant $(His)_6$-Ssa1p's from the pRS317-

TABLE 2

Doubling times of strains expressing wild-type or mutant *SSA1* at 26° in SC −ura gal

Strain	Doubling time (in hours)	% expression[a]
AJMY01 (pYES2)	3	NA
AJMY02 (*SSA1*)	4.9	100
AJMY03 (*ssa1-K69Q*)	7	33
AJMY04 (*ssa1-G198D*)	7.2	20
AJMY05 (*ssa1-G199D*)	6.3	46
AJMY06 (*ssa1-G226D*)	6.2	28
AJMY07 (pYES2)	5.5	NA
AJMY08 (*SSA1*)	4.8	100
AJMY09 (*ssa1-K69Q*)	9.5	47
AJMY10 (*ssa1-G198D*)	8.1	27
AJMY11 (*ssa1-G199D*)	5.2	39
AJMY12 (*ssa1-G226D*)	5.6	33

[a] The % expression is the amount of mutant Ssa1p expressed from pYES2, compared to wild-type Ssa1p expressed from pYES2 (normalized to 100%) in the same strains, as quantified by phosphorimage analysis of immunoblots incubated with antipentahistidine primary antibody and ^{125}I-labeled anti-mouse secondary antibody. NA, not available.

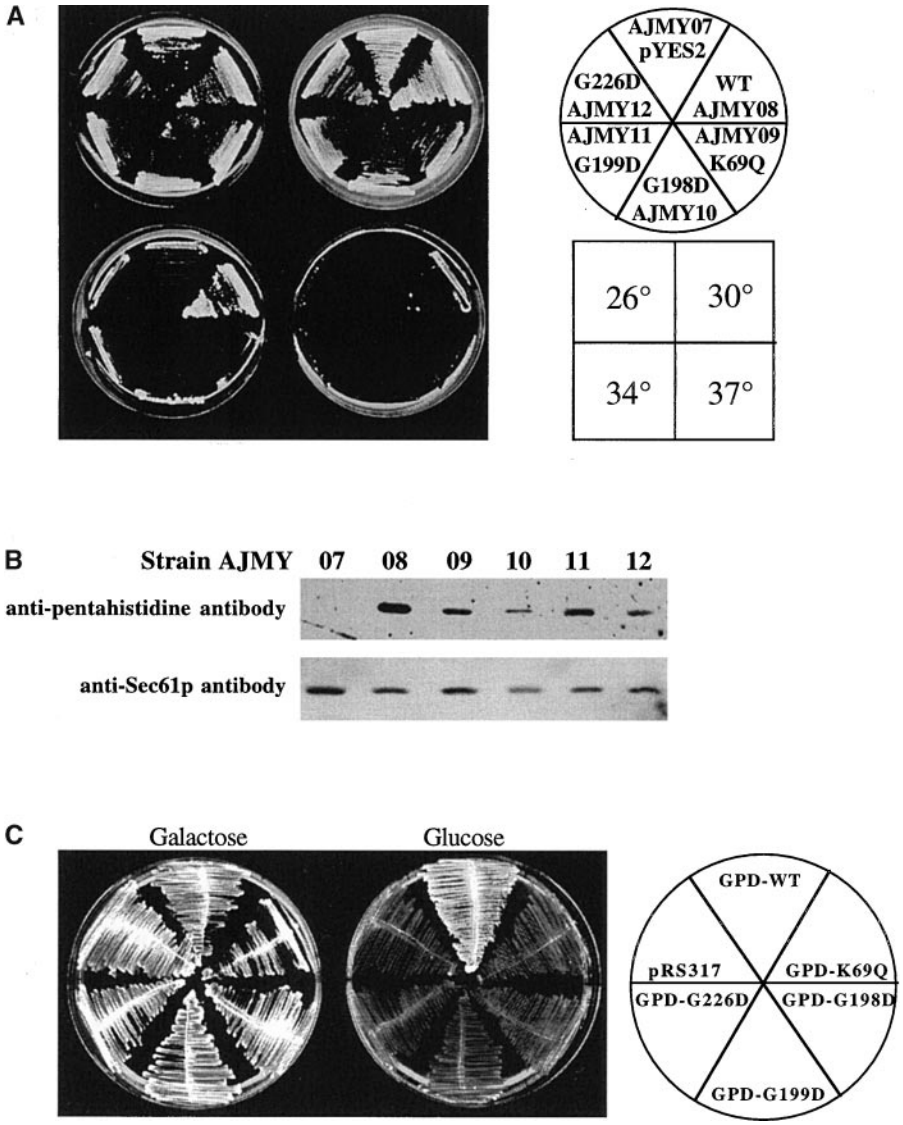

FIGURE 2.—The mutant Ssa1p's do not rescue the growth of the *ssa1-45* temperature-sensitive strain and result in a null phenotype. (A) Strain JB67 was transformed with the galactose-inducible expression vector, pYES2, lacking insert (AJMY07) or containing $(His)_6$-*SSA1* (AJMY08), $(His)_6$-*ssa1-K69Q* (AJMY09), $(His)_6$-*ssa1-G198D* (AJMY10), $(His)_6$-*ssa1-G199D* (AJMY11), or $(His)_6$-*ssa1-G226D* (AJMY12) (see Table 1). Growth on galactose-containing medium was examined after 4 days at the indicated temperatures. (B) Protein expression was confirmed by immunoblot analysis with antipentahistidine antibody, and anti-Sec61p antibody was used as a loading control. (C) Strain AJMY28A [which contains pYES2-$(His)_6$-*SSA1* as the only source of Ssap] was transformed with pRS317 lacking insert or constitutively expressing wild-type or mutant $(His)_6$-Ssa1p's (GPD-WT, GPD-K69Q, GPD-G198D, GPD-G199D, and GPD-G226D). Growth on medium containing either glucose or galactose, as indicated, was assessed after 3 days at 26°.

