The function of the yeast molecular chaperone Sse1 is mechanistically distinct from the closely related Hsp70 family

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Abstract

The Sse1/Hsp110 molecular chaperones are a poorly understood subgroup of the Hsp70 chaperone family. Hsp70 can refold denatured polypeptides via a carboxyl-terminal peptide binding domain (PBD), which is regulated by nucleotide cycling in an amino-terminal ATPase domain. However, unlike Hsp70, both Sse1 and mammalian Hsp110 bind unfolded peptide substrates but cannot refold them. To test the in vivo requirement for interdomain communication, SSE1 alleles carrying amino acid substitutions in the ATPase domain were assayed for their ability to complement sse1Δ yeast. Surprisingly, all mutants predicted to abolish ATP hydrolysis (D8N, K69Q, D174N, D203N) complemented the temperature sensitivity of sse1Δ and lethality of sse1Δ sse2Δ cells, whereas mutations in predicted ATP binding residues (G205D, G233D) were non-functional. Complementation ability correlated well with ATP binding assessed in vitro.

The extreme carboxyl-terminus of the Hsp70 family is required for substrate targeting and heterocomplex formation with other chaperones, but mutant Sse1 proteins with a truncation of up to 44 carboxyl-terminal residues not included in the PBD were active. Remarkably, the two domains of Sse1 when expressed in trans functionally complement the sse1Δ growth phenotype and interact by coimmunoprecipitation analysis. In addition, a functional PBD was required to stabilize the Sse1 ATPase domain, and stabilization also occurred in trans. These data represent the first structure-function analysis of this abundant but ill-defined chaperone, and establish several novel aspects of Sse1/Hsp110 function relative to Hsp70.
Introduction

Cells respond to heat shock by induction of a specific set of genes that allow them to cope with and recover from the stress. Many of these heat shock proteins (HSPs) function as molecular chaperones, binding unfolded proteins and preventing aggregation or facilitating their refolding (1). One of the most well-studied classes of chaperones is the Hsp70 family. *Saccharomyces cerevisiae* possesses 14 Hsp70 homologs, distributed in the cytosol, mitochondria, and endoplasmic reticulum (2). Hsp70s promote folding of nascent polypeptides, facilitate translocation of proteins across membranes, and protect the cell from protein-denaturing stresses. All Hsp70s share a common domain architecture consisting of an amino-terminal ATPase domain and carboxyl-terminal peptide binding domain (PBD$^1$). The PBD is responsible for binding unfolded peptide substrates and is regulated by the nucleotide binding status of the ATPase domain (3). The PBD of the *E. coli* Hsp70 homolog DnaK is composed of a series of eight β-strands (β) that form a peptide-binding cleft, followed by a series of α-helices that form the “lid” (α subdomain) thought to regulate entry and exit of the substrate (4).

In *E. coli*, the DnaK folding cycle is regulated by DnaJ and GrpE, which stimulate the ATPase activity and act as a nucleotide exchange factor, respectively (5). DnaJ homologs are found in all cellular compartments of eukaryotes while GrpE homologs have only been found in mitochondria (6).

The eukaryotic cytoplasmic Hsp110/Sse1 and ER-resident Grp170/Lhs1 proteins are divergent members of the Hsp70 chaperone family (7,8). Hsp110 and Grp170 proteins are significantly larger than Hsp70, possessing a loop region between the β and α- subdomains, as well as an extended tail. Hsp110 has been shown to bind denatured...
proteins \textit{in vitro} but is unable to actively refold them and instead acts as a "holdase," maintaining substrate polypeptides in a folding-competent state (9,10). No endogenous substrates have been identified for the Hsp110s to date, however overexpression of Hsp110 increases thermotolerance in Chinese hamster ovary (CHO) cells (9). More recently overexpression of the Hsp110 family member Hsp105α was shown to suppress protein aggregation and subsequent apoptosis in COS-7 cells expressing the polyglutamine tract-containing truncated androgen receptor (tAR), suggesting a potential role for Hsp110 in the prevention of protein plaque-associated pathologies (11).

\textbf{SSE1} and its close paralog \textbf{SSE2} are the \textit{Saccharomyces cerevisiae} members of the Hsp110 subfamily. \textbf{SSE1} was identified biochemically as a calmodulin binding protein and genetically as a high copy suppressor of the hyperactive PKA mutant \textit{ira1Δ} (12,13). Yeast lacking \textbf{SSE1} are slow growing and slightly temperature sensitive. Deletion of \textbf{SSE2} results in no observable growth defects and deletion of both \textbf{SSE} genes was reported to be equivalent to deletion of \textbf{SSE1} alone (12,13). Mammalian Hsp110 does not complement the deletion of \textbf{SSE1} in yeast (J. Subjeck, personal communication; data not shown), suggesting distinct cellular substrate or cofactor specificities. \textit{In vitro}, Sse1 binds denatured luciferase and accelerates its refolding upon addition of yeast cytosol (14,15). Sse1 also participates in Hsp90 signal transduction -- its absence leads to the derepression of the yeast heat shock transcription factor, Hsf1, and loss of glucocorticoid receptor (GR) signaling, two established roles for Hsp90 (16). Recently, \textbf{SSE1} was isolated as a high copy suppressor of a mutated form of the cytosolic Hsp40, Ydj1, possibly due to the participation of both proteins in Hsp90-dependent functions (15).
The apparent lack of folding activity for Sse1/Hsp110 calls into question the role of nucleotide cycling in the ATPase domain. Several Hsp70 ATPase domain residues have been demonstrated to be vital for ATP binding and hydrolysis in multiple mammalian and yeast homologs (17-21). The Sse1 ATPase domain is 53% similar to Hsc70, while Ssa1 and Hsc70 share 87% similarity; many of the substitutions between the chaperones are within the ATP binding pocket. For example, a glutamate residue at position 175 in Hsc70 is replaced by aspartate in Sse1, while aspartate 206 of Hsc70, proposed to be a proton acceptor during the hydrolysis cycle, is replaced by threonine in Sse1 and glutamine in CHO Hsp110 (20,22). Recombinant mammalian Hsp110 expressed in E. coli was shown to bind ATP only in the absence of the PBD, and furthermore neither Hsp110 nor Sse1 have been reported to hydrolyze ATP (10).

