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Overexpression of Yeast Hsp110 Homolog Sse1p Suppresses *ydj1-151* Thermosensitivity and Restores Hsp90-dependent Activity

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The *Saccharomyces cerevisiae* heat-shock protein (Hsp)40, Ydj1p, is involved in a variety of cellular activities that control polypeptide fate, such as folding and translocation across intracellular membranes. To elucidate the mechanism of Ydj1p action, and to identify functional partners, we screened for multicopy suppressors of the temperature-sensitive *ydj1-151* mutant and identified a yeast Hsp110, *SSE1.* Overexpression of Sse1p also suppressed the folding defect of v-Src kinase in the *ydj1-151* mutant and partially reversed the α-factor translocation defect. *SSE1*-dependent suppression of *ydj1-151* thermosensitivity required the wild-type ATP-binding domain of Sse1p. However, the Sse1p mutants maintained heat-denatured firefly luciferase in a folding-competent state in vitro and restored human androgen receptor folding in *sse1* mutant cells. Because the folding of both v-Src kinase and human androgen receptor in yeast requires the Hsp90 complex, these data suggest that Ydj1p and Sse1p are interacting cochaperones in the Hsp90 complex and facilitate Hsp90-dependent activity.

INTRODUCTION

Molecular chaperones are required to catalyze a number of cellular activities, including protein folding, transport, and degradation. These activities can be highly interrelated. For example, chaperones that fail to refold denatured proteins may redirect the polypeptide to proteolytic machines in the cell (reviewed in Wickner *et al.*, 1999). In addition, chaperones can prevent premature folding or aggregation to deliver nascent polypeptides to translocation complexes localized at the endoplasmic reticulum (ER) or mitochondrial membranes (reviewed in Rassow and Pfanner, 1996; Rapoport *et al.*, 1999). Finally, specific molecular chaperones may deliver protein substrates to other chaperones. Notably, chaperone "bucket-brigades" were observed when the protein-folding activities of the DnaK-DnaJ-GrpE and GroEL/GroES complexes were first uncovered, and during the translocation and subsequent folding of proteins in the mitochondria (Manning-Krieg *et al.*, 1991; Langer *et al.*, 1992; reviewed in Frydman, 2001). In another example, the mammalian heat-shock protein (Hsp)110 molecular chaperone acts as a "holdase," binding to unfolded proteins and preventing their aggregation until ATP-dependent refolding is catalyzed by the Hsp70 and Hsp40 chaperones (Oh *et al.*, 1997). Not surprisingly, the expression of mammalian Hsp110 is induced by heat, and overexpression of Hsp110 confers thermotolerance to Rat-1 and HeLa cells. Subsequent studies determined that the holdase activity of mammalian Hsp110 requires its putative peptide-binding and C-terminal domains, but not the ATP-binding domain (Oh *et al.*, 1999).

The cytoplasmic Ydj1 protein in the yeast *Saccharomyces cerevisiae* is a member of the Hsp40 family of molecular chaperones (Caplan and Douglas, 1991; Atencio and Yaffe, 1992) that activate the ATPase activities of cognate Hsp70 chaperones through the J-domain, an ~70-amino acid motif that interacts directly with Hsp70 (reviewed in Cheetham and Caplan, 1998; Kelley, 1998). Regulation of Hsp70 ATPase activity in turn modulates the affinity and on/off rates of Hsp70 polypeptide clients. Some Hsp40s, like Ydj1p, can also bind directly to polypeptides (Cyr, 1995). Because of their multiple partners and biochemical activities, it is anticipated that a specific DnaJ homolog plays many critical roles in the cell. Consistent with this notion, yeast containing the *ydj1-151* temperature-sensitive mutation accumulate untranslocated preproteins in the cytoplasm at the nonpermissive temperature (Caplan *et al.*, 1992) and are defective for the translation of heterologous proteins (Brodsky *et al.*, 1998) and cyclin-dependent phosphorylation (Yaglom *et al.*, 1996).

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Ydj1 also appears to be important for Hsp90 function. For example, mutations in *YDJ1* compromise v-Src folding and the activity of steroid hormone receptors, both of which also require Hsp90 activity (reviewed in Caplan, 1999). Furthermore, mutation of both *YDJ1*- and Hsp90-encoding genes in the same cell results in a synthetic lethal defect (Kimura *et al.*, 1995), which suggests a functional interaction between the chaperones. Because Ydj1p and Hsp90 do not appear to form a stable bimolecular complex (Kimura *et al.*, 1995; Chang *et al.*, 1997), Hsp70 and other chaperones may "bridge" Hsp90 and Ydj1p. Consistent with this view, Hsp90 is recruited to some polypeptides via Hop, which binds to the ADP-bound form of Hsp70, and to which Ydj1p is also bound (Kosano *et al.*, 1998). Additionally, in recent work it has been shown that entry of the avian progesterone receptor into the Hsp90 chaperoning pathway is initiated by binding of the Hsp40 homologs Ydj1p or Hdj2p (Hernández *et al.*, 2002).

Whereas much information on the mechanism of Ydj1p action has been obtained from these genetic and biochemical studies, the Hsp110 family of molecular chaperones in yeast has been relatively ill-defined. Deletion of one of the genes encoding an Hsp110 chaperone in *S. cerevisiae*, known as *SSE1*, results in poor viability at every temperature examined (Mukai *et al.*, 1993; Shirayama *et al.*, 1993). Although it is not clear why Sse1p supports optimal cell growth, purified yeast Sse1p, like mammalian Hsp110, exhibits holdase activity (Brodsky *et al.*, 1999). In addition, Sse1p interacts biochemically and genetically with the yeast Hsp90 complex (Liu *et al.*, 1999), and yeast lacking the Hsp90 homologs Hsc82 (constitutively expressed at high levels and moderately stress inducible) and Hsp82 (constitutively expressed at low levels but heat inducible) are inviable (Borkovich *et al.*, 1989; Nathan and Lindquist, 1995). However, it is unknown whether holdase activity and/or the action of Sse1p on Hsp90-dependent functions are essential for cell growth.