based plasmids was confirmed by immunoblotting extracts from an unrelated wild-type strain transformed with the pRS317-GPD constructs (data not shown). In summary, although the mutant Ssa1 proteins are expressed at a somewhat lower level than wild-type Ssa1p, these results indicate that the Ssa1p mutants confer a dominant slow-growth phenotype, are null, and that the hexahistidine tag does not affect the ability of wild-type Ssa1p to support cell growth.

The G199D mutant dominantly affects post-translational protein translocation *in vivo*: The depletion or mutation of *SSA1* in strains lacking other *SSA*s prevents the post-translational translocation of the yeast mating pheromone precursor, ppαf, into the ER (DESHAIES *et al.* 1988; BECKER *et al.* 1996). To test whether the mutant Ssa1p's supported the translocation of ppαf *in vivo*, strains containing galactose-inducible wild-type *SSA1* and constitutively expressing *SSA1*, *ssa1-K69Q*, or *ssa1-G199D* were assessed for ppαf accumulation before and during conditions of glucose repression. We focused on the *in vivo* and *in vitro* activities of the two mutant proteins that are expressed to the highest levels in all strains examined (K69Q and G199D; see above). The four point mutations are functionally equivalent when studied in the context of yeast BiP (MCCLELLAN *et al.* 1998).

First, we recapitulated the results originally obtained with strain MW141 (DESHAIES *et al.* 1988) in which ppαf accumulation was observed 10 hr after depletion of Ssa1p (Figure 3A, row 1). The same result was obtained with strain AJMY28A1, which contains only pYES2-$(His)_6$-*SSA1* and a control vector lacking insert (Figure 3A, row 2). Ssa1p levels decreased significantly after 14 hr in both of these strains (to 5% of the initial level for MW141 and to 9% of the initial level for AJMY28A1; data not shown). In contrast, the presence of constitutively expressed *SSA1* prevented the accumulation of ppαf (Figure 3A, row 3). Strikingly, neither mutant protein prevented the accumulation of ppαf when wild-type Ssa1p was depleted (Figure 3A, rows 4 and 5). In fact, a significant amount of ppαf accumulated when Ssa1-

