The Yeast Hsp110 Sse1 Functionally Interacts with the Hsp70 Chaperones Ssa and Ssb*

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There is growing evidence that members of the extended Hsp70 family of molecular chaperones, including the Hsp110 and Grp170 subgroups, collaborate in vivo to carry out essential cellular processes. However, relatively little is known regarding the interactions and cellular functions of Sse1, the yeast Hsp110 homolog. Through co-immunoprecipitation analysis, we found that Sse1 forms heterodimeric complexes with the abundant cytosolic Hsp70s Ssa and Ssb in vivo. Furthermore, these complexes can be efficiently reconstituted in vitro using purified proteins. Binding of Ssa or Ssb to Sse1 was mutually exclusive. The ATPase domain of Ssa1 was found to be critical for interaction as inactivating point mutations severely reduced interaction with Ssa and Ssb. Sse1 stimulated Ssa1 ATPase activity synergistically with the co-chaperone Ydj1, and stimulation required complex formation. Ssa1 is required for post-translational translocation of the yeast mating pheromone α-factor into the endoplasmic reticulum. Like ssa mutants, we demonstrate that sse1Δ cells accumulate prepro-α-factor, but not the co-translationally imported protein Kar2, indicating that interaction between Sse1 and Ssa is functionally significant in vivo. These data suggest that the Hsp110 chaperone operates in concert with Hsp70 in yeast and that this collaboration is required for cellular Hsp70 functions.

Cells respond to protein-denaturing stresses such as heat by rapidly inducing expression of a wide array of heat shock genes. Chief among these are the molecular chaperones, highly conserved proteins that associate with and protect unfolded proteins, preventing their aggregation and supporting refolding (1). Perhaps the most abundant and well characterized chaperones are the heat shock protein 70 (Hsp70) family, found in all cell types from bacteria to eukaryotes (2, 3). Hsp70s assist in protein refolding by binding of exposed hydrophobic surfaces of a substrate to a C-terminal peptide binding domain. Cycles of substrate binding and release are brought about by transition between low and high affinity binding states regulated by nucleotide occupancy in the N-terminal ATPase domain (4).

In eukaryotic cells Hsp70 class chaperones can be divided into three subfamilies: 1) DnaK-like, 2) Hsp110, and 3) Grp170 (5). The budding yeast, Saccharomyces cerevisiae, possesses 14 Hsp70 homologs with family members present in the cytoplasm, endoplasmic reticulum (ER), and mitochondria (6). The most well studied Hsp70s in yeast are the cytoplasmic Ssa proteins (stress seventy A), encoded by the differentially expressed SSA1–4 genes. Ssa1–4 (collectively referred to as “Ssa”) perform largely redundant functions and the presence of at least one SSA gene is required for viability (7). Ssa chaperones are involved in cellular processes such as translation, translocation of proteins across cellular membranes, and general protein folding (8). Cells depleted of Ssa exhibit multiple cellular defects, including: (i) growth arrest in G1/M phase, (ii) accumulation of precursor proteins destined for the ER and mitochondria, (iii) abnormal nuclear distribution, and (iv) aberrant microtubule formation (9, 10). Ssa ATPase activity, and therefore protein folding capacity, is in turn governed by modulatory proteins such as the Dna1 homolog, Ydj1, which stimulates ATPase activity up to 10-fold, and Fes1, recently identified as a nucleotide exchange factor (11, 12). In addition, Sti1 and Cns1, two tetratricopeptide repeat proteins associated with the Hsp90 chaperone complex, were recently demonstrated to strongly activate Ssa1 (13, 14). Another pair of Hsp70 proteins in yeast, functionally distinct from the Ssa chaperones, are encoded by SSB1 and SSB2. Ssb1/2 are associated with both the ribosome and nascent chains emerging from the ribosomal exit channel (15). Strains lacking SSB1/2 are cold-sensitive and exhibit enhanced sensitivity to translational inhibitors, indicative of a role in translation (16). Thus far, Ssb has not been shown to bind peptide substrates in vitro, nor is it stimulated by classic Hsp70 substrates (17). However, the ribosome-associated complex (RAC), a heterodimer composed of the Hsp70 Ssz1 and the J-protein Zuo1, potently and specifically activates SsATPase activity (18–20).