To begin to address this question and to better characterize the yeast homolog of the Hsp110 family (reviewed in Easton *et al.*, 2000), we used both genetic and biochemical methods. First, we found that overexpression of Sse1p rescues the thermosensitivity of yeast containing the *ydj1-151* mutation. Purification and functional analyses of wild-type and mutant forms of Sse1p indicate that the presumptive ATP-binding domain is dispensable for the in vitro holdase activity of the chaperone. However, yeast overexpressing holdase-proficient Sse1p mutants cannot rescue the *ydj1-151* growth defect. These results indicate that the contribution of Sse1p to cell growth arises at least in part from facilitating Hsp90-dependent functions, and more generally that demonstrations of chaperone holdase activity might not correspond to an essential in vivo activity.

MATERIALS AND METHODS

Yeast Strains and Culture Conditions

Yeast strains used in this study were as follows: RSY607, *MATα ura3-52 leu2-3,112 PEP4::URA3*; W303, *MATα ade2-1 his3-11 leu2-3,112 ura3-1 trp1-1 can1-100*; ACY17b, *MATα ade2-1 his3-11 leu2-3,112 ura3-1 trp1-1 can 1-100 ydj1-2::HIS3 LEU2::ydj1-151* (Caplan *et al.*, 1992); E0020, *MATα his3 leu2 ura3 trp1 sse1::HIS3*; E0030, *MAT***a**/*MATα his3/his3 leu2/leu2 ura3/ura3 trp1/trp1 sse1::HIS3/sse1::URA3* (Shirayama *et al.*, 1993); and JGY014a, a haploid spore of E0030, *MAT***a** *his3 leu2 ura3 trp1 sse1::URA3* (this study). Standard media and methods for the growth, manipulation, and transformation of yeast were used (Kaiser *et al.*, 1994). For serial dilution assays, saturated liquid cultures grown at 26°C in synthetic complete medium lacking uracil (SC-ura) were adjusted to an optical density measured at 600 nm (OD_{600}) of 3.2, serially diluted 10-fold in sterile water, plated on SC-ura plates containing either galactose or glucose as the carbon source, and incubated at the indicated temperatures for 4 d. Yeast lysates for immunoblot analysis were prepared as described previously (Brodsky *et al.*, 1998).

Genetic and Molecular Methods

To screen for multicopy suppressors of the *ydj1-151* temperature-sensitive phenotype, strain ACY17b was transformed by the lithium-acetate method with a *URA3*-marked yeast genomic library in plasmid YEp24 (Carlson and Botstein, 1982), obtained from Dr. John Woolford (Carnegie Mellon University, Pittsburgh, PA). Transformants were selected for growth on SC-ura at 26°C and replicates were incubated at either 26 or 37°C for 2 d. Approximately 22,000 transformants were screened, and plasmids from 60 strains in which suppression of the temperature sensitivity of *ydj1-151* was apparent were reisolated and retransformed into ACY17b to verify suppression. Of these, 13 plasmids were obtained that suppressed the *ydj1-151* temperature sensitivity upon retransformation and that fell into three distinct groups as assayed by restriction digest. DNA sequence analysis of a representative insert from each group was performed using the Sequenase kit (Amersham Biosciences, Piscataway, NJ) and revealed that the common open reading frame (ORF) in each clone was *SSE1* (Figure 1A). Suppression of *ydj1-151* temperature sensitivity by *SSE1* was verified by subcloning a *Bam*HI-*Cla*I fragment containing *SSE1* from one of the original, isolated clones (pJG8.1) into the high copy vector pRS426 (Sikorski and Hieter, 1989) to create pJG816. Retransformation of pJG816 into yeast strain ACY17b resulted in growth at permissive and semipermissive temperatures (Figure 1B). *SSE1* on a single copy *CEN* vector was created by introducing the ~2.1-kb *Bam*HI-*Sal*I fragment from pJG816 into pRS314 (Sikorski and Hieter, 1989).

To construct plasmids for the expression and purification of wild-type and mutant hexahistidine-tagged forms of Sse1p, an *SSE1*-containing DNA fragment flanked by *Bam*HI and *Xba*I sites was generated by polymerase chain reaction (PCR) amplification from pJG816 by using primers 5′ CATTTGGATCCAAGATGAGTACTCCATTTGGTTTAG 3′ and 5′ GCTGCCTGCAGATTAGTCCATGTCAAC 3′, the PCR product was treated with *Bam*HI and *Xba*I, gel-purified using the QiaQuick kit (QIAGEN, Valencia, CA), and the fragment was cloned into pTrcHisA (Invitrogen, Carlsbad, CA), resulting in addition of a hexhistidine (His_6) tag to the N-terminus of *SSE1*. The tagged *SSE1* was then excised from pTrcHisA by digestion with *Nco*I, the overhangs were filled in with Klenow (Roche Applied Science, Indianapolis, IN), and the DNA was digested with *Kpn*I and gel purified as described above. The resulting DNA fragment was ligated into the high copy, galactose-inducible expression vector pYES2 (Invitrogen) that had been digested with *Hin*dIII, filled in with Klenow, and similarly digested with *Kpn*I. This resulted in the construction of pJG010 (P_{GAL}-His_6*SSE1*).