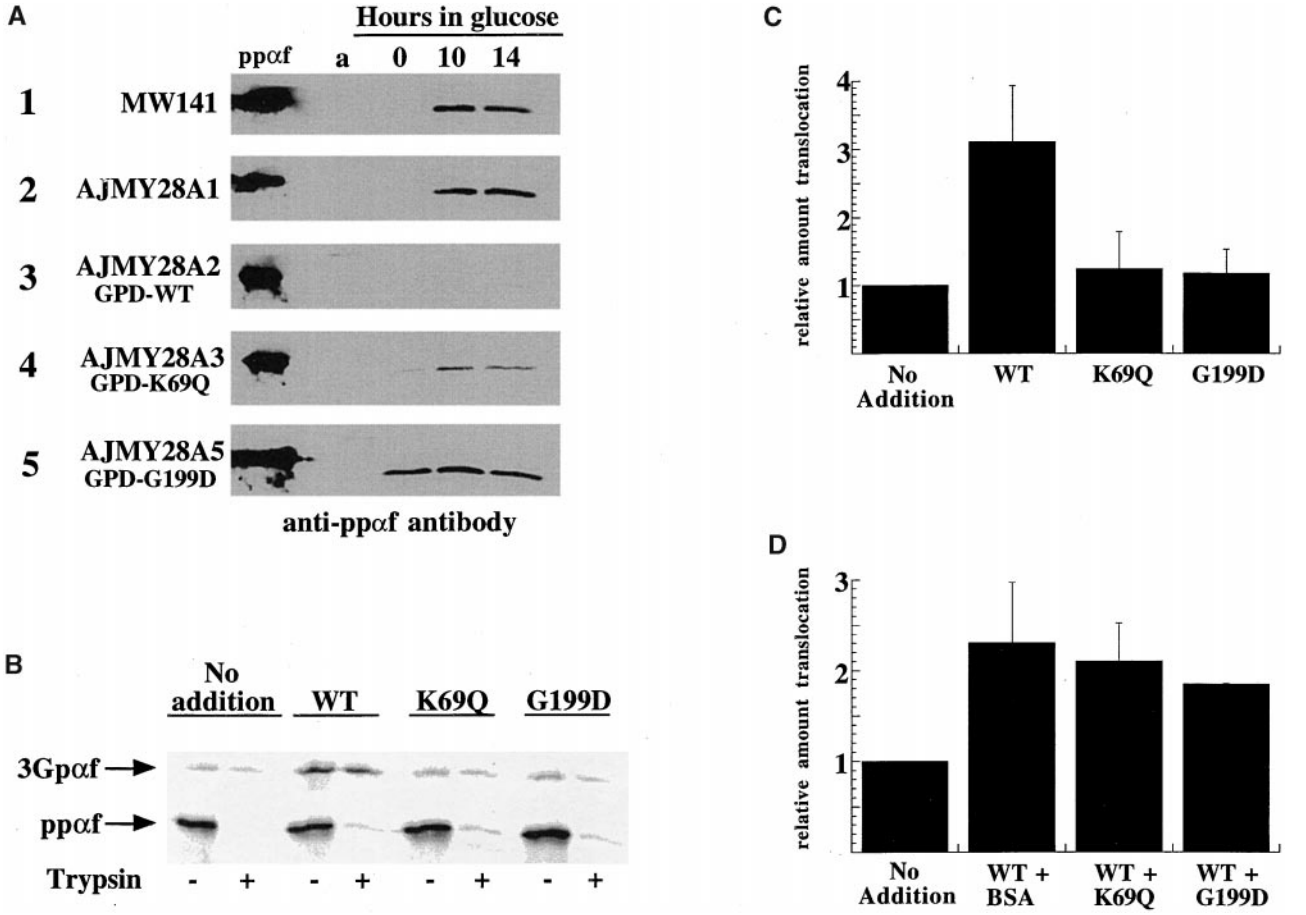

FIGURE 3.—The K69Q and G199D mutants cannot support ppαf translocation. (A) Strains containing galactose-driven *SSA1* and no vector (MW141, row 1), vector lacking insert (AJMY28A1, row 2), or a vector constitutively expressing wild-type (row 3), K69Q (row 4), or G199D (row 5) $(His)_6$-Ssa1p's were grown in the appropriate synthetic medium containing galactose and then diluted into glucose-containing medium at time 0. Equivalent amounts of cells were harvested at each time point. The lane marked ppαf contains 1 μg of purified $(His)_6$-ppαf (BUSH *et al.* 1991), which migrates more slowly than endogenous ppαf, as a positive control for the anti-ppαf antibody. The lane marked **a** represents yeast protein extract from a *MAT***a** strain as a negative control. (B) The results of *in vitro* translocation reactions containing 5 μg of yeast cytosol and 5 μg of the indicated $(His)_6$-Ssa1p are shown. The positions of ppαf and triply glycosylated, signal-sequence-cleaved pαf (3Gpαf) are indicated. (C) The mean "relative amount translocation," as determined by quantifying trypsin-protected, triply glycosylated, signal-sequence-cleaved ppαf from three independent *in vitro* translocation assays, ±SD, are shown. The relative amount translocation in the absence of Ssa1p was set to 1. (D) The mean relative amount translocation, ±SD, as determined in C, is shown for *in vitro* translocation experiments containing either 10 μg of BSA (No Addition) or 5 μg of wild-type $(His)_6$-Ssa1p and 5 μg of either BSA, $(His)_6$-Ssa1-K69Qp, or $(His)_6$-Ssa1-G199Dp. The relative amount translocation that occurred in the absence of Ssa1p was set to 1.

G199Dp was expressed, even before wild-type Ssa1p was depleted (Figure 3A, row 5: compare ppαf signal at time 0 to that observed in rows 1–4). Ppαf accumulation was also evident, but to a lesser extent, when Ssa1-K69Qp was expressed in the presence of wild-type Ssa1p (Figure 3A, row 4).