Unlike the ubiquitous DnaK Hsp70 subfamily, the Hsp110 molecular chaperones are found only in eukaryotic cells. The defining characteristics of the Hsp110 family are an extended linker region (~98 residues versus ~9 in Hsp70) separating the β-sandwich peptide binding fold from the α-helical lid domain, and an extended C-terminal tail region whose structure and function is unknown (5). Importantly, Hsp110 cannot actively refold proteins but is able to hold thermally denatured model substrates such as luciferase in a protected state such that they can be more efficiently renatured by folding-competent chaperones such as Hsp70 (21). Because of this property, Hsp110 is classified as a “holdase,” similar to Hsp90 and many of its co-chaperones, rather than a “foldase.” In contrast to the Hsp70s, very little is known about Hsp110 in vivo functions. Overexpression of mammalian Hsp110 in Rat-1 and HeLa cells confers increased thermoresistance (22). Recently, overexpression of Hsp110 was found to suppress cell toxicity caused by induction of the polyglutamine tract-containing truncated androgen receptor (tAR) in COS-7 cells (23). These findings are consistent with the general chaperone activity demonstrated in vitro, but to date, no endogenous cellular targets have been identified.

The Hsp110 family is represented in S. cerevisiae by the Sse1 and Sse2 proteins. Loss of SSE1 renders cells slow growing and temperature-resistant (24).
sensitive, whereas loss of SSE2 causes no observable phenotypes (24). However, in some strain backgrounds SSE1 and SSE2 may constitute an essential gene pair, as inactivation of both genes is lethal (25). Like mammalian Hsp110, Sse1 acts as a holdase in vitro, binding to denatured substrates, preventing aggregation and enhancing renaturation by folding-competent chaperones (26). Sse1 was isolated as a calmodulin-binding protein and is required for modulation of protein kinase A signaling (24, 25, 27). Sse1 also functions as an Hsp90 co-chaperone and is required for full function of several client proteins (28). However, the molecular role Sse1 plays in the Hsp90 chaperone cycle has not been elucidated. Sse1 was recently shown to function in vivo by a mechanism likely distinct from that of Hsp70; multiple ATPase domain mutations previously demonstrated to inactivate both Kar2 and Ssa1 had no phenotype effect on Sse1 (29). Moreover, the ATPase and peptide binding domains of Sse1 exhibit trans-complementation in vivo and functionally interact, suggesting that the chaperone may exist in higher order complexes (29). Hsp110 in mammalian cells has been reported to form large complexes (400–700 kDa) with Hsp70 and Hsp25 chaperones (30, 31). In addition, the Hsp105α isoform was recently demonstrated to inhibit ATPase activity of Hsc70 (32). However, the functional significance of this interaction in vivo is unknown. Similarly, a recent report demonstrated that Kar2 and the Grp170 subfamily member, Lhs1 interact and reciprocally regulate both ATPase activities. This biochemical effect has functional significance, as mutation of Lhs1 severely impairs the ability of Kar2 to support post-translational translocation of prepro-α-factor (ppαF) (33).

In this report, we demonstrate that Sse1 exists in heterodimeric complexes with the Ssa and Ssb chaperones. Using purified proteins, we show that the dimeric complexes are able to form in vitro in the absence of additional yeast components. The ATPase domain of Sse1 appears to be important for this interaction, as several inactivating point mutants in this domain block complex formation. Sse1 stimulates Ssa1 ATPase activity in steady-state assays synergistically with Ydj1, and this stimulation requires stable complex formation. Similar to cells depleted of Ssa, sse1Δ cells accumulate ppαF, indicating a potential role for Sse1 with Ssa in mediating protein translocation. Together, these results demonstrate a functional interaction between cytosolic Hsp110 and Hsp70 protein chaperones in yeast.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—Yeast strains used in this study were W303 (MATa ura3-52 trp1 leu2-3,112 his3-11,15 ade2-1 can1-100), sse1Δ (W303 sse1Δ::kanMX), ssa1/2Δ (MATa ssa1::HIS3 ssa2::LEU2, lys1, lys2, leu2-3,112, ura3-52, his3-11,15, trpl-Δ1), JN516 (MATa his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 SSA1 ssa2::LEU2 ssa3::TRP1 ssa4::LVS2) and JB67 (MATa his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 ssa1::1-45 ssa2::LEU2 ssa3::TRP1 ssa4::LVS2). Strains RSY1293 (MATa can 1-100 leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 sec61::HIS3 [pDQ1]) and RSY1295 (RSY1293 except (psec61-41)) were obtained from R. Schekman (UC Berkeley) (34). Synthetic complete (SC) medium lacking the appropriate nutrient for plasmid selection was purchased from BIO101 (Carlsbad, CA). Standard yeast propagation and transformation procedures were employed. Isogenic MATa variants of W303 and sse1Δ strains were generated by mating type switching with the pGALHO plasmid (35). FLAG-Sse1 and mutant derivatives were overexpressed in BL21 Escherichia coli (B F-, ompT, hsdS (Tr− Mγ−), gal, dec) (29). Wild-type and mutant FLAG-Sse1 proteins were expressed in yeast from the low copy plasmid p416TEF (36).