Plasmids containing site-directed mutations in *SSE1* were PCR amplified from pJG010 by using the QuickChange kit (Stratagene, La Jolla, CA). The primers used to construct pJG015 (P_{GAL}-His_6*SSE1–1*) were 5′ CCAAACCAATAATTCTTTGCAAGTTGGCGACAGTG 3′ and 5′ CACTGTCGCCAACTTGCAAAGAATTATTGGTTTGG 3′, resulting in amino acid substitution K69Q. To construct pJG026 (P_{GAL}-His_6*SSE1-2*) primers 5′CGAAGTCCCTACCATCAAAATGCTTGTCGCAGGC 3′ and 5′GCCTGCGACAAGCATTTTGATGGTAGGGACTTCCG 3′ were used, resulting in amino acid substitution G233D. Primers 5′ CGCCTCTAGATTAGTCCATGTCAACATCACC 3′ and 5′ GCGGATCCATGCATCATCATCATCATCATGGGTGCCGCCTTTATTTGCGCCATTCACTCTCC 3′ were used to construct the His_6-tagged truncation mu-

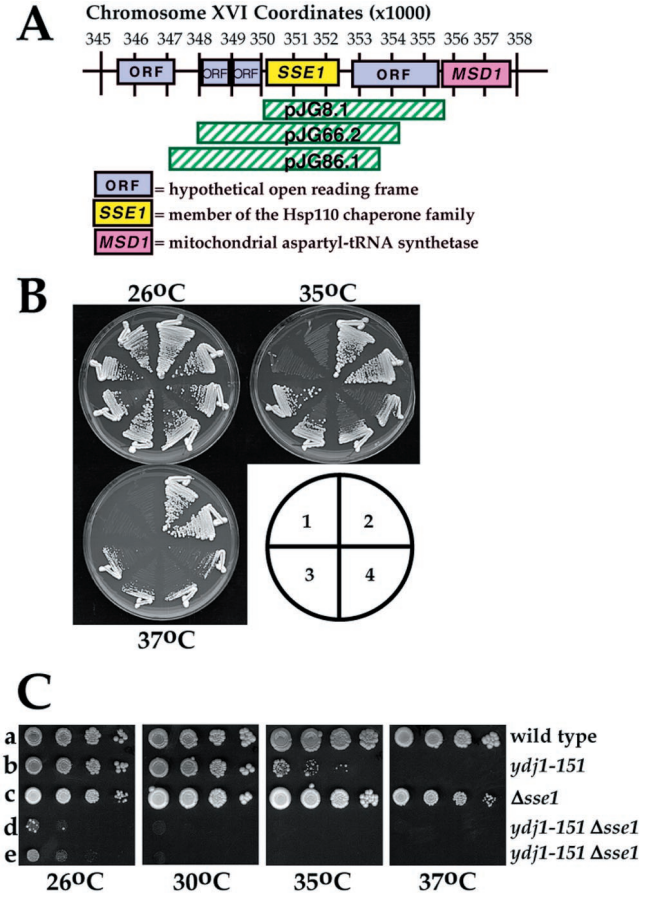

Figure 1. *SSE1* is a multicopy suppressor of *ydj1-151* temperature sensitivity and interacts with *YDJ1*. (A) Map of chromosome XVI in *S. cerevisiae* depicting the approximate positions of the DNA inserts that rescued *ydj1-151* thermosensitivity, all of which included *SSE1*. (B) *Ydj1-151* cells were transformed with a multicopy YEp24 yeast vector either 1) lacking or 2) containing *YDJ1*, 3) pJG8.1 (original complementing clone), or 4) pJG816 (*SSE1*) were struck onto SC-ura medium and were incubated at the indicated temperatures for 4 d. The growth of two transformants from each strain is shown. (C) Serial dilutions of W303 wild-type parental yeast (a), ACY17b *ydj1-151* cells (b), JGY014a *sse1*Δ cells (c), or yeast deleted for *SSE1* and containing the *ydj1-151* allele (d and e) were spotted onto complete medium and incubated at the indicated temperatures for 4 d.

tant (Sse375-694p) lacking the ATP binding domain (pSse CTD). The resulting mutations were confirmed by DNA sequence analysis.

Diploid yeast containing one copy of the *ydj1-151* allele and deleted for one copy of *SSE1* were obtained from a cross between ACY17b and JGY014a. Sporulation was induced and haploid progeny were obtained and analyzed genetically using established methods (Kaiser *et al.*, 1994).

Antibodies

An anti-Sse1p antiserum was generated by Cocalico Biologicals (Reamstown, PA) against a peptide corresponding to amino acids 663–684 in Sse1p and that included a cysteine added at the C terminus (AAMAEKLAAQRKAEAEKKEEKKC; synthesized by the University of Pittsburgh Peptide Synthesis Facility, Pittsburgh, PA) and to which bovine serum albumin was conjugated using 4-succinimidyloxycarbonyl-methyl-α[2-pyridyldithio]toluene (Pierce Chemical, Rockford, IL). Antibodies against prepro-α factor (ppαF) were obtained from Dr. Randy Schekman (University of California, Berkeley, Berkeley, CA), and antibodies against yeast BiP (Brodsky and Schekman, 1993), Ydj1p (Caplan and Douglas, 1991), and Sec61p (Stirling *et al.*, 1992) were described previously. Polyclonal antibody against Hsp90 was a kind gift from Dr. Susan Lindquist (Massachusetts Institute of Technology, Cambridge, MA); anti-penta-histidine antibody was from QIAGEN, and anti-phosphotyrosine and anti-Src antibodies were obtained from Upstate Biotechnology (Lake Placid, NY).