The mutant Ssa1p's do not stimulate ppαf translocation *in vitro*: Previous *in vitro* studies demonstrated that purified Ssa1p, in the presence of limiting amounts of yeast cytosol, stimulates the post-translational translocation of wheat-germ-translated ppαf (CHIRICO *et al.* 1988; DESHAIES *et al.* 1988; BRODSKY *et al.* 1993), a reaction that also requires hydrolyzable ATP (WATERS and BLOBEL 1986). To examine whether the mutant Ssa1p's support ppαf translocation *in vitro*, the proteins were purified from yeast using a combination of ion-exchange and metal-affinity chromatography (see MATERIALS AND METHODS). As shown in Figure 3, B and C, the addition of wild-type $(His)_6$-Ssa1p stimulated the translocation of ppαf into yeast microsomes approximately threefold, as demonstrated by the appearance of triply glycosylated and signal-sequence-cleaved pαf (3Gpαf) that is protected from trypsin degradation. This level of stimulation is identical to that previously observed using the same conditions employed in this study (BRODSKY *et al.* 1993). We found, however, that Ssa1-K69Qp and Ssa1-G199Dp were unable to stimulate the post-translational translocation of ppαf, consistent with the results obtained in Figure 3A.

We next examined whether we could recapitulate *in*

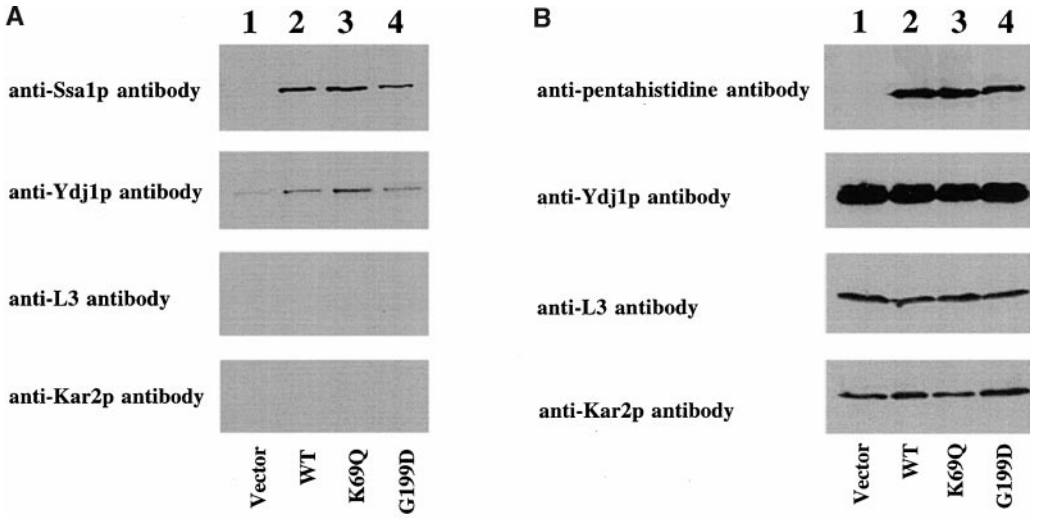

FIGURE 4.—Ydj1p associates with wild-type and mutant Ssa1p's. Strain JN516 was transformed with pRS426 lacking insert (lane 1, AJMY36) or constitutively expressing $(His)_6$-*SSA1* (lane 2, AJMY37), $(His)_6$-*ssa1-K69Q* (lane 3, AJMY38),or $(His)_6$-*ssa1-G199D* (lane 4, AJMY39), and protein extracts were prepared and examined for Ydj1p-Ssa1p interaction as described in MATERIALS AND METHODS. Immunoblots of bound (A) and unbound (B) fractions with the indicated antibodies are shown. As negative controls, we failed to observe association between Ssa1p and the L3 ribosomal subunit or Kar2p/BiP, an ER lumenal protein.

vitro the dominant inhibition of protein translocation we observed *in vivo* for Ssa1-G199Dp. To this end, *in vitro* translocation was assayed such that the total amount of protein was constant but mixtures of wild-type and mutant proteins were present. Figure 3D shows that the mutant protein did not abrogate the ability of wild-type Ssa1p to stimulate ppαf translocation *in vitro*. Possible explanations for the failure of Ssa1-G199Dp to exhibit dominance *in vitro* are presented in the DISCUSSION.