**Immunoblot Analysis**—SDS-PAGE and immunoblot analysis was performed as described (29). Anti-Sse1 polyclonal antibody was used at 1:2000 dilution (obtained from J. Brodsky, University of Pittsburgh). Anti-Ssa1/2 polyclonal antibody was used at 1:5000 dilution. Anti-Ssb1/2 polyclonal antibody was used at 1:5000 dilution (both obtained from E. Craig, University of Wisconsin). M2 monoclonal antibody (recognizes FLAG epitope, Sigma) was used at 1:1000 dilution. Anti-Kar2 polyclonal antibody was used at 1:1000 dilution (obtained from J. Gaut, University of Michigan). Anti-ppαF antibody was used at 1:2000 dilution (obtained from R. Schekman, UC Berkeley). Anti-phosphoglycerate kinase (PGK) monoclonal antibody (Molecular Probes, Inc., Eugene, OR) was used at 1:1000 dilution.

**Immunoprecipitation Analysis and Native-PAGE**—For immunoprecipitation of FLAG-Sse1 complexes from yeast, soluble protein extracts were prepared from 20 ml of mid-log phase (A600 = 0.5) cultures of the appropriate strains expressing the wild-type and mutant FLAG-Sse1 fusions using the glass bead lysis method exactly as described (29). 50 μl of a 1:1 slurry of M2 resin (Sigma) in TEGN (20 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 10% glycerol, 50 mM NaCl) were added to protein extracts (~1 mg of total protein). The volume was adjusted to 800 μl with TEGN followed by incubation with mixing at 4 °C for 2 h. Resin was then collected by centrifugation and washed four times with TEGN. For elution of bound proteins, 50 μl of 200 μg/ml FLAG peptide in TEGN was added to the resin pellet and incubated at room temperature for 15 min. For native PAGE, 6 × native sample buffer (0.35 M Tris-HCl, pH 6.8, 36% glycerol) was added to eluted protein samples. For SDS-PAGE, 2 × SDS-PAGE sample buffer was added to eluted protein samples and boiled for 3 min. Native-PAGE was carried out using a previously described imidazole/HEPES (I/H) buffer system (34 mM imidazole, 35 mM HEPES) (37). Briefly, 6% polyacrylamide gels made with 1:1 buffer were pre-run at 4 °C for 90 min followed by addition of fresh buffer and loading of samples. Samples were resolved at 90 V for 3 h. Gels were then either stained with Coomassie Blue dye or proteins were transferred to nitrocellulose for immunoblot analysis.

**Protein Purification**—Ssa1 was either purified from yeast strain JN516 using the method of Cyr et al. (12) or according to Wegele et al. (13). For purification of His6-Ssb1, plasmid p416TEF His6-SSB1 (kindly provided by E. Craig) was transformed into yeast strain W303 for expression. Protein was purified as described by Pfund et al. (15) with a 5-ml Talon resin column (BD Biosciences, Palo Alto, CA) used for metal affinity chromatography in place of His-bind resin. Aliquots of purified protein at a concentration of 0.5 mg/ml were snap-frozen in liquid nitrogen and stored at −80 °C. FLAG-Sse1 and the K69Q and G233D mutant proteins were overexpressed in E. coli as described. E. coli extracts containing overexpressed FLAG-Sse1 proteins were passed over 2 ml of M2 resin (Sigma) columns using gravity flow and washed with 15 ml of TEGN followed by elution with 10 ml of TEGN + 100 μg/ml FLAG peptide. Eluted proteins were buffer-exchanged using Amicon Ultra Centrifugal Filter Devices (Millipore) into Buffer 88 (20 mM HEPES pH 6.8, 150 mM KOAc, 5 mM MgOAc, and 250 mM sorbitol) and applied to a 2.5-ml Q-Sepharose column and washed and eluted as described (38). Eluted proteins were again buffer exchanged with Amicon Ultra Centrifugal Filter Devices (Millipore) into Buffer 88 (20 mM HEPES pH 7.4, 200 mM KCl, 5 mM MgCl₂, 5 mM β-mercaptoethanol, 2.5% glycerol) and applied to a 160-ml Sephacryl S-300 column. FLAG-Sse1-containing fractions were buffer-exchanged, concentrated to 2 mg/ml using Amicon filters into Sse1 storage buffer (50 mM Tris-Cl, pH 7.4, 50 mM NaCl, 0.8 mM DTT, 2 mM MgCl₂, and 5% glycerol), aliquoted, and snap-frozen in liquid nitrogen. The YD1 open reading frame was cloned into pFLAG and overexpressed in E. coli as described above with FLAG-Sse1. FLAG-
Ydj1 protein extracts were applied to a 2-ml M2 resin column, washed, and eluted as above. Eluted proteins were buffer exchanged with Amicon filters into Buffer A (20 mM MOPS, pH 7.5, 0.5 mM EDTA, 10 mM DTT) and applied to a 10-ml DE52 column (Whatman). Column was washed with 25 ml of Buffer A followed by elution with a 30-ml 0–300 mM NaCl gradient in Buffer A. FLAG-Ydj1 containing fractions were concentrated and buffer exchanged into Standard Buffer II and applied to a 160 mM Sephacryl S-300 column. Ydj1 containing fractions were concentrated and washed with 2 mg/ml into Buffer C (10 mM ATP, and 1 mM NaCl gradient in Buffer A. FLAG-Ydj1 containing fractions were concentrated and buffer exchanged into Standard Buffer II and applied to a 160 mM Sephacryl S-300 column. Ydj1 containing fractions were concentrated and washed with 2 mg/ml into Buffer C (10 mM ATP, and 1 mM NaCl, 10 mM DTT, 10% glycerol), aliquoted, and snap-frozen in liquid nitrogen.