To learn more about this abundant yet ill-defined chaperone, we have undertaken a molecular genetic analysis of Sse1 in baker’s yeast. Residues known to be involved in ATP binding and hydrolysis in the Hsp70s were mutagenized in Sse1 and assayed for complementation of sse1Δ phenotypes. Mutants that were able to bind ATP affinity resin also complemented sse1Δ, while mutants that did not bind were non-functional in vivo. Deletion analysis of the carboxyl-terminus revealed that Sse1 can tolerate removal of 44 residues with no effect on function, suggesting that this region is not involved in chaperone-chaperone communication as it is in Hsp70. Mutations abrogating the β-strand or α-helical subdomains of the PBD substantially destabilized the protein. Remarkably, the ATPase domain and PBD were found to function and interact when expressed in trans, indicating direct interdomain communication between the two domains. These
results demonstrate significant differences in the cellular function of Sse1 versus the classical Hsp70s.
Experimental procedures

Strains and plasmids- All strains are isogenic derivatives of *Saccharomyces cerevisiae* strain W303 (*MATα ade2-1 trp1 can1-100 leu2-3,-112 his3-11,-15 ura3*). The *sse1Δ::kan*R strain was previously described (16). Construction details of strain *sse1Δ::kan*R Δ *sse2::LEU2* will be described elsewhere2. Synthetic complete (SC) media lacking the appropriate nutrient for plasmid selection was purchased from BIO101 (Carlsbad, CA). Standard yeast propagation and transformation procedures were employed (23). The *SSE1* ORF was amplified by PCR from pYEp24*SSE1* and cloned into p414TEF, p416TEF, and p426GPD yeast expression vectors as listed in Table 1 (16,24). *Spe* I (5’) and *Xho* I (3’) restriction sites were incorporated by PCR into all constructs to facilitate subcloning. Amino-terminal FLAG and HA epitope-tagged *SSE1* clones were described previously (16). *SSE1*K69Q and *SSE1*G233D point mutants were described previously (15). *SSE1*D8N, *SSE1*D174N, *SSE1*D203N, and *SSE1*G205D point mutants and the HA-*SSE1*Δ394-419 deletion mutant were made by the PCR overlap extension method using primers incorporating the appropriate mutation or deletion (25). ATPase domain fragments were made by PCR incorporating a stop codon after residue 393 using p416TEF HA-*SSE1* or p416TEF FLAG-*SSE1* as template. Amino-terminal peptide binding domain truncations were constructed by PCR incorporating a start codon in front of residue 394, 419, 444, or 469. Carboxyl-terminal truncations were constructed by PCR incorporating a stop codon after residue 683, 649, 590, or 505 using p416TEF HA-*SSE1* as template. To facilitate subcloning, a *Spe* I site was added to pFLAG-MAC (Sigma, St. Louis, MO) in frame and upstream of the *Xho* I site by PCR, creating pFLAG-*Spe*. Wild type and ATPase point mutant *SSE1* alleles were then subcloned by
ligation of a Spe I/Xho I fragment into similarly digested pFLAG-Spe. Sequences for all oligonucleotides used in this report are available upon request.

**Immunoblot analysis**-Cells were pelleted and resuspended in TEGN buffer (20 mM Tris-pH 7.9, 0.5 mM EDTA, 10% glycerol, 50 mM NaCl) + protease inhibitors (aprotinin 2 µg/mL, pepstatin A 2 µg/mL, leupeptin 1 µg/mL, PMSF 1 mM, chymostatin 2 µg/mL) (Roche Diagnostics Corp., Indianapolis, IN) followed by addition of glass beads. The samples were then agitated using a Vortex mixer in four rounds of 1.5 min each followed by 1.5 min on ice. The lysate was then cleared by centrifugation at 4,500 x g. Proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunodetection. 12CA5 monoclonal antibody recognizing the HA epitope was purchased from Roche Diagnostics. Anti-phosphoglycerate kinase antibody was purchased from Molecular Probes, Inc. (Eugene, OR). M2 anti-FLAG antibody was purchased from Sigma. Anti-Sse1 polyclonal antibody was previously described (15). Proteins were detected by addition of appropriate horseradish peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse secondary antibodies (Bio-Rad, Hercules, CA) followed by ECL chemiluminescence. An Alpha Innotech Corp. (San Leandro, CA) FluorChem 8800 Imaging System and AlphaEaseFC software were used for image capture and analysis.

**ATP binding assay**-*E. coli* strain BL21 (B F-, ompT, hsdS (rB-, mB-), gal, dcm) was transformed with pFLAG constructs for overexpression of wild type and mutant proteins. Strains were grown overnight in LB + ampicillin (100 µg/mL) at 37°C, subcultured to OD<sub>600</sub>=0.1 in 150 mL and grown to OD<sub>600</sub>=0.5 at which time cells were induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to 0.5 mM and shifted to 30°C for 4 h.
Cells were then pelleted by centrifugation and resuspended in 15 mL TEGN + protease inhibitors (aprotinin 2 µg/mL, pepstatin A 2 µg/mL, leupeptin 1 µg/mL, PMSF 1 mM, chymostatin 2 µg/mL). The cell suspension was passed through a French pressure cell twice at 1,200 psi (SLM Instruments, Urbana, IL). Unbroken cells and debris were pelleted by centrifugation at 7,500 x g for 10 min. The medium speed supernatant was then centrifuged at 100,000 x g for 30 min, glycerol was added to the soluble fraction to a final concentration of 25%, and the samples were aliquoted and stored at –80˚ C.