The mutant Ssa1p's associate with, but are not activated by, Ydj1p: It has been suggested that interaction between Ydj1p and Ssa1p is required for post-translational protein translocation (CAPLAN *et al.* 1992; BECKER *et al.* 1996). To determine whether Ssa1p, Ssa1-K69Qp, and Ssa1-G199Dp associate with Ydj1p, we employed a procedure established by OKA *et al.* (1998) in which metal affinity precipitation was used to isolate $(His)_6$-Ssa1p · Ydj1p complexes. Immunoblot analysis of Talon resin-bound protein fractions indicated that wild type, Ssa1-K69Qp, and Ssa1-G199Dp interact with Ydj1p (Figure 4A, lanes 2–4). Immunoblot analyses using ^{125}I-labeled protein A were quantified and revealed no difference in the ratio of Ydj1p:Ssa1p, whether wild-type or mutant Ssa1p was expressed (data not shown). In addition, the amount of Ydj1p was increased ~4.7-, ~4.9-, and ~1.6-fold in lanes 2, 3, and 4, respectively, over the negative control (Figure 4A, lane 1). Immunoblot analysis of unbound fractions showed no significant differences in the level of Ydj1p in cells expressing wild-type or mutant Ssa1p (Figure 4B). These data suggest that the mutant Ssa1p's associate with Ydj1p *in vivo*.

We next wanted to determine whether Ydj1p interacts productively with the mutant Ssa1p's and stimulates their ATPase activity (CYR *et al.* 1992; ZIEGELHOFFER *et al.* 1995; SRINIVASAN *et al.* 1997; MCCLELLAN *et al.* 1998). Because these mutations lie in the ATPase domain, we first examined whether the Ssa1-K69Qp and Ssa1-G199Dp mutants hydrolyze ATP. Figure 5A shows the results obtained with wild-type $(His)_6$-Ssa1p (open circles). The average specific activity calculated at 30 min was ~0.77 nmol of ATP hydrolyzed per minute per milligram of Ssa1p (nmol · min^{-1} · mg^{-1}). This corresponds well to published ATPase values for Ssa1p (CYR *et al.* 1992; ZIEGELHOFFER *et al.* 1995; MCCLELLAN *et al.* 1998) and human hsp70 (RAJAPANDI *et al.* 1998), showing that the hexahistidine tag does not affect ATP hydrolysis by Ssa1p. When Ydj1p was present (Figure 5A, filled circles), the ATPase activity of Ssa1p was stimulated approximately fivefold, similar to what was observed previously for Ssa1p (MCCLELLAN *et al.* 1998). Figure 5, B and C, present the results obtained for the two mutant proteins. Ssa1-K69Qp exhibited a specific activity of ~0.59 nmol · min^{-1} · mg^{-1} and the specific activity of Ssa1-G199Dp was ~0.9 nmol · min^{-1} · mg^{-1} (open circles). These values are higher compared to the specific activities observed for these same amino acid changes in yeast BiP (~0.03 and ~0.06 nmol · min^{-1} · mg^{-1} for K69Qp and G199Dp, respectively; MCCLELLAN *et al.* 1998) and the analogous mutation in human hsp70 (<10% of what we observe; RAJAPANDI *et al.* 1998), but correlate well with the activity of the mammalian BiP mutant corresponding to Ssa1-G199Dp (~1.5 nmol · min^{-1} · mg^{-1}; WEI *et al.* 1995). This discrepancy may arise from the fact that the hsp70s in these other studies were purified from bacteria and not from their native organism, as was done in this study. Regardless, these results demonstrate that Ydj1p failed to stimulate the ATPase activities of the Ssa1p mutants (filled circles). In accordance with our results, mutation of mammalian hsc70 residue K71 (analogous to K69 in Ssa1p) resulted in defective interaction with Ydj1p (RAJAPANDI *et al.* 1998).