\[\text{ATPase Assay—} \text{All reactions were performed in a volume of 20} \mu\text{l for 45 min at 30} \degree \text{C in ATPase assay buffer (50 mM HEPES-KOH, 150 mM KCl, 2 mM MgCl}_2, 10 mM DTT). For the Sse1 titration experiment, 3} \mu\text{g of Ssa1 was preincubated with the indicated molar ratio of FLAG-Sse1 on ice for 60 min, followed by addition of 6} \mu\text{g of FLAG-Ydj1, 50} \mu\text{M ATP, and 1} \mu\text{Ci of [\alpha-32P]ATP. For the steady-state ATPase assay, reactions contained 3} \mu\text{g of Ssa, 6} \mu\text{g of FLAG-Sse1 (or mutant versions), 6} \mu\text{g of FLAG-Ydj1 in the combinations indicated. Reactions were stopped with 20} \mu\text{l of Stop Buffer (2} \text{M formic acid, 1} \text{M LiCl) on ice. 1-}\mu\text{l aliquots were then spotted in duplicate on PEI-cellulose TLC plates and resolved in 1 M formic acid and 0.5 M LiCl. TLC plates were developed by phosphorimage analysis using a Storm 840 Imager and ImageQuant software (Amersham Biosciences). Spontaneous ATP hydrolysis and background hydrolysis from the Ydj1 preparation were subtracted. Percent conversion of ATP to ADP was then calculated. For Fig. 5A, values were normalized to the reaction containing Ssa1 and Ydj1 alone. In Fig. 5B, values were normalized to the reaction containing Ssa1 alone. Basal (unstimulated) activity of Ssa1 was near the limit of detection in our assay system with the protein amounts utilized. In pilot experiments, increasing amounts of Ssa1 produced greater ATPase activity, whereas increasing amounts of wild-type or mutant Sse1 proteins did not. Reconstitution of Sse1-Hsp70 Complexes in Vitro—For reconstitution studies, 200} \mu\text{g of E. coli protein extract containing overexpressed FLAG-Sse1 was combined with 6} \mu\text{g of purified Ssa1 or} \text{His}_6\text{-Ssb1, 50} \mu\text{M of a 1:1 slurry of M2 resin and adjusted to 800} \mu\text{l with TEGN followed by incubation at 4} \degree \text{C for 2 h. Resin was washed four times and eluted as described above. The eluate was divided into two equal portions and prepared for native- and SDS-PAGE as described above. To determine if Ssa and Ssb compete for binding to Sse1, 100} \mu\text{g of FLAG-Sse1 and 200} \mu\text{g of Ssa1 were incubated together on ice for 2 h and precipitated with M2 resin. The resin was washed and split evenly into five tubes: 0, 10, 25, 100, or 200} \mu\text{g of His}_6\text{-Ssb1 were added and incubated for 2 h on ice.}

Resin-bound complexes were washed and eluted with 100} \mu\text{g/ml FLAG peptide. The eluate was analyzed by SDS-PAGE and Coomassie Blue staining.}