ATP-agarose binding was performed essentially as described (10). Briefly, ATP-agarose (Sigma-A2767) and nucleotide-free agarose were stored as a 1:1 slurry in buffer B (20 mM Tris-HCl, 20 mM NaCl, 0.1 mM EDTA, 2 mM dithiothreitol). A total of 1 mg of total protein was added to 200 µL of ATP-agarose or nucleotide-free agarose slurry and incubated at 4˚C overnight with mixing. The beads were then washed six times with Buffer B, eluted with SDS-PAGE sample buffer, and proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblot analysis.

**Immunoprecipitation analysis-sse1Δ** cells harboring p416TEF FLAG-SSE1-ATPase1-393 and p414TEF SSE1-CTD394-693 or p416TEF and p414TEF SSE1-CTD 394-693 were grown in SC-Ura-Trp to mid-log phase at 30˚C. Total soluble protein was then obtained by glass bead lysis. Protein extracts were incubated with 40 µL anti-FLAG M2 resin for 2 h at 4˚C. Resin was then pelleted and washed five times with TEGN buffer followed by elution with SDS-PAGE sample buffer at 65˚C for 10 min. Proteins were then separated by SDS-PAGE followed by immunoblot analysis.
Cycloheximide chase analysis-sse1Δ cells harboring either p416TEF HA-SSE1 or p416TEF HA-SSE1Δ394-419 were grown in SC-Ura medium to mid-log phase at which time cycloheximide was added to a final concentration of 200 µg/mL (t=0). Samples were taken at 0, 2, 4, and 6 h, pelleted and stored at -80°C. Soluble protein was obtained by glass bead lysis and separated by SDS-PAGE followed by transfer to nitrocellulose and immunoblotting. Quantitation was performed using real time 16-bit CCD image acquisition and AlphaEaseFC image analysis software. Half-life was determined by normalization of the Sse1 signal to PGK at each time point and fitting the data to a single exponential curve. The experiment was performed twice with similar results.

β-Galactosidase Assay- Liquid β-galactosidase assays for Hsf1 activity were performed exactly as described (16).
Results

Sse1 ATPase domain mutants are functional-Previous research has shown that the Hsp70 ATPase activity is essential for function both in vivo and in vitro. Extensive biochemical and genetic studies have been carried out using point mutations in the ATPase domain to determine which residues are important in the ATP binding and hydrolysis cycle of Hsp70s (17-21). For example, several residues in bovine Hsc70 are important for ATP hydrolysis and mutations caused dramatic decreases in $k_{cat}$ and moderate increases in $K_m$ values (20,21). Equivalent mutations in the yeast cytosolic Hsp70, Ssa1, and ER lumenal Hsp70 Kar2/BiP, were shown genetically and biochemically to render the protein non-functional (18,19). Notably, the ATPase domain of Sse1 shares significant sequence homology with other Hsp70s (36% identity with the Ssa1 ATPase domain). In order to study the role of the N-terminal ATPase domain in Sse1 function, we mutagenized residues known to be involved in the ATP binding and hydrolysis cycle of these other Hsp70s. The residues we targeted for mutation were D8N (D10 in bHsc70), K69Q (K71), D174N (E175), D203N (D199), G205D (G201), and G233D (G229). It was reported previously that Hsf1-regulated genes are derepressed in sse1Δ (16). Because SSE1 is a known target gene of Hsf1 we decided to place wild type and mutant SSE1 constructs under control of the heterologous TEF1 promoter (12,24). Immunoblotting experiments showed that expression levels of these proteins were approximately two-fold higher than that expressed from the native SSE1 promoter (data not shown).

Two different complementation assays were employed to assess function of the Sse1 mutants. In the first, the mutants were assayed for the ability to rescue the lethality
of an $sse1\Deltasse2\Delta$ strain upon loss of a Yep24SSE1 plasmid on 5-FOA, shown in Fig. 1A. It was previously reported that deletion of SSE2 in a $sse1\Delta$ strain did not show any additional phenotypes (12). However, our lab recently found that $SSE1$ and $SSE2$ constitute an essential gene pair\(^2\). Surprisingly, we found that the D8N, K69Q, D174N, D203N, and G205D mutants all allowed for the efficient loss of pYep24SSE1. G233D was the only point mutant unable to permit loss of wild type $SSE1$ in the $sse1\Deltasse2\Delta$ strain background. A construct consisting of the carboxyl-terminal peptide binding domain (PBD) lacking the ATPase domain was also unable to complement $\Deltasse1\Deltasse2$ (data not shown). We then assayed for the ability of the mutants to complement the temperature sensitive phenotype of the $sse1\Delta$ mutant. Fig. 1B shows that the D8N, K69Q, D174N, and D203N mutants complemented this phenotype while the G205D and G233D mutants did not. All of the mutants are expressed at or near the levels of wild type Sse1 under control of the $TEF1$ promoter (Fig 1C).