Pulse-Chase and Immunoprecipitation Assays

The accumulation of the precursor form of the yeast mating prepheromone (ppαF) was used to assay for a posttranslational translocation defect (Deshaies and Schekman, 1987; Caplan *et al.*, 1992). In brief, *ydj1-151* mutant yeast were transformed with either vector (pRS426) or a high copy plasmid containing *SSE1* (pJG816), grown at 26°C, and then radiolabeled with 0.1 mCi/ml of "EXPRE^{35}S^{35}S" ^{35}S-Methionine and Cysteine Labeling Mix (PerkinElmer Life Sciences, Boston, MA) at either 26 or 37°C for 10 min. Labeling was stopped by the addition of cycloheximide to a final concentration of 0.2 mg/ml, aliquots were removed at the indicated time points and extracts were prepared as described previously (Morrow and Brodsky, 2001). Anti-ppαF or anti-Kar2p (BiP) antibody was added to extracts containing ~5×10^6 cpm of ^{35}S, and the mixture was incubated overnight at 4°C before immune complexes were precipitated for 2 h at room temperature with protein A-Sepharose CL-4B (Amersham Biosciences) in IP wash buffer (50 mM Tris-Cl pH 7.4, 0.2% SDS, 150 mM NaCl, 1% Triton X-100, and 5 mM EDTA). The resulting immunoprecipitates were washed (Rothblatt *et al.*, 1989), the proteins were resolved by SDS-PAGE, and results were obtained by PhosphorImager analysis (Fuji Medical Systems, Stamford, CT).

Analysis of v-Src Activity in Yeast

v-Src activity was assayed essentially as described (Dey *et al.*, 1996). Yeast cultures (100 ml) were grown at 30°C in minimal medium containing 2% raffinose (wt/vol) to OD_{600} = 0.2, and v-Src expression was induced by the addition of 2% galactose (wt/vol). After a 6-h induction period, cells were harvested and resuspended in protein extraction buffer (20 mM HEPES pH 7.5, 100 mM KCl, 0.1 mM EDTA pH 7.5, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml protease inhibitor cocktail). The resuspended cells were broken in the presence of 1.5 g of 0.4-mm-diameter glass beads by two 60-s bursts in a mini-BeadBeater (Biospec Products, Bartlesville, OK) at 4°C, with cooling on ice for 60 s between bursts. The crude lysate was cleared at 14,000 × g for 10 min at 4°C. Protein concentrations were measured using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA).

The levels of tyrosine phosphorylation and v-Src protein were assayed using anti-phosphotyrosine and anti-Src monoclonal antibodies. Lysates (20 μg of total protein) were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Filters were washed briefly with Tween 20/Tris-buffered saline (TTBS) (20 mM Tri-HCl pH 7.5, 0.5 M NaCl, and 0.05% Tween 20) and blocked overnight with TTBS containing 5% nonfat dry milk. Filters were incubated with the respective primary antibodies diluted 1:1000 in antibody dilution buffer (1× phosphate-buffered saline, 3% bovine serum albumin, 0.05% Tween 20, and 0.1% thimerosal) for 2 h. Filters were washed three times for 10 min in TTBS. Filters were then treated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G [for anti-phosphotyrosine and anti-Src] or goat anti-rabbit [for anti-Ydj1]) for 1 h, and washed and treated with the Super Signal West Pico chemiluminescence reagent (Pierce Chemical) and exposed to x-ray film.

Viability of the strains expressing galactose-regulated v-Src was analyzed by inoculating a sample of log phase cultures onto medium containing either glucose or galactose and incubating the plates at 30°C for 3 d.

Androgen Receptor-Hormone Binding Assay

Yeast cells were grown in selective medium containing 2% galactose (wt/vol) to an OD_{600} = 0.2 at 30°C, and 1-ml aliquots were used for each assay. The binding was performed essentially as described (Caplan *et al.*, 1995). In brief, [^{3}H]R1881 (DuPont, Wilmington, DE) diluted 1:5 with unlabeled R1881 to a final concentration of 100 nM plus or minus 20 μM of unlabeled R1881 (200-fold excess) was added to the cells, which were incubated for 90 min at 25°C with shaking, washed three times with ice-cold water, and the amount of associated radioactivity was assessed in 10 ml of scintillation fluid. Nonspecific counts were calculated from the samples containing 200-fold excess of unlabeled R1881 and subtracted from the radioactivity for samples incubated in the absence of excess hormone.

Purification of Sse1p

Sse1p and the mutant and truncated versions of this protein were purified from yeast by using the galactose-inducible expression vectors described above. Yeast strain W303 was transformed with the appropriate plasmid, cells were grown overnight at 26°C in 1 liter of SC-ura containing 2% raffinose as the carbon source, washed, and then transferred to 4 liters of SC-ura containing 2% galactose. Cells were harvested in mid/late-log phase (OD_{600} of ~2.5) and were converted to spheroplasts (Brodsky and Schekman, 1993). Spheroplasts were resuspended in 25 ml of ice-cold lysis buffer (50 mM HEPES pH 7.4, 300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each pepstatin A and leupeptin), an equal volume of glass beads was added, and the cells were agitated with four 1-min pulses on a Vortex mixer at the highest setting with cooling of the sample on ice between each pulse. The lysate was cleared by a 5-min centrifugation at 5,000 $\times$ *g*, followed by a 10-min centrifugation at 11,000 $\times$ *g*, and the final supernatant was applied to a 5-ml Ni^{2+}-nitrilotriacetic acid-agarose column (QIAGEN). The column was washed with 5 volumes of lysis buffer and then sequentially with 5 volumes of lysis buffer containing each of the following: 1) 1% Triton, 2) 5 mM ATP, and 3) 1 M NaCl. Bound proteins were eluted with lysis buffer containing 500 mM imidazole. Peak fractions containing His_6-tagged protein as assessed by SDS-PAGE and staining with Coomassie brilliant blue, and by immunoblot analysis using anti-Sse1p antibody were diluted 1:3 with buffer 88 (20 mM HEPES pH 6.8, 150 mM KOAc, 5 mM MgOAc, and 250 mM sorbitol) and applied to a 5-ml Q-Sepharose column (Amersham Biosciences). The column was washed with buffer 88 containing 0.4 M KOAc and eluted with a 12- $\times$ 12-ml gradient of buffer 88 containing 0.4 M KOAc to buffer 88 containing 2 M KOAc. Fractions containing the purified proteins as determined by SDS-PAGE and staining with Coomassie brilliant blue were dialyzed against 1000 volumes of dialysis buffer (50 mM Tris-Cl pH 7.4, 50 mM NaCl, 0.8 mM dithiothreitol, 2 mM $MgCl_2$, and 5% vol/vol glycerol) overnight at 4°C, and the protein in the dialysate was concentrated in Centricon 30 microconcentrators (Millipore, Bedford, MA) following the manufacturer's instructions. Protein concentration was determined using the Bio-Rad Protein Assay reagent, with bovine serum albumin as the standard. The purity of each protein was estimated to be >95% and aliquots of the purified proteins were snap-frozen in liquid nitrogen and stored at −80°C.