\[\text{ppof Accumulation Assay—} \text{Yeast strains JN516, JB67, and MAT\alpha mating type W303 and see1Δ were grown at 24} \degree \text{C in 50 ml of YPD to mid-log phase} (A_{600} = 0.5). 25 ml were harvested and stored at −80 \degree \text{C} (\tau_0). The remaining culture was then shifted to 39 \degree \text{C for 30 min and harvested. Soluble protein was prepared by glass bead lysis as described previously (29). Proteins were resolved by 12% SDS-PAGE, transferred to nitrocellulose and subjected to immunoblot analysis. In a separate experiment to investigate if co-translational import of Kar2 was affected in an see1Δ mutant, JN516 and JB67 were grown at 30 \degree \text{C to mid-log phase, half the culture shifted to 37 \degree \text{C for 2 h, and extracts prepared from both cultures, while extracts were prepared from W303 and see1Δ strains grown at 30} \degree \text{C. As a control, a cold-sensitive see61-41 mutant and its isogenic parent strain were shifted to 17 \degree \text{C for 2 h to block all ER translocation. Extracts from all cultures were resolved on the same 8\% SDS-PAGE gel and immunoblotted with anti-Kar2 antibody.}

\[\text{RESULTS}

\text{Ssa1/2 and Ssb1/2 Co-immunoprecipitate with Sse1—} \text{As part of ongoing studies to elucidate the roles of Sse1 in yeast, we performed immunoprecipitation experiments to identify interacting proteins. For these experiments, a strain expressing a functional FLAG-tagged SSE1 allele was constructed. We have previously demonstrated that this modified protein is abundantly expressed, allowing facile immunoprecipitation and visualization by gel staining with Coomassie Blue dye (29). FLAG-Sse1 was affinity-purified using M2-agarose resin from soluble whole cell extracts, and proteins resolved by 10\% SDS-PAGE. As shown in Fig. 1A, three bands were detected that were not present in the negative control lane lacking FLAG-Sse1. The band with the slowest mobilization of Sse1, the most likely candidates were the Ssa and Ssb Hsp70 in mammalian cells (30, 31). Therefore, we speculated that the possible roles of Sse1 in yeast, we performed immunoprecipitation experiments to identify interacting proteins. For these experiments, a strain expressing a functional FLAG-tagged SSE1 allele was constructed. We have previously demonstrated that this modified protein is abundantly expressed, allowing facile immunoprecipitation and visualization by gel staining with Coomassie Blue dye (29). FLAG-Sse1 was affinity-purified using M2-agarose resin from soluble whole cell extracts, and proteins resolved by 10% SDS-PAGE. As shown in Fig. 1A, three bands were detected that were not present in the negative control lane lacking FLAG-Sse1. The band with the slowest mobilization of Sse1, the most likely candidates were the Ssa and Ssb Hsp70 in mammalian cells (30, 31). Therefore, we speculated that the possible roles of Sse1 in yeast, we performed immunoprecipitation experiments to identify interacting proteins. For these experiments, a strain expressing a functional FLAG-tagged SSE1 allele was constructed. We have previously demonstrated that this modified protein is abundantly expressed, allowing facile immunoprecipitation and visualization by gel staining with Coomassie Blue dye (29). FLAG-Sse1 was affinity-purified using M2-agarose resin from soluble whole cell extracts, and proteins resolved by 10% SDS-PAGE. As shown in Fig. 1A, three bands were detected that were not present in the negative control lane lacking FLAG-Sse1. The band with the slowest mobilization of Sse1, the most likely candidates were the Ssa and Ssb Hsp70 chaperones. To test this possibility, immunoblot analysis of the precip...}
Figure 2. Sse1 independently forms heterodimeric complexes with Ssa1/2 and Ssb1/2. A, FLAG-Sse1 expressed from the plasmid p416TEF in the indicated strains was immunoprecipitated under native conditions, the immunocomplexes resolved by SDS-PAGE and Coomassie Blue-stained to visualize proteins. B, samples from A were resolved using native gel electrophoresis, followed by Coomassie Blue staining to visualize protein complexes. A set of protein chromatographic standards (Sigma) was used to estimate relative molecular mass. C, samples from A were resolved on replicate native gels, transferred to nitrocellulose membrane, and probed with the indicated antisera.

Sse1 Complexes with Hsp70 Chaperones in Yeast

Sse1 forms separate, likely heterodimeric complexes with Ssa1/2 and Ssb1/2. A single band was visible from ssb1/2Δ extracts, co-migrating with the faster migrating complex and demonstrating that the slower migrating species corresponds to the Sse1-Ssb1/2 complex (Fig. 2B). Surprisingly, a different pattern of complexes was obtained from the ssa1Δ-45 strain: a slower migrating band replaced the Sse1-Ssa1/2 band, and the band corresponding to Sse1-Ssb1/2 decreased in intensity. The reduced mobility of Sse1-Ssa1-45 is not likely to be caused by an additional protein present in the complex, because no additional proteins were seen when analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 2A, lane 3). Instead, the altered migration may be caused by a different conformation assumed by the Sse1-Ssa1-45 complex. This altered conformation may also result in differential affinity for Sse1 in vivo, perhaps ultimately limiting the amount of Sse1-Ssb complex.