The ability of the G205D mutant to function as the sole source of $SSE1$ in $sse1\Deltasse2\Delta$ at 30°C and the failure of this mutant to complement the temperature sensitivity of $sse1\Delta$ suggested that this was a temperature sensitive allele. This hypothesis was tested by assaying the growth of $sse1\Deltasse2\Delta$ mutants harboring a plasmid expressing either wild type $SSE1$ or $sse1$-G205D at 30°C and 37°C. As shown in Fig. 1D, the inability of $sse1\Deltasse2\Delta$ expressing G205D to grow at 37°C illustrates that $sse1$-G205D is a temperature sensitive allele. Comparative Western blots of samples grown at 30°C and 37°C showed that the mutant protein is stably expressed in both cases, indicating that G205D renders the Sse1 protein temperature sensitive for function, but not for stability (data not shown).
Sse1, Hsp90 and the cyclophilin Cpr7 function similarly to repress Hsf1 under non-stress conditions (26). Using a reporter containing the lacZ gene under control of the SSA3 promoter, which contains the Hsf1-responsive Heat Shock Element (HSE), it was shown that sse1Δ cells derepress Hsf1 under steady-state conditions (16,27). Utilizing this reporter, we assayed the SSE1 ATPase mutants for the ability to repress expression of Hsf1 target genes under non-stress conditions (30˚C, mid-log phase). Fig. 2 illustrates the results of this experiment. The D8N, K69Q, D174, and D203N mutants were able to repress expression of the reporter to near wild type levels. In contrast, G205D, G233D, and the peptide binding domain fragment (PBD) all exhibited β-galactosidase activity ranging from ~2.5 - 3.5 times higher than wild type, equivalent to the empty vector control. Taken together, these genetic data suggest that ATP binding is required for Sse1 function in vivo.

Sse1 and complementing ATPase mutants bind ATP in vitro—Although the ATPase domain shares significant homology with other Hsp70s, Sse1 has not been reported to bind or hydrolyze ATP. Mammalian Hsp110 binds ATP only in the absence of the peptide binding domain, but has not been reported to actively hydrolyze ATP, indicating that this subfamily of chaperones might function differently than Hsp70s (10). To establish the nucleotide binding capabilities of wild type Sse1, we incubated total yeast protein extracts with ATP immobilized on agarose followed by elution of all proteins bound to the resin. Using this technique we were unable to detect Sse1 binding to the resin, while Ssa1 bound efficiently, demonstrating the efficacy of the procedure (data not shown). To test the possibility that a component in yeast extract prevented Sse1 from
binding ATP, we overexpressed Sse1 and Ssa1 in *E. coli* and assayed nucleotide binding. Wild type Sse1 or Ssa1 produced in *E. coli* efficiently bound ATP-agarose, and binding to ATP-agarose was significantly more efficient than the non-specific binding to nucleotide-free agarose (Fig. 3A, compare lanes 3 and 4 to 5 and 6, respectively). These results demonstrate that Sse1 binds ATP in the absence of yeast cytosolic components. This assay was then utilized to determine if the point mutants had altered nucleotide binding capabilities (Fig. 3B and Table 2). K69Q bound at levels near wild type Sse1, whereas D174N and D203N bound ATP but at a lower efficiency. No binding to ATP affinity resin above background could be observed for the G205D and G233D mutants, suggesting that these mutants lost the ability to bind ATP *in vitro*. Conclusions cannot be drawn about D8N because of the large amount of protein bound to both ATP- and nucleotide free- agarose. The decrease in the ability of the lysine and aspartate mutants to bind ATP was not unexpected as Wilbanks *et al.* showed that the equivalent bovine Hsc70 mutants have an increased *K*<sub>m</sub> for ATP compared to wild type protein (21). Taken together with the functional assays, these results further demonstrate that Sse1 must retain some ability to bind ATP in order to function.

*Overexpression of SSE1 is toxic*—Early in our studies we observed that high-level overexpression of *SSE1* was detrimental to wild type yeast, leading to extremely slow growth at 30°C (data not shown). While the cellular targets of Sse1 that lead to this toxicity are unknown, we sought to use this effect as another assay of Sse1 function. The ATPase mutant alleles were subcloned into the high-copy plasmid p426GPD and transformed into the W303 background. The results for K69Q and G233D are shown in Fig. 4A and the results for all mutants are summarized in Table 2. The serial dilutions
illustrate that overexpression of K69Q, like wild type SSE1, compromised cell growth while G233D overexpression had no observable growth effect. Other mutants showed differential effects when overexpressed in wild type yeast. D8N was equally as toxic as wild type while D203N had a moderate effect and D174N had a mild effect. G205D, like G233D, had no deleterious effects when overexpressed. To test whether differences in toxicity were due to differences in expression levels of the mutant constructs, Western blot analysis was performed. The results of this experiment confirmed that the examined proteins were overexpressed to similar levels (Fig 4B and data not shown). Overall, ATP binding by the Sse1 mutants appears to correlate with overexpression toxicity (Table 2). For example, wild type Sse1, K69Q, and D203N are best able to bind ATP in vitro and are the most toxic when overexpressed, whereas G205D, G233D and the PBD alone do not slow growth when overexpressed and do not bind ATP.

*Mild overexpression of SSE1 mutants suppresses ydj1-151 temperature sensitivity*-Ydj1 is one of two yeast cytosolic Hsp40 homologs and has been shown to regulate the chaperone activity of Ssa1 by stimulating its ATPase activity (28). Because overexpression of SSE1 in ydj1-151 yeast partially suppresses the temperature sensitivity of this strain, the ability of the ATPase point mutants to suppress ydj1-151 was tested as another assay of Sse1 function (Table 2) (15). We found that wild type SSE1 suppressed temperature sensitivity at 35°C when expressed under control of the TEF1 promoter. Of the six point mutants, only K69Q suppressed as well as wild type SSE1. D203N suppressed slightly less well than wild type and D8N poorer still. D174N, G205D, G233D, and the PBD fragment were inactive in this assay. As with overexpression
toxicity, suppression of \textit{ydl1-151} temperature sensitivity correlated well with the ability to bind ATP.