In Vitro Luciferase Refolding and Aggregation Assays

The holdase activity of Sse1p was assessed both by following its ability to retain thermally denatured firefly luciferase in a folding competent conformation, which upon addition of cytosol and ATP resulted in active luciferase (Oh *et al.*, 1997; Brodsky *et al.*, 1999), and by its ability to temper the aggregation of thermally inactivated luciferase as assessed by spectroscopy (Oh *et al.*, 1997). In brief, the folding assay was assembled by denaturing 150 nM of firefly luciferase (Sigma-Aldrich, St. Louis, MO) in luciferase assay buffer (25 mM HEPES pH 7.8, 5 mM MgOAc, 50 mM KCl, 5 mM β-mercaptoethanol, and 1 mM ATP) by heating to 42°C in the absence or presence of the indicated molar ratio of purified wild-type or mutant Sse1p for 30 min. Refolding was then assayed in an Analytical Luminescence Laboratory luminometer (BD PharMingen, San Diego, CA) with luciferin (BD PharMingen) as the substrate upon the addition of an ATP-regenerating system and cytosol at 26°C as published (Brodsky *et al.*, 1999). Triplicate aliquots were removed at the indicated time points, and a parallel reaction was "mock denatured" at 26°C and the level of luciferase activity in this control reaction was set to 100%. To assay luciferase aggregation, a final concentration of 1.9 μM luciferase was preincubated in luciferase assay buffer or at a 1:5 molar ratio of Sse1p in buffer at room temperature for 15 min before it was diluted 12.5-fold into prewarmed (42°C) luciferase assay buffer and stirred. The absorbance was measured at 320 nm on a 14DS UV-VIS-IR spectrophotometer (AVIV, Lakewood, NJ) with a water-jacketed cuvette holder equilibrated to 42°C.

RESULTS

SSE1 *Overexpression Suppresses the* ydj1-151 *Temperature-sensitive Growth Defect*

To better understand how Ydj1p exerts its pleiotropic effects in the cell, we initiated a genetic screen to isolate multicopy suppressors of the *ydj1-151* temperature-sensitive phenotype. As described in MATERIALS AND METHODS, three classes of complementing clones were isolated, each containing the *SSE1* ORF; retransformation of only the *SSE1* ORF on a multicopy plasmid also rescued *ydj1-151* thermosensitivity (Figure 1B), as did the introduction of *SSE1* on a *CEN* single copy vector (our unpublished data). In either case, the expression of Sse1p was approximately three- to fourfold greater than endogenous levels as determined using anti-Sse1p antiserum (our unpublished data). *SSE1* encodes one of the two yeast Hsp110s, but only Sse1p is required for optimal cell growth at all temperatures (Mukai *et al.*, 1993; Shirayama *et al.*, 1993). Pertinent to this study, Sse1p has been found associated with the yeast Hsp90 complex (Caplan, 1999; Liu *et al.*, 1999), and strains deleted for *SSE1* are hypersensitive to Hsp90 inhibitors and defective for glucocorticoid receptor function (Liu *et al.*, 1999).

To examine whether *SSE1* and *YDJ1* interact, we mated a strain lacking the Sse1p-encoding gene ("*sse1*Δ") with *ydj1-151* cells, selected for diploids, and then dissected haploid progeny after nitrogen starvation. We then compared the growth characteristics of an isogenic wild-type strain, *sse1*Δ and *ydj1-151* cells, and two progeny containing both mutations (Figure 1C). At every temperature examined, a strong synthetic effect on the growth of *sse1*Δ *ydj1-151* cells compared with the single mutant and wild-type strains was observed. Such synthetic interactions suggest that the *YDJ1* and *SSE1* gene products interact and/or that they function in similar pathways.

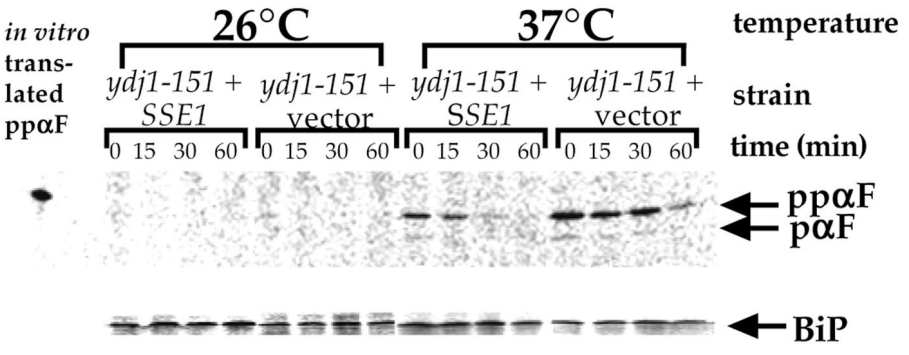

Figure 2. *SSE1* partially suppresses the ppαF translocation defect in *ydj1-151* yeast. *Ydj1-151* yeast containing either pJG816 or a vector lacking insert were labeled with [^{35}S]methionine and chased for the indicated times at either 26 or 37°C, and ppαF and BiP were immunoprecipitated from cell extracts as described in MATERIALS AND METHODS. The positions of ppαF that migrates similarly to in vitro translated ppαF in the first lane and pαF are indicated. BiP serves as a control for the efficiencies of the radiolabeling and immunoprecipitation in this experiment. The decrease in ppαF intensity over time arises from translocation and intracellular degradation of the accumulated material.