Immunoblot analysis after native-PAGE further confirmed the identities of the Sse1-Hsp70 complexes (Fig. 2C). As expected, anti-Sse1 antibody produced a positive signal corresponding to all complexes visible on the Coomassie Blue-stained native-PAGE. Anti-Ssa1/2 antibody recognized only the faster migrating band from both ssa1Δ and ssb1/2Δ FLAG-Sse1 immunoprecipitates. In addition, the antiserum recognized the up-shifted band specific to ssa1Δ-45 (Fig. 2C, lanes 3 and 6). A fainter signal with the same migration was seen in the ssb1/2Δ strain (Fig. 2C, lane 5), further evidence that the altered migration of the Sse1-Ssa1-45 complex is caused by an alternative conformation that can be assumed in Sse1-Ssa1 complexes but is exaggerated with the ssa1Δ-45 mutation. Anti-Ssb1/2 antiserum specifically recognized the slower migrating complex from ssb1/2Δ extracts (Fig. 2C, lanes 7 and 9, respectively). No signal was detected using this antiserum on immunoprecipitates from ssb1/2Δ extracts.

Inactivating Sse1 ATPase Point Mutants Reduce Interaction with Ssa and Ssb—We previously characterized several point mutants in the ATPase domain of Sse1 for the ability to rescue the phenotypes associated with sse1Δ cells (29). sse1-K69Q was able to complement sse1Δ phenotypes whereas sse1-G233D was not. The sse1-G205D allele was determined to be temperature sensitive in vivo, capable of complementation at 30 °C but not 37 °C. Both glycine mutants, but not the K69Q mutant, are defective in nucleotide binding as demonstrated by inability to bind ATP-agarose in vitro. To ask whether Sse1 function correlated with the ability to form the Sse1-Hsp70 complexes, we tested FLAG-tagged versions of these mutants for their ability to interact with Ssa and Ssb by co-immunoprecipitation analysis (Fig. 3). Immunoprecipitates of wild-type and mutant Sse1 proteins were resolved by SDS-PAGE and Coomassie Blue-stained or subjected to immunoblot analysis (Fig. 3, A and B, respectively), or resolved using native-PAGE (Fig. 3C). Sse1-K69Q interacted with Ssa as well as wild-type Sse1. However, the Coomassie Blue-stained gel indicates that the ability of Sse1-K69Q to precipitate Ssb was reduced. Both the Sse1-G205D and Sse1-G233D proteins were substantially reduced in their abilities to interact with either Hsp70, but as shown by immunoblot in Fig. 3B, complex formation with these mutant proteins was not completely abrogated.

The wild-type and mutant Sse1 precipitates were also subjected to native PAGE analysis (Fig. 3C). As expected, wild-type Sse1 showed two bands corresponding to Sse1-Ssa and Sse1-Ssb complexes. Only the band corresponding to the Sse1-K69Q-Ssa complex was observed, in agreement with the reduced amount of Ssb interacting with this mutant as seen on the SDS-PAGE Coomassie Blue-stained gel. The mobility of the single band detected with Sse1-G205D and Sse1-G233D on native
PAGE was reduced slightly compared with the Sse1-Ssa complex from wild-type cells. As these mutants do not precipitate Ssa or Ssb efficiently, this band could correspond to aberrantly migrating mutant Sse1 monomers. Alternatively, this new species may be caused by homodimerization of the Sse1 mutants that may only occur when Sse1 cannot interact with Ssa or Ssb. Taken together, these data indicate that a functional Sse1 ATPase domain is required for interaction with Ssa and Ssb.

Sse1-Ssb1 and Sse1-Ssa1 Complexes Can Be Reconstituted in Vitro—Although we did not observe additional proteins in the Sse1-Hsp70 complexes, it was formally possible that other factors are required for complex assembly at substoichiometric levels. To determine if Sse1-Hsp70 complexes could be formed in the absence of other yeast proteins, we purified small amounts of both Ssa1 and Ssb1 from yeast for in vitro reconstitution studies using established protocols, as shown in Fig. 4A. Purified Ssa1 or His6-Ssb1 was mixed with E. coli protein extract containing overexpressed FLAG-Sse1, followed by FLAG immunoprecipitation. The immunoprecipitates were then analyzed for the ability to form complexes by SDS- and native-PAGE (Fig. 4B and C). Both Ssa1 and His6-Ssb1 were efficiently co-precipitated with Sse1 only when FLAG-Sse1 was present, indicating that complex formation can occur in the absence of any other yeast components. Similar results were obtained when FLAG-Sse1 was highly enriched by FLAG affinity chromatography prior to complex formation, suggesting that no E. coli proteins are assisting in complex formation (data not shown). In addition, no exogenously added ATP or ADP was required for complex formation in vitro, although the nucleotide status of the purified proteins was unknown (data not shown).