\textit{The carboxyl-terminal tail of Sse1 is not required for function}-Most eukaryotic cytosolic Hsp70s possess a conserved “EEVD” motif at their extreme carboxyl terminus that is required for functional interactions with tetratricopeptide (TPR) repeat-containing proteins like Sti1/Hop, which mediate their interaction with Hsp90 (29,30). While Sse1 does not possess this motif, it does have a concentration of negatively charged residues at the carboxyl-terminus. Therefore, we constructed a series of carboxyl-terminal deletion mutants lacking the final 10, 44, 103 and 188 residues to assess the importance of this region for Sse1 function (Fig. 5A).

Deletion mutant Δ10 removes the final 10 residues of Sse1 and mutant Δ44 lacks the entire region following the predicted \(\alpha\)-helical “lid” domain of the PBD, the latter corresponding to the variable domain of eukaryotic Hsp70s (7). Mutant Δ103 lacks the majority of the \(\alpha\)-helical domain and the entire variable domain. Mutant Δ188 removes the predicted unstructured loop region, specific to the Hsp110 family, as well as the \(\alpha\)-helical domain and the variable domain. These mutants were assayed for their ability to complement the temperature sensitive phenotype of \textit{sse1Δ} and repress Hsf1. The Δ10 and Δ44 mutants complemented both of these phenotypes equally as well as wild type \textit{SSE1} (Fig. 5B,D). Both of these mutants were expressed and stable, as demonstrated by Western blot analysis in Fig. 5C. These data indicate no obvious functional role for the variable domain of Sse1. In contrast, the Δ103 and Δ188 mutants were unable to complement the \textit{sse1Δ} phenotypes and unlike the Δ10 and Δ44 mutants, could only be detected by long exposures of the Western blot, indicating that these truncations may
destabilize Sse1 (data not shown). This suggests that the predicted α-helical “lid” of the peptide binding domain is important for Sse1 stability and function.

*Sse1 stability is dependent on a functional peptide binding domain*-To test the idea that the PBD may be required for Sse1 stability, a deletion mutant was constructed that disrupted the β-sheet region of the peptide binding domain by removing two of the eight predicted β-sheets. We used an HA-tagged version of Sse1 to aid in immunodetection of the deletion mutant protein, designated HA-Sse1Δ394-419 (Fig. 6A). As expected, this mutant was unable to complement sse1Δ phenotypes, whereas wild type HA-Sse1 functioned normally (data not shown). Importantly, like the Δ103 and Δ188 mutants, this mutant was found to be poorly expressed by Western blot analysis (Fig. 6B). To determine more directly if the observed poor expression of HA-Sse1Δ394-419 was the result of delayed synthesis or protein instability, we performed a cycloheximide chase. To block protein synthesis, cycloheximide was added to growing cultures of sse1Δ harboring either HA-Sse1 or HA-Sse1Δ394-419. When samples from the four time points were analyzed by Western blot, quantified and fitted to an exponential decay curve, we found that wild type HA-Sse1 exhibited a half-life of 12.5 h (Fig. 6C). However, levels of HA-Sse1Δ394-419 were substantially reduced over the time course with a calculated half-life of 3 h, indicating post-translational destabilization.

To begin to localize the stabilization determinants in Sse1, we attempted to express the ATPase domain alone in yeast and found it to be extremely unstable (Fig. 7). However, we reasoned that co-expression of the peptide binding domain might stabilize the ATPase domain and therefore examined the stability of the two domains expressed in *trans*. The full length peptide binding domain (PBD) as well as several mutants that
removed 25, 50, and 75 residues from the amino terminus of the PBD were co-expressed with the ATPase domain of SSE1 (Fig. 7A). Expression levels of the ATPase domain were very low in all cases except where the full length PBD was co-expressed (Fig. 7B). In contrast, all of the PBD deletion mutant fragments were readily detectable. In other experiments we expressed the ATPase fragment in wild type cells to determine if endogenous Sse1 could stabilize this domain. We found that the ATPase domain was equally unstable in wild type cells as it was in sse1Δ cells (data not shown). Together, these data indicate that the stability of the ATPase domain is dependent on a full length peptide binding domain, and furthermore that this interaction can occur in trans. In addition, the PBD remains stable in the absence of the ATPase domain. However, truncation of the carboxyl-terminal 188 amino acid residues in the context of the PBD alone (i.e., residues 394-505), but not the carboxyl-terminal 103 residues (394-590), resulted in destabilization (data not shown). Therefore, although PBD stability is largely independent of the presence of the ATPase domain in trans, complete removal of the linker region, α-subdomain and variable region is not tolerated.

*Sse1 domains can function and interact in trans*- The protein binding and folding activity possessed by the peptide binding domain of Hsp70s is regulated by the nucleotide binding status of the ATPase region, implying interdomain communication. Several studies have shown evidence for such communication using intrinsic tryptophan fluorescence or intramolecular suppression of mutations in distinct domains (31-35). The data presented in Fig. 7 showing that stability of the ATPase domain depends on the peptide binding domain likewise suggest Sse1 interdomain communication and perhaps interaction. We tested this hypothesis by transforming sse1Δ cells with plasmids bearing
one or both of the Sse1 domains and testing for complementation of the temperature sensitivity phenotype. The ATPase and peptide binding domains expressed in trans were able to complement the slow growth phenotype of sse1Δ yeast, indicating that these independent domains are able to function similarly to wild type, full-length protein when expressed separately (Fig. 8A). This complementation did not occur when either domain was expressed alone. In addition, trans complementation was poorer at 37°C, indicating that the trans interaction may be weaker than the cis interaction (data not shown).

The dependence of a full length PBD on ATPase domain stability and the fact that these domains function when expressed in trans suggested an in vivo interaction. This was investigated by performing coimmunoprecipitation experiments. A full length PBD was co-expressed with or without a FLAG-tagged ATPase domain in sse1Δ yeast followed by immunoprecipitation with the M2 anti-FLAG antibody. We found that the PBD coimmunoprecipitated with FLAG-ATPase but did not precipitate when FLAG-ATPase was absent (Fig. 8B), indicating interaction between the two Sse1 domains.