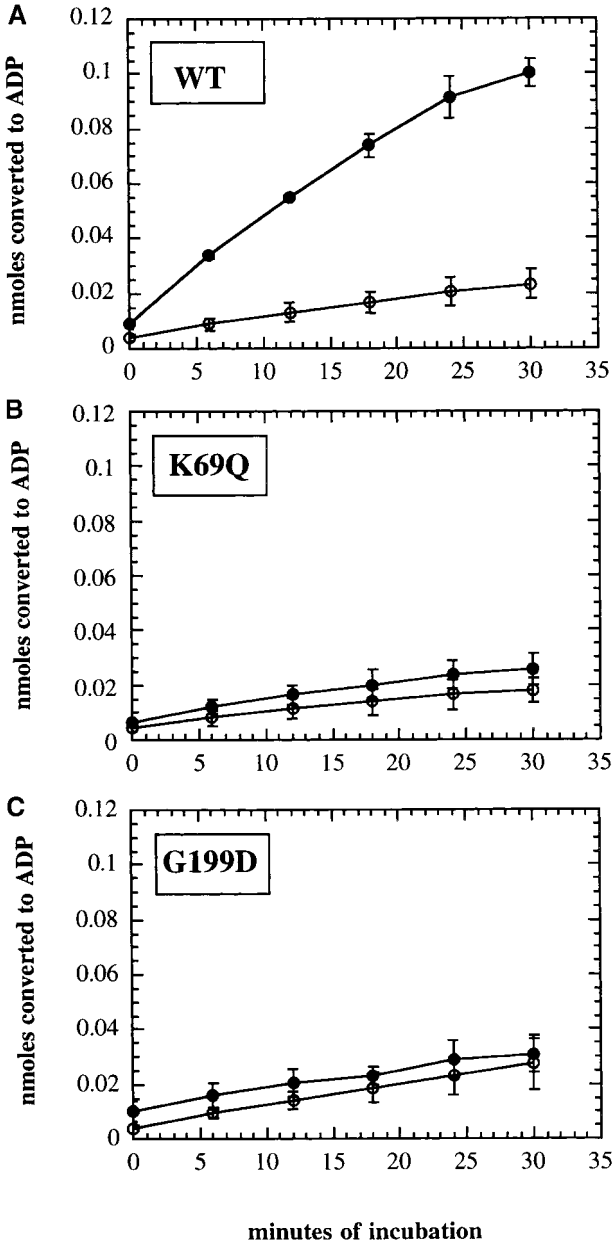

FIGURE 5.—Ydj1p cannot activate the ATPase activity of mutant Ssa1p. Reactions containing 1 μg of (A) $(His)_6$-Ssa1p, (B) $(His)_6$-Ssa1-K69Qp, or (C) $(His)_6$-Ssa1-G199Dp were assayed for ATPase activity in the presence (solid circles) or absence (open circles) of 2 μg of Ydj1p at 30° (see MATERIALS AND METHODS). Results shown are the means of reactions performed in triplicate with two different preparations of each Ssa1 protein, ±SD.

Mutant Ssa1p's cannot bind a permanently unfolded polypeptide substrate: The proposed role of Ssa1p in the process of protein translocation also depends upon its ability to bind to unfolded precursor proteins and maintain them in an extended and translocation-competent conformation (CHIRICO *et al.* 1988). To determine whether the Ssa1p mutants bind an extended polypeptide, we used an ^{125}I-labeled, permanently unfolded polypeptide substrate (^{125}I-CMLA). CMLA has been extensively utilized as a substrate for hsp70s (see, for example, PALLEROS *et al.* 1991; CYR *et al.* 1992; FOURIE *et al.* 1994; FREEMAN *et al.* 1995). When binding was assessed by native PAGE, we found that wild-type Ssa1p bound proficiently to ^{125}I-CMLA (Figure 6A, lane 2). In lanes 3 and 4, which contain Ssa1-K69Qp and Ssa1-G199Dp, respectively, little binding was observed. Quantified results from several assays showed that the mutant Ssa1p's are clearly defective for ^{125}I-CMLA binding (Figure 6B). As these mutant Ssa1p's harbor amino acid substitutions in the ATPase domain, we suggest that the coupling between the hsp70 ATPase and peptide-binding domains (FLYNN *et al.* 1989; SCHMID *et al.* 1994; FUNG *et al.* 1996; DAVIS *et al.* 1999) is altered. These data suggest that the inability of the mutant Ssa1p's to support protein translocation may be due, in part, to their failure to bind unfolded polypeptides.

DISCUSSION

We report here that Ssa1 proteins containing point mutations in the ATP-binding pocket cannot support post-translational translocation into the yeast ER either *in vivo* or *in vitro*. To explore the molecular basis of this translocation defect, the wild-type and mutant Ssa1p's were purified. In established assays, the mutant proteins associated with their cognate DnaJ homologue, Ydj1p, but failed to interact productively because Ydj1p was unable to stimulate their ATPase activities. One hypothesis to explain these data is that Ydj1p fails to interact properly with the mutated ATPase domain, precluding stimulation of Ssa1p's ATPase activity. This hypothesis is built upon mutational and NMR analyses that demonstrated that the J domain of DnaJ interacts with the ATPase domain of DnaK, while some portion of DnaJ interacts with the DnaK substrate binding domain (GÄSSLER *et al.* 1998; GREENE *et al.* 1998; SUH *et al.* 1998). Mutant protein expression compromised the ability of wild-type Ssa1p to support post-translational protein translocation *in vivo*, and the mutant Ssa1p's, unlike wild-type protein, were unable to bind to an unfolded polypeptide substrate. The mutant Ssa1p's also caused a dominant slow-growth phenotype and were unable to support life as the only cellular Ssap.