Effect of Sse1p Overexpression on Prepro-α Factor Translocation and v-Src Folding in ydj1-151 Yeast

Previous studies showed that Ydj1p is important for the posttranslational translocation of the yeast mating prepheromone ppαF into the ER (Caplan *et al.*, 1992). Thus, we analyzed whether overexpression of Sse1p in *ydj1-151* yeast suppressed the ppαF translocation defect by using a pulse-chase and immunoprecipitation approach (see MATERIALS AND METHODS). A pulse-chase analysis was conducted in *ydj1-151* cells containing either the Sse1p overexpression vector or a vector lacking insert at permissive (26°C) and nonpermissive (37°C) temperatures, and the fate of ppαF was followed. As shown in Figure 2, ppαF accumulated in the *ydj1-151* strain shifted to 37°C regardless of whether or not Sse1p was overexpressed. However, the level of ppαF that accumulated was lowered two- to eightfold when the *SSE1* encoding vector was present, suggesting a limited rescue of this phenotype. The defect in ppαF translocation in yeast containing a temperature-sensitive mutation in Ssa1p, *ssa1-45* (Becker *et al.*, 1996), was not rescued by overexpression of Sse1p (our unpublished data).

We also tested whether Sse1p overexpression rescued the v-Src folding defect in *ydj1-151* yeast. Expression of v-Src protein leads to tyrosine phosphorylation of many yeast proteins, but this is completely abolished in the *ydj1-151* and several other *ydj1* mutants (Kimura *et al.*, 1995; Dey *et al.*, 1996). Because the endogenous levels of phosphotyrosine are low in yeast, v-Src activity can be measured by immunoblot analysis by using an anti-phosphotyrosine monoclonal antibody. In the absence of v-Src expression, this antibody appears unreactive when incubated with a whole cell lysate (Figure 3A, lane 9). In contrast, upon v-Src expression in wild-type cells, many yeast proteins become phosphorylated on tyrosine, resulting in the presence of multiple, phosphorylated substrates (Figure 3A, lanes 1–4). In the *ydj1-151* strain, there is no visible phosphotyrosine activity and v-Src is undetectable (Figure 3A, lane 5). However, the introduction of *SSE1*-encoding plasmids completely restored both v-Src levels and the phosphotyrosine activity (Figure 3A, e.g. compare lanes 5 and 6). We conclude that Sse1p overexpression rescues the v-Src folding defect in the *ydj1-151* mutant.

We corroborated these data by examining the effect of v-Src expression on the viability of the *ydj1-151* strain (Dey *et al.*, 1996). Expression of v-Src in wild-type cells is lethal regardless of whether Sse1p is overexpressed (Figure 3B, lanes 3 and 4). In contrast, growth is apparent in *ydj1-151* yeast expressing v-Src when the *SSE1*-encoding plasmid is lacking (Figure 3B, lane 2), but expression of v-Src is lethal when the *SSE1*-encoding plasmid is present (Figure 3B, lane

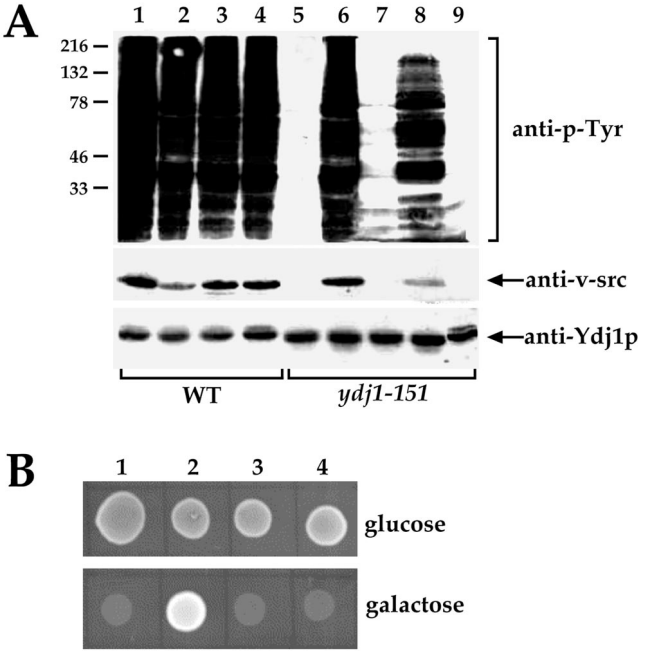

Figure 3. *SSE1* rescues v-Src activity in the *ydj1-151* mutant. (A) Wild-type and *ydj1-151* cells were transformed with a single copy vector (pRS314) either harboring (lanes 2 and 6) or lacking *SSE1* (lanes 1 and 5), or a multicopy vector (pRS424) either harboring (lanes 4 and 8) or lacking *SSE1* (lanes 3 and 7). All cells also contained a galactose-inducible v-Src expression vector, except those in lane 9, which contained only pRS424-*SSE1*. Cells were grown at 30°C, and extracts were prepared and analyzed for phosphotyrosine (anti-p-Tyr) and v-Src (anti-v-src) levels as described in MATERIALS AND METHODS. Ydj1p serves as a loading control. (B) *ydj1-151* (lanes 1 and 2) or wild-type (lanes 3 and 4) yeast transformed with the pRS314-*SSE1* expression vector (lanes 1 and 3) or the vector lacking the *SSE1* insert (lanes 2 and 4) and containing the galactose-regulated v-Src expression vector were inoculated onto glucose- or galactose-containing medium and incubated at 30°C for 3 d.