Native-PAGE further confirmed the in vitro complex formation demonstrated in Fig. 4B. FLAG-Sse1 immunoprecipitates from wild-type yeast cells were included to compare the in vitro assembled complexes with endogenous complexes (lane 1). FLAG-Sse1 alone (lane 2) migrated at an apparent molecular mass in between that of the two complexes. Again, it is unclear whether this species represents an Sse1 monomer or homodimer. Purified Ssa1 migrated faster than the Sse1-Ssa1/2 complex from yeast (lane 4). The in vitro assembled Sse1-Ssa1 complex approximately co-migrated with the yeast complex, distinct from either purified protein alone (lane 3). Similarly, purified Ssb1 migrated slower than Sse1 alone but when complexed with Sse1 resulted in another distinct species similar to the Sse1-Ssb1 yeast complex (lanes 6 and 5, respectively).

Sse1 appeared to bind Ssa1 and Ssb1 with equivalent efficacy. To ask whether the Hsp70s share a common binding site on Sse1, we performed a competition experiment. Sse1-Ssa1 complexes were preformed by binding and isolation using anti-FLAG agarose resin and then challenged with increasing amounts of purified His6-Ssb1 (see “Experimental Procedures”). Total Ssa1 or Sse1-FLAG associated proteins were then re-isolated after multiple washes of the resin, eluted with FLAG peptide and resolved on 8% SDS-PAGE. The amount of Ssa1 complexed with Sse1 diminished when increasing amounts of purified His6-Ssb1 were added (see “Experimental Procedures”).
Cns1 have all been shown to regulate Ssa ATPase activity in vitro. Given the strong and stable interaction between Ssa and Sse1, we hypothesized that Sse1 may likewise influence nucleotide hydrolysis as a means to regulate chaperone activity. To assess effects of Sse1 on the Ssa1 ATPase activity, we performed steady state ATPase assays using purified proteins. Addition of Sse1 to Ssa1 and Ydj1 at increasing molar ratios resulted in increased total ATP hydrolysis relative to Ssa1 and Ydj1 alone (Fig. 5A). Stimulation reached a maximum of ~3-fold at a 1:1 ratio of Ssa1 to Ssa1, consistent with our predicted heterodimer stoichiometry. To further investigate this stimulatory effect, we asked whether complex formation was required using the previously described ATPase domain mutant proteins (Fig. 5B). Relative to activity observed with Ssa1 alone, Sse1 and Ydj1 stimulated Ssa1 ~9-fold. These data indicate that the binding sites for Sse1 and Ydj1 are likely different and that both co-chaperones can synergize to hyperactivate Ssa1. To verify that the increase in ATPase activity was attributed to Ssa1 and not activation of a cryptic ATPase activity in Sse1, we utilized the K69Q mutant of SSE1. This mutation has been previously demonstrated to abolish hydrolysis of both Ssa1 and Kar2 Hsp70s, but has no effect on SSE1 complementation or the ability to form Sse1-Ssa heterodimers. Consistent with genetic and biochemical results, addition of Sse1-K69Q and Ydj1 to Ssa1 resulted in ATPase levels indistinguishable from those obtained with wild-type Sse1. Purified Sse1-G233D, which is incapable of binding Ssa1 in vitro, failed to further stimulate Ydj1-activated Ssa1 ATPase activity. Sse1 must therefore be able to form stable heterodimers with Ssa1 in order to enhance the latter ATPase activity.

**ppaF Accumulates in sse1Δ Cells**—One established role for Ssa in yeast is to assist in post-translational protein translocation into the ER. The mating pheromone precursor, ppaF, requires Ssa for post-translational translocation into the ER and subsequent processing and secretion (39). Strains expressing the ssa1-45 allele as the sole source of Ssa chaperone have previously been shown to accumulate ppaF at the non-permissive temperature (9). If Sse1 collaboration is required for Ssa1 to function in translocation, then sse1Δ cells would be expected to at least partially phenocopy ssa1 mutant phenotypes. To test this hypothesis, we used an isogenic MATα wild-type and sse1Δ pair of strains and an isogenic set of wild-type and ssa1-45 strains to test for ppaF accumulation. All strains were incubated at 24 °C until cultures reached mid-log phase (A500 = 0.5) at which time half of the culture was harvested whereas the other half was shifted to 39 °C for 30 min. Soluble protein was obtained and resolved by 12% SDS-PAGE followed by immunoblot analysis using antisera specific for ppaF or PGK (load control). The results of this experiment are shown in Fig. 6A. As expected we found that neither wild-type strain accumulated ppaF at either temperature whereas the ssa1-45 strain exhibited significant ppaF accumulation at 39 °C. Interestingly, sse1Δ cells accumulated an intermediate amount of ppaF at both temperatures. To verify that this translocation block was not a general, nonspecific feature of sse1Δ mutants, we assessed the co-translational import of the ER luminal Hsp70 Kar2. As a control, the cold-sensitive sec61Δ mutant sec61-41 was utilized to inactivate the ER translocon, resulting in the accumulation of pro-Kar2 that has not been processed to remove its signal peptide. As shown in Fig. 6B, a small amount of pro-Kar2 was evident after inactivation of Sec61. In contrast, neither inactivation of Ssa1 nor deletion of Sse1 affected Kar2 import. These results indicate that Sse1 specifically collaborates with Ssa chaperones in the process of post-translational protein translocation as part of a functional protein complex.