There are two models to account for the functional interaction seen between the Sse1 domains; 1) Sse1 is a monomer and the two domains associate tightly in an intramolecular interaction or 2) Sse1 is predominantly dimeric, with the possibility of intermolecular domain interactions. To distinguish between these possibilities, HA- and FLAG-tagged versions of Sse1 were co-expressed in sse1Δ cells and coimmunoprecipitation experiments were attempted. We were unsuccessful in several attempts to detect HA-Sse1 coimmunoprecipitating with FLAG-Sse1 (data not shown). Together, these data suggest that Sse1 does not function in a homo-multimeric state in yeast.
Discussion

In this report we present evidence that the yeast Hsp110 homolog, Sse1, functions in vivo by a mechanism distinct from its Hsp70 relatives. Several mutations known to abrogate ATP binding and hydrolysis in Hsp70 were introduced into the ATPase domain of Sse1 and had no effect on in vivo function as assayed by the ability to complement the growth and Hsf1-repression phenotypes of sse1Δ yeast. Non-complementing ATPase point mutants were also unable to bind ATP-agarose affinity resin while functional mutants retained the ability to bind, suggesting that nucleotide binding is critical for Sse1 function. We also showed that the stability of Sse1 depends on an intact peptide binding domain. Mutations in the peptide binding domain significantly destabilized the protein, suggesting a novel auto-chaperoning activity. Unexpectedly, the ATPase domain and PBD expressed in trans were able to complement sse1Δ yeast and were found to interact directly in vivo by coimmunoprecipitation, providing evidence for interdomain communication.

Extensive studies have been performed with Hsp70 ATPase domain point mutants, showing that the cycle of ATP binding and hydrolysis is essential for function. Mutation of residues in Hsp70 corresponding to Sse1 D8, K69, D174, D203, G205, and G233 were previously demonstrated to be nonfunctional in vivo, in vitro or both. However, Sse1 mutants D8N, K69Q, D174N, and D203N remained functional in vivo as judged by complementation of multiple sse1Δ phenotypes. An interesting correlation was observed in which mutants that complemented sse1Δ phenotypes were able to bind ATP-agarose, albeit less efficiently than wild type. Only mutants that were unable to bind ATP-agarose did not function in vivo, suggesting that Sse1 must bind nucleotide to
function. To date there are no published reports of a member of the Hsp110 family hydrolyzing ATP. Our genetic evidence suggests that if Sse1 has the ability to hydrolyze ATP, it likely plays a minor functional role. The potential lack of, or exceedingly weak, ATPase activity is consistent with the Hsp110/Sse1 family’s inability to actively refold denatured protein substrates. Hsp110/Sse1 instead acts as a “holdase,” allowing for more efficient refolding in the presence of Hsp70 (14). Indeed, Hsp110 binds more efficiently to denatured peptides than does Hsp70 (9). This cooperativity with Hsp70 could reflect the in vivo role of the Hsp110/Sse1 subfamily of chaperones. Hsp110 has been found in a multi-chaperone complex with Hsp70, but the significance of the Hsp110-Hsp70 interaction in vivo remains to be investigated (36). Together these data support a model wherein Hsp110 quickly recognizes a denatured protein substrate, binds it, and acts cooperatively with the “foldase”-competent Hsp70 to effect renaturation.

One of the most intriguing aspects of this study is the observed trans complementation and coimmunoprecipitation of the ATPase and peptide binding domains of Sse1, strongly suggestive of interdomain communication. It is known that the ATPase domain of Hsp70 regulates the affinity for substrate of the peptide binding domain, although the exact nature of this regulation is unknown. For example, Davis et al showed that a lethal mutation in the PBD of the yeast mitochondrial Hsp70, Ssc1, could be suppressed by a compensatory mutation in the ATPase domain (32). The same intragenic suppression was found to occur in DnaK, suggesting conservation through evolution (32). However, physical contact has not been directly demonstrated between the ATPase and PBD. In addition, there is no evidence that Ssa1 stability depends on the peptide binding domain. It is feasible that the 28-residue loop region in Ssel, which
separates the $\beta$ and $\alpha$ subdomains and is absent in Hsp70, allows the peptide binding domain to fold back in order to make contact with and “chaperone” the ATPase domain, as depicted in Fig. 9 (10). The \textit{in vivo} role for this novel type of interdomain communication in a molecular chaperone is unclear, but it may function as a regulatory mechanism to control access of the PBD to substrate. The inherent instability of the ATPase domain raises the possibility that substrates could be targeted for degradation via association with Sse1. However, we do not have data to support such a mechanism, and the stability of intact Sse1 is inconsistent with a significant population of the chaperone being degraded with faster kinetics.

The interdomain contact suggests that Sse1 possibly exists in multimeric complexes, not unlike mammalian Hsc70, BiP, and yeast Kar2, which have all been demonstrated to form homomultimers in the cell (19,37,38). In support of Sse1 existing in one or more cytosolic protein complexes, the PBD was unable to stabilize the ATPase domain when independently coexpressed in \textit{E. coli} (data not shown), consistent with a model wherein the two halves associate with each other in the context of another scaffolding protein. The ATPase domain of Sse1 expressed in wild type cells remains unstable, suggesting that full length Sse1 is unable to functionally interact with a separate Sse1 ATPase domain. However, even if Sse1 is present in complexes, it most likely consists of one molecule of Sse1 per complex or we would have observed coimmunoprecipitation of differentially tagged proteins. A possible explanation lies in the observed association of Sse1 with the Hsp90 molecular chaperone complex (16). For example, the two domains of Sse1 could associate with Hsp90 independently. Sse1 lacks the conserved EEVD motif found in cytosolic Hsp70s that mediates association with
Sti1/HOP in the Hsp90 system, and our results demonstrate that protein sequence distal to the α-helix domain of the PBD can be deleted, suggesting that the Sse1-Hsp90 interaction is of a different nature than that of Ssa1-Hsp90. Alternatively, the genetic interaction between Sse1 and Ydj1 may reflect a protein-protein interaction that could also occur between the ATPase and peptide binding domains, respectively (15). Experiments examining the multimeric state of Sse1, the role of nucleotide binding in modulating these interactions and the identification of Sse1 binding partners are in progress.
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Footnotes