Taken together, these results suggest a model in which the nonproductive interaction of mutant Ssa1p with Ydj1p precludes the productive interaction of polypeptide-bound wild-type Ssa1p with Ydj1p. In corroboration with this model, CAPLAN *et al.* (1992) showed that yeast containing the *ydj1-151* temperature-sensitive mutation accumulated untranslocated ppαf at the nonpermissive temperature, and purified Ydj1-151 protein was unable to enhance the ATPase activity of Ssa1p. However, these authors did not investigate whether Ydj1-151p could release a polypeptide substrate from Ssa1p.

Alternatively, Ssa1p may act as a dimer, and nonfunctional mixed dimers of wild-type and mutant protein

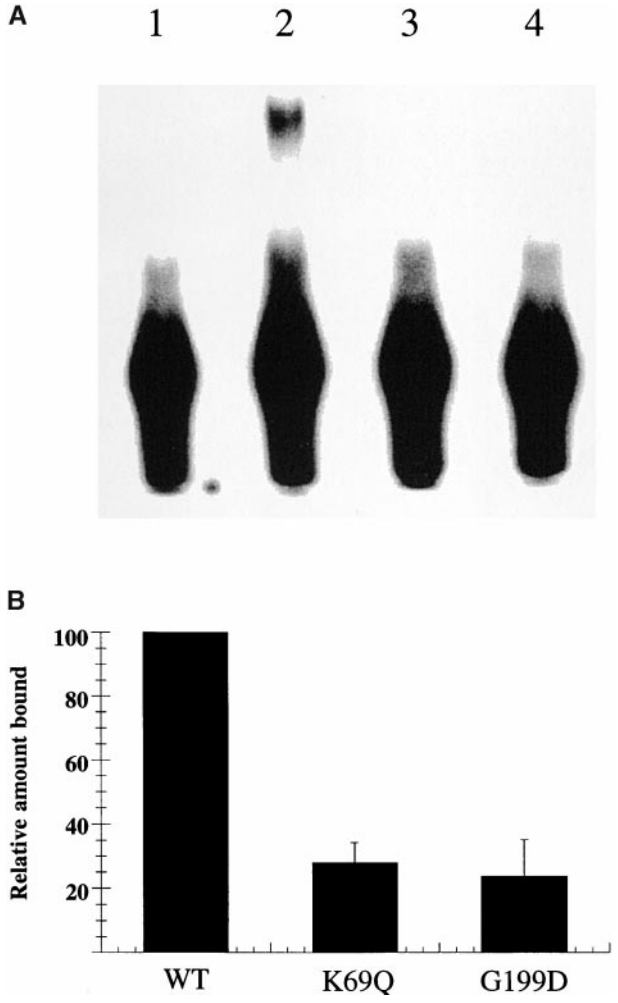

FIGURE 6.—Mutant Ssa1p's are defective for polypeptide binding. (A) A total of 2 μg of wild-type Ssa1p (lane 2), $(His)_6$-Ssa1-K69Qp (lane 3), or $(His)_6$-Ssa1-G199Dp (lane 4) were incubated with ^{125}I-CMLA and binding was assessed by native 5–15% gradient PAGE and phosphorimage analysis. Lane 1 lacks Ssa1p. (B) The means of four independent binding assays, ±SD, are shown with the amount of binding exhibited by wild-type Ssa1p set to 100.

may form. In support of this second hypothesis, complementing mutant alleles of *SSA1* were previously uncovered, leading NICOLET and CRAIG (1991) to suggest that Ssa1p functions as a dimer. In addition, we found that the wild-type and two mutant Ssa1p's purified in this study resolve as monomers and dimers by native PAGE and exhibit identical elution profiles by gel filtration, indicating that they exist in similar oligomeric states (data not shown). Regardless of which model is correct, the results of this study coalesce previous genetic (BECKER *et al.* 1996) and biochemical (CAPLAN *et al.* 1992; CYR *et al.* 1992; ZIEGELHOFFER *et al.* 1995; LU and CYR 1998) evidence that Ssa1p and Ydj1p interact and that polypeptide binding by Ssa1p is essential and suggest further that Ssa1p and Ydj1p act in concert, rather than in parallel pathways, to support post-translational protein translocation.

This report represents the first genetic and biochemical description of the importance of the ATP-binding pocket of Ssa1p in protein translocation. Other reports examining the effect of alterations in the ATPase domain of Ssa1p are that of NICOLET and CRAIG (1991) and studies from the Chirico laboratory (LIU *et al.* 1996; CHIRICO *et al.* 1998; HERMAWAN and CHIRICO 1999). NICOLET and CRAIG (1991) created several point mutations in the extreme N terminus (D8N, T11A, C15R, C15S, and C15G) and found that their expression inhibited the growth and could not rescue the temperature sensitivity of *ssa1 ssa2* and *ssa1 ssa2 ssa4* strains. Interestingly, two combinations of mutant alleles (D8N and C15G or D8N and C15S) could rescue the temperature sensitivity of these strains. As noted above, this result prompted the suggestion that Ssa1p may function as a dimer. In addition, strains expressing the mutant proteins accumulated ppαf upon depletion of wild-type Ssa1p, and the analysis of cell extracts from these strains suggested that the mutant proteins were competent for both ATP- and peptide-conjugated agarose binding (NICOLET and CRAIG 1991). In accordance with our results, expression of these mutant Ssa1p's inhibited cell growth (NICOLET and CRAIG 1991).