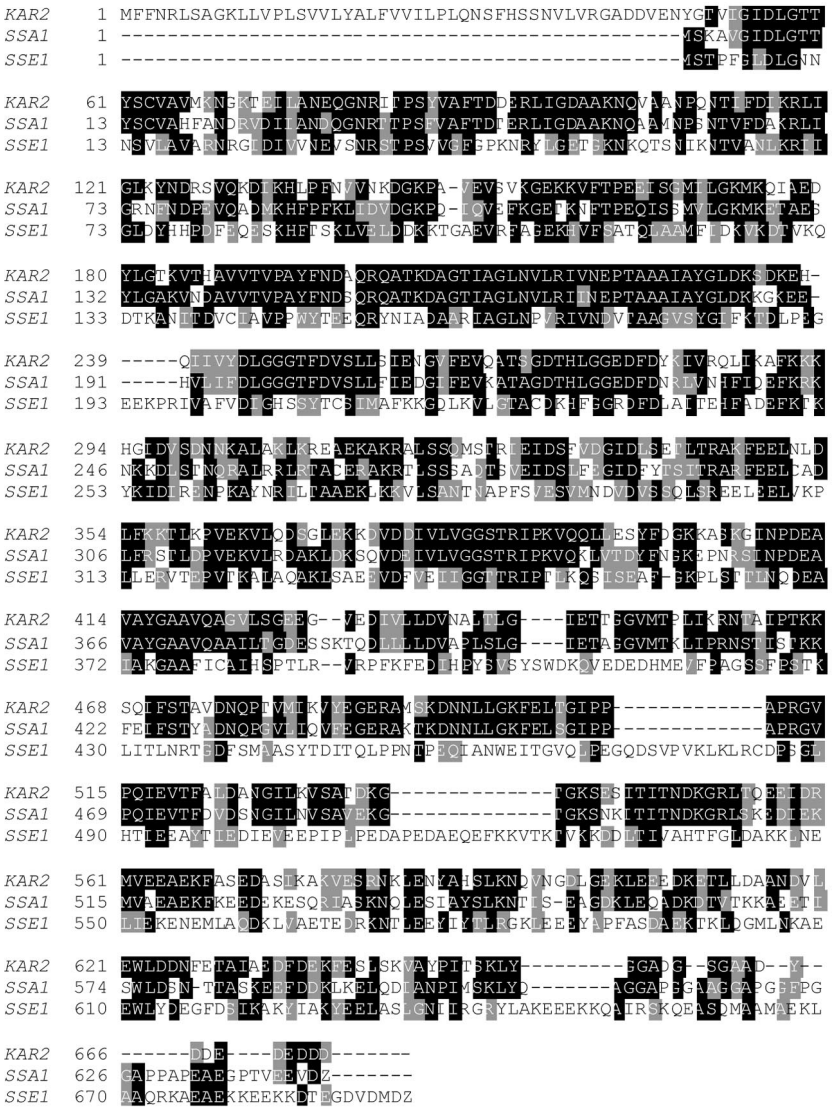

Figure 4. Amino acid sequence alignment of the Kar2p, Ssa1p, and Sse1p molecular chaperones. Protein coding sequences were aligned using CLUSTALW (Thompson *et al.*, 1994), and blackened and shaded residues correspond to positions containing at least two identities or two similarities, respectively. Dashes indicate gaps in the sequence alignment. The *sse1-1* mutation is K69Q in the *SSE1* numbering system, the *sse1-2* mutation is G233D, and the Sse1p CTD starts at position 375.

1), indicating further that overexpression of Sse1p rescues a *ydj1-151* physiological defect.

Rescue of ydj1-151 *Temperature Sensitivity Requires the ATP-binding Domain of Sse1p*

The data presented above show that *SSE1* rescues the Ydj1 requirement for v-Src activity but is less able to suppress a posttranslational translocation defect. We suggest that Sse1p substitutes for Ydj1p in a restricted manner but does not globally bypass Ydj1p function. Consistent with this view is the finding that *SSE1* overexpression cannot suppress the temperature-sensitive lethal phenotype of a *ydj1*Δ mutant (Fewell and Brodsky, unpublished data). Because Sse1 is an Hsp70-like chaperone that interacts with Hsp90, the suppression of the *ydj1-151* mutant defects may be restricted to Hsp90-dependent function (Figure 3). In one scenario, Ydj1-151p might become stabilized or functional in the Hsp90 complex only when multiple copies of Sse1p are present. Furthermore, by virtue of its homology to the Ssa1p hsp70, which interacts with Ydj1p in an ATP-dependent manner (Caplan *et al.*, 1992; Cyr and Douglas, 1994; Becker *et al.*, 1996; Lu and Cyr, 1998; McClellan and Brodsky, 2000), the ATP-binding domain of Sse1p may also be essential for *ydj1-151* growth at elevated temperatures.

We have been unable to show that Sse1p exhibits ATPase activity (our unpublished data), although Sse1p and mammalian Hsp110 are predicted to possess the structural elements necessary for ATP binding. However, these motifs are poorly conserved with respect to Hsp70 and Grp170, and it remains unclear whether the ATP-binding domain is required for all Sse1p/Hsp110 activities (Chen *et al.*, 1996; Oh *et al.*, 1999; Easton *et al.*, 2000). Thus, to examine whether the nucleotide-binding domain of Sse1p is important for the suppression of *ydj1-151* thermosensitivity, we site-directed amino acid substitutions at conserved residues found in