1. The abbreviations used in this manuscript are: PBD, peptide binding domain; HSP, heat shock protein; CHO, Chinese hamster ovary

2. A. Trott, L. Shaner and K.A. Morano, manuscript in preparation

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Figure legends

Figure 1: Sse1 ATPase domain point mutants complement phenotypes associated with sse1Δ and sse1Δ sse2Δ mutants. (A) sse1Δ sse2Δ cells harboring pYEp24SSE1 were transformed with the indicated mutant SSE1 alleles expressed from p414TEF. The strains were grown on 5-FOA plates for 3 d at 30˚C to select for loss of pYEp24SSE1. (B) sse1Δ yeast were transformed with p414TEF constructs from (A). Serial dilutions were spotted onto SC-Trp plates and incubated at 37˚C for 2 d. (C) Protein extracts were obtained from strains in (B) and subjected to Western blot analysis with anti-Sse1 antiserum to confirm protein expression. PGK represents the loading control, SSE1, wild type SSE1 protein; vector, empty vector control. (D) sse1Δ sse2Δ cells harboring p414TEFSSE1 or p414TEFsse1-G205D were grown on YPD plates and incubated for 2 d at 30˚C or 37˚C, as indicated.

Figure 2: Repression of Hsf1 transcriptional activity under non-stress conditions by SSE1 mutant alleles. sse1Δ cells expressing the indicated mutant alleles of SSE1 expressed from p414TEF were transformed with pCM64-SSA3-lacZ (URA3). The strains were grown to mid-log phase in SC-Ura-Trp media followed by determination of β-galactosidase activity as described in Experimental Procedures.

Figure 3: ATP-agarose binding by Sse1 mutant proteins. (A) FLAG-fusions of Ssa1 and Sse1 were produced in E. coli and assayed for their ability to bind ATP-agarose as described in Experimental Procedures. Total cell extracts (lanes 1 and 2) were applied to ATP-agarose (lanes 3 and 4), or nucleotide-free agarose (lanes 5 and 6) and incubated at
4°C overnight. Reactions were washed with buffer B and eluted proteins were resolved on 10% SDS-PAGE followed by transfer to nitrocellulose. FLAG-fusions were detected using M2 antibody. (B) Mutant Sse1 FLAG-fusions were obtained and assayed for the ability to bind ATP affinity resin and detected as described in (A). Lanes 1, 4, 7, 10, 13, 16 and 19 represent total protein loaded onto resin, lanes 2, 5, 8, 11, 14, 17 and 20 protein eluted from ATP-agarose, and lanes 3, 6, 9, 12, 15, 18 and 21 protein eluted from nucleotide-free agarose.

**Figure 4: Growth inhibition by overexpression of functional SSE1.** Wild type strain W303 was transformed with high copy plasmids expressing wild type and mutant SSE1 alleles. (A) Serial dilutions were spotted on SC-Ura plates and incubated for 2 d at 30°C. (B) Overexpression of wild type and mutant Sse1 proteins was confirmed by Western analysis using anti-Sse1 antiserum.

**Figure 5: Sse1 can tolerate deletion of 44 residues from the carboxyl-terminus.** (A) Truncations of HA-Sse1 were generated that removed 10, 44, 103, and 188 residues from the carboxyl-terminus. (B) Truncated alleles expressed from p414TEF were assayed for complementation of sse1Δ temperature sensitivity. The indicated strains were spotted on SC-Trp and incubated for 2 d at 37°C. (C) Expression of truncated proteins was confirmed by Western blot using anti-HA antibody. Mutants Δ103 and Δ188 cannot be seen in this image but were visible with longer exposure (data not shown). (D) Strains in (B) were transformed with the reporter plasmid pCM64-SSA3-lacZ and β-galactosidase assays were performed as in Fig. 2.
Figure 6: Disruption of the peptide binding domain destabilizes Sse1. (A) An in-frame deletion of residues 394-419 was generated in HA-tagged Sse1, removing two of eight β-strands of the β subdomain. (B) Wild type HA-Sse1 and HA-Sse1Δ394-419 were expressed from p416TEF in sse1Δ. Protein was extracted from mid-log phase cultures and resolved by SDS-PAGE. Sse1 proteins were detected using HA antibody. Equal loading was confirmed by anti-PGK signal. (C) Cycloheximide chase analysis was employed to demonstrate instability of HA-Sse1Δ394-419. Cultures from (B) were grown to mid-log phase in SC-Ura at which time cycloheximide was added to a final concentration of 200 µg/ml (time = 0) Aliquots were taken at 0, 2, 4, and 6 h and protein was prepared and detected as in (B). Half-lives of wild type and mutant proteins were determined by spot densitometry normalizing the Sse1 signal to PGK as described in Experimental Procedures. To facilitate visual comparison of Sse1 stability, protein loads were increased two-fold and exposure time adjusted approximately 3-5 fold for the HA-Sse1Δ394-590 mutant.