Chirico and co-workers undertook a biochemical analysis and examined the effects of *N*-ethylmaleimide (NEM) modification of three cysteine residues (C15, C264, and C303) in Ssa1p. Covalent modification of Ssa1p compromised ATP-agarose binding, ATP hydrolysis, and protein translocation *in vitro* (LIU *et al.* 1996). Conformational analyses demonstrated that NEM modification promotes the formation of higher order oligomers. In the presence of ATP, unmodified Ssa1p was largely monomeric (~93%) while only ~47% of NEM-Ssa1p was monomeric (CHIRICO *et al.* 1998). Additional studies showed that NEM-Ssa1p dominantly inhibits protein folding (HERMAWAN and CHIRICO 1999), consistent with the proposal above that toxic wild-type:mutant Ssa1p dimers may arise.

It is intriguing that identical point mutations in BiP, with which Ssa1p is 63% identical, result in dominant lethality (MCCLELLAN *et al.* 1998). BiP is required for both co- and post-translational protein translocation (BRODSKY *et al.* 1995). The expression of mutant BiP may decrease the translocation of essential secretory proteins, as well as wild-type BiP, into the lumen of the ER, possibly blocking the translocation pore (SANDERS *et al.* 1992) and arresting cell growth. In contrast, Ssa1p may function only in the post-translational translocation pathway (CAPLAN *et al.* 1992; BECKER *et al.* 1996) and dominant lethal effects may be overcome by an increased dependence on the co-translational pathway. There are also notable differences in the activities of the mutant BiP and Ssa1 proteins. For example, the purified BiP mutants bind CMLA (our unpublished ob-

servations) whereas the Ssa1p mutants do not (Figure 6). As such, the BiP mutants may exhibit dominant effects as a result of illegitimate substrate binding *in vivo*.

We were surprised by our inability to recapitulate *in vitro* the dominant effect of the mutant Ssa1p's on protein translocation that was observed *in vivo*. However, it is possible that the translocation substrate, ppαf, is prebound to wheat germ chaperones that block the dominant effects of the mutant Ssa1p's, since the mutant Ssa1p's are unable to bind an unfolded polypeptide *in vitro* (Figure 6). Thus, only wild-type Ssa1p can free ppαf from the wheat germ chaperones and target the preprotein to the translocation complex. Alternatively, if the dominant effect *in vivo* arises from the formation of nonfunctional wild-type:mutant Ssa1p dimers, it is possible that these dimers form only *in vivo* and not in the context of the *in vitro* translocation reaction.

Although the mutant Ssa1 proteins were not dominant in this *in vitro* assay and were genetically null, they produce measurable phenotypes. First, their expression slows cell growth by greater than approximately twofold, although even higher levels of wild-type Ssa1p do not (Figure 1B and Table 2). Second, the expression of Ssa1-G199Dp and, to a lesser extent, Ssa1-K69Qp, in the presence of wild-type Ssa1p, attenuates ppαf translocation *in vivo* (Figure 3A). Third, to varying degrees, the mutant proteins interact with Ydj1p *in vivo* (Figure 4A), in contrast to the Ssa1-134 protein characterized previously (Oka *et al.* 1998). As noted above, there are at least two scenarios in which the dominance exhibited by these mutants may arise.

In conclusion, our discovery of Ssa1p mutants that confer a phenotype on a particular cellular process while not acting as dominant lethal mutants provides a valuable tool to further assay the dependence of other processes on hsp70 function. For example, it has been shown that Ssa1p plays a role in the folding of multisubunit enzymes (Crombie *et al.* 1994; Kim *et al.* 1998), that Ssa1p overexpression can rescue some nuclear transport defects (Shulga *et al.* 1996), and that Ssa1p stimulates the uptake of a cytosolic protein by the vacuole (Horst *et al.* 1999). Although a role for Ssa1p in peroxisomal transport has not been established, it is intriguing that a J domain-containing protein, whose cognate hsp70 remains unreported, has been implicated in peroxisomal import (Djp1p; Hettema *et al.* 1998). The importance of Ssa1p in the folding of other proteins or in cellular transport processes, such as peroxisomal import, can now be examined by the expression of these nonlethal but potentially dominant *SSA1* alleles.

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