**DISCUSSION**

In this report, we have used co-immunoprecipitation and native gel electrophoretic approaches to show that Sse1 forms heterodimeric complexes with the Ssa and Ssb cytosolic Hsp70 chaperones in vivo and in vitro. The majority, if not all, of the cellular Sse1 pool appears to be
present in these chaperone complexes. A recent genome-wide determination of relative protein abundance derived from immunoblot analysis of TAP-tagged proteins suggests that the ratio of Ssa to Sse chaperones is ∼9:1, and Ssb to Sse ∼4:1 (40). Therefore, based on our findings that the complexes are stable and likely exist in 1:1 ratios, the total cellular pool of Sse can only partner with a fraction of Ssa or Ssb chaperones at a given time. This model is consistent with results obtained by Yam et al. (43) demonstrating that Sse1 in cytosolic extracts migrates nearly exclusively within a 150–220 kDa range with Ssa and Ssb by gel filtration (see accompanying article). Significantly, it appears that the Hsp110-Hsp70 interaction is conserved in the higher eukaryotes. Several studies have demonstrated that mammalian Hsp110 forms higher order complexes with Hsp70, although the biological significance in these systems is unknown (30–32).

We have found that Sse1 activates the slow ATPase activity of Ssa1 by a modest degree, ∼3-fold. However, Sse1 potentiated further activation by Ydj1, demonstrating that the two activators operate independently and are likely to synergize within the cell. This result is particularly intriguing given the identification of SSE1 as a multicopy suppressor of both the protein translocation and Hsp90 signaling defects of a temperature-sensitive ydj1-151 allele (41). Our identification of a partial block in prepro-αF translocation in sseΔ cells suggests that like Ydj1, Sse1 is required for optimal Ssa chaperone function in vivo. The Hsp90 co-chaperone Sti1, the yeast Hsp ortholog, was recently shown to bind Ssa1 with relatively low affinity but to enhance ATPase activity up to 200-fold (42). In contrast, our data suggest that Sse1 and Ssa1 form stable complexes with presumably lower dissociation constants, and that Sse1 only moderately activates Ssa1. Perhaps these differences reflect that fact that Sti1 likely encounters Ssa1 transiently in the context of the Hsp90 system, whereas Sse1 appears to be a stable binding partner.

Our results are in contrast with recent findings that the mammalian Sse1 homolog Hsp105α inhibits ATP hydrolysis by Hsc70, whereas its own latent ATPase activity is concomitantly activated (32). However, in another example of Hsp70-type chaperone collaboration, the Grp170 homolog Lhs1 was shown to enhance nucleotide exchange when complexed with presumably lower dissociation constants, and that Sse1 only moderately activates Ssa1. Perhaps these differences reflect that fact that Sse1 likely encounters Ssa1 transiently in the context of the Hsp90 system, whereas Sse1 appears to be a stable binding partner.

An important question that we have not yet addressed is what domains of Sse1 and Ssa/Ssb are required for interaction. As demonstrated in Fig. 3, two ATPase domain point mutants lost the ability to interact with Ssa and Ssb. We previously showed that these same mutants failed to bind ATP in vitro, and were unable to complement phenotypes associated with ssaΔ cells. Therefore, the ability of Sse1 to interact with Ssa and Ssb correlates well with function in vivo, and ability to stimulate ATPase activity in vitro. In support of this observation, we have found that an N-terminal hexahistidine-tagged variant of Ssa that incompletely complements the ssa1 Δ45 mutant fails to bind Sse1 (data not shown).3 The Hsp70 proteins also do not co-purify with the Sse1 peptide binding domain expressed alone (data not shown), suggesting a requirement for the ATPase domain for complex formation. Moreover, Sse1 cannot be affinity-purified from yeast cytosolic extracts with ATP-agarose resin, while it can be efficiently isolated in this manner when expressed recombinantly in E. coli (29). Although it is prema-

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