Figure 7: The ATPase domain of Sse1 is unstable in the absence of a full-length peptide binding domain. (A) Amino-terminal truncations of the peptide binding domain were generated in 25 residue increments. The PBD truncations were expressed from p414TEF in sse1Δ cells harboring p416TEF HA-SSE1-ATPase1-393. (B) Protein was prepared and resolved using SDS-PAGE. HA-Sse1-ATPase was detected with anti-HA antibody and the truncated Sse1-PBDs were detected with anti-Sse1 antibody that
recognizes an epitope at the extreme C-terminus (15). Equal loading was confirmed with anti-PGK.

**Figure 8:** Sse1 domains expressed in *trans* physically interact and complement the *sse1Δ* mutant. (A) *sse1Δ* cells expressing the indicated constructs were spotted onto SC-Ura-Trp plates and incubated at 30°C for 2 d. (B) FLAG-tagged ATPase domain was immunoprecipitated (described in Experimental Procedures) and Sse1 domains were detected by Western analysis as in Fig. 7. Lanes 1 and 2 are total cell extracts from *sse1Δ* cells expressing FLAG-*SSE1*-ATPase and *SSE1*-PBD or *SSE1*-PBD alone, respectively. Lanes 3 and 4 represent the corresponding immunoprecipitations.

**Figure 9:** Model for Sse1 function. The data presented here suggest a model whereby the Sse1 peptide binding domain interacts with and “chaperones” the intrinsically unstable ATPase domain. Sse1 must then undergo conformational changes, perhaps mediated by changes in occupancy of the nucleotide binding domain (hexagon), that free the peptide binding domain (α and β modules) to associate with substrate proteins in the cell.
| Plasmid                                | Reference  |
|----------------------------------------|------------|
| pYep24 SSE1                            | (12,16)    |
| p414TEF SSE1                           | This study |
| p414TEF SSE1/D8N                       | This study |
| p414TEF SSE1/K69Q                       | This study |
| p414TEF SSE1/D174N                      | This study |
| p414TEF SSE1/D203N                      | This study |
| p414TEF SSE1/G205D                      | This study |
| p414TEF SSE1/G233D                      | This study |
| p416TEF SSE1                           | This study |
| p416TEF SSE1/D8N                       | This study |
| p416TEF SSE1/K69Q                       | This study |
| p416TEF SSE1/D174N                      | This study |
| p416TEF SSE1/D203N                      | This study |
| p416TEF SSE1/G205D                      | This study |
| p416TEF SSE1/G233D                      | This study |
| p426GPD SSE1                            | This study |
| p426GPD SSE1/D8N                       | This study |
| p426GPD SSE1/K69Q                       | This study |
| p426GPD SSE1/D174N                      | This study |
| p426GPD SSE1/D203N                      | This study |
| p426GPD SSE1/G205D                      | This study |
| p426GPD SSE1/G233D                      | This study |
| p414TEF SSE1-CTD_{394-693}              | This study |
| p414TEF SSE1-CTD_{419-693}              | This study |
| p414TEF SSE1-CTD_{444-693}              | This study |
| p414TEF SSE1-CTD_{469-693}              | This study |
| p416TEF HA-SSE1                         | This study |
| p416TEF HA-SSE1-ATPase1-393             | This study |
| p416TEF FLAG-SSE1                       | This study |
| p416TEF FLAG-SSE1-ATPase1-393           | This study |
| p416TEF HA-SSE11-683                    | This study |
| p416TEF HA-SSE11-649                    | This study |
| p416TEF HA-SSE11-590                    | This study |
| p416TEF HA-SSE11-505                    | This study |
| p416TEF HA-SSE1_{A394-419}              | This study |
| pCM64-SSA3-lacZ                         | (27)       |
| pFLAG-MAC                              | Sigma      |
| pFLAG-Spe                               | This study |
| pFLAG-Spe-SSA1                         | This study |
| pFLAG-Spe-SSE1                         | This study |
| pFLAG-Spe- SSE1/D8N                    | This study |
| pFLAG-Spe- SSE1/K69Q                    | This study |
| pFLAG-Spe- SSE1/D174N                   | This study |
| pFLAG-Spe- SSE/D203N   | This study |
|------------------------|------------|
| pFLAG-Spe- SSE/G205D   | This study |
| pFLAG-Spe- SSE/G233D   | This study |
Table 2: Characteristics of mutant SSE1 alleles

| SSE1 allele | overexpression toxicity\(^{a}\) | ATP-agarose/agarose binding ratio\(^{b}\) | \(ydj1-151'^{s}\) suppression\(^{c}\) |
|-------------|-----------------|-----------------|-----------------|
| wild type   | +++             | 0.65            | +++             |
| D8N         | +++             | 1.0             | +               |
| K69Q        | +++             | 0.45            | +++             |
| D174N       | +               | 0.07            | –               |
| D203N       | ++              | 0.10            | ++              |
| G205D       | –               | < 0.05          | –               |
| G233D       | –               | < 0.05          | –               |
| PBD         | –               | N.D.            | –               |

*\(^{a}\) The degree of growth inhibition of W303 yeast was rated from none (-) to severe (+++).

*\(^{b}\) Data obtained from real-time luminescence counting of the blots shown in Fig. 3.

*\(^{c}\) The degree of growth of the \(ydj1-151'^{s}\) strain at 35°C as an indicator of genetic suppression was rated from none (-) to robust (+++).

ATP-agarose binding was similar to agarose binding, suggesting non-specific interaction.
A

B

PBD fragment:  --  I  II  III  IV
HA-Sse1_{1-393}:  +  +  +  +  +
A

$sse1\Delta$

HA-Sse1

vector

HA-ATPase$_{1-393}$

PBD$_{394-693}$

HA-ATPase$_{1-393}$ + PBD$_{394-693}$

30°C

B

|                | extract | IP (α-FLAG) |
|----------------|---------|-------------|
| FLAG-ATPase$_{1-393}$ | +       | +           |
| PBD$_{394-693}$       | +       | +           |

FLAG-ATPase

PBD

1 2 3 4
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