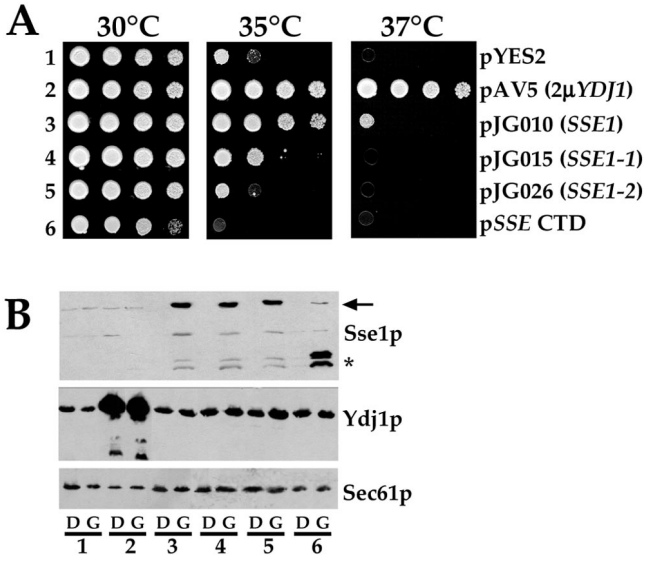

Figure 5. *sse1* mutants are unable to rescue *ydj1-151* thermosensitivity. (A) Cultures of *ydj1-151* yeast transformed with a galactose-inducible expression vector lacking an insert (1), or containing the gene encoding wild-type Sse1p (3), Sse1-1p (4), Sse1-2p (5), or the Sse1p-CTD (6) were spotted onto SC-ura medium supplemented with galactose and incubated for 4 d at the indicated temperatures. Lane 2 contains *ydj1-151* yeast with a multicopy, constitutive *YDJ1* expression vector (pAV5). (B) Overnight cultures of each strain were grown in SC-ura supplemented with either glucose (D) or galactose (G) at 26°C, cell extracts were prepared, and immunoblots were analyzed using anti-Sse1p, anti-Ydj1p, or anti-Sec61p antiserum. The arrow indicates the migration of full-length Sse1p, and the asterisk denotes the positions of Sse1p CTD (upper band) and a degradation product (lower band). The numbers correspond to those in A.

Sse1p (K69Q and G223D) and the yeast cytoplasmic and luminal Hsp70 molecular chaperones Ssa1p and BiP, respectively (Figure 4; Craven *et al.*, 1997; Easton *et al.*, 2000); strains expressing Ssa1p and BiP containing these mutations are defective for ATP hydrolysis and accumulate untranslocated ppαF, and expression of the BiP mutants is dominantly lethal (McClellan *et al.*, 1998; McClellan and Brodsky, 2000). We also deleted the ATP-binding domain (residues 1–374) in Sse1p and introduced a new translation start site to express the C-terminal domain (CTD) of Sse1p. Galactose-inducible expression constructs either lacking or containing wild type or the Sse1p mutants were transformed into the *ydj1-151* mutant, transformants were restruck, and overnight cultures were serially diluted onto selective medium containing galactose (Figure 5A). As anticipated, *ydj1-151* yeast grew poorly at 37°C (Figure 5A, row 1) unless they contained a multicopy vector that constitutively expressed wild-type Ydj1p (Figure 5A, row 2). We found that cells containing the wild-type *SSE1* construct, but not the *sse1* mutant constructs, grew significantly better on galactose-containing medium at 35 and 37°C (Figure 5A, compare row 3 with rows 4–6). To verify that the wild-type and mutant proteins were expressed, total protein extracts were prepared from each strain and the levels of Sse1p, Ydj1p, and the ER membrane protein Sec61p (as a loading control) were determined by immunoblot analysis by using anti-Sse1p, anti-Ydj1p, and anti-Sec61p antisera, respectively. As displayed in Figure 5B, endogenous Sse1p was barely visible unless cells contained wild-type (lane 3) or the Sse1p mutants (lanes 4–6) on the galactose-inducible expression vector and were grown on galactose (G). In this experiment, the steady-state levels of Ydj1-151p in the cells appeared constant, regardless of whether wild-type or mutant Sse1p was expressed. In other experiments, expression of the Sse1p mutants reduced the steady-state levels of Ydj1-151p approximately twofold (our unpublished data; see DISCUSSION); as anticipated, significantly greater amounts of Ydj1p were present when a constitutive 2-μ (YEp24-based) Ydj1p-expression vector was present (Figure 5B, lane 2).

Although the Sse1-1 (K69Q), Sse1-2 (G233D), and CTD Sse1p mutant proteins were expressed in vivo, it was possible that they were inactive. To examine whether this was the case, we asked whether expression of the mutants in an *sse1*Δ background rescued the binding of a synthetic hormone to the androgen receptor heterologously expressed in yeast, because androgen receptor folding in yeast requires Hsp90 complex activity (Fang *et al.*, 1996). First, we showed that wild-type cells lacking androgen receptor exhibited background levels of hormone binding (Figure 6, lane 1), whereas *sse1*Δ yeast expressing wild-type *SSE1* bound significant amounts of hormone when the receptor was present (lane 2). In contrast, *sse1*Δ mutant cells containing a vector lacking the insert showed an approximate twofold decrease in hormone binding, but maximal binding was restored by the expression of the Sse1-1p and Sse1-2p mutants. We note that the steady-state levels of the Sse1p mutants in this experiment were higher than wild type; thus, it is possible that restoration of maximal hormone binding required greater amounts of the mutant than wild-type protein. Nevertheless, because the Sse1-1 and Sse1-2 mutant proteins were unable to rescue *ydj1-151* thermosensitivity when the same expression system was used (Figure 5A), we conclude that the mutant proteins are at least partially active in vivo, but that this activity is insufficient to rescue *ydj1-151* defects. In contrast, expression of the Sse1p CTD failed to restore hormone binding in *sse1*Δ cells (Figure 6B), and *ydj1-151* yeast expressing the CTD fragment grew less well than *ydj1-151* yeast containing an empty vector (Figure 5A), suggesting that this mutant may be partially dominant in these backgrounds. Overall, these data suggest that a wild-type ATP-binding domain of Sse1p is important for restoration of growth of *ydj1-151* yeast at the nonpermissive temperature, but that the ATP-binding domain may not be required for Hsp90 complex function.

Sse1 Mutant Proteins Retain a Thermally Denatured Polypeptide in a Folding-competent Conformation

We previously demonstrated that purified, wild-type Sse1p "holds" thermally denatured firefly luciferase in a conformation that facilitates cytosol-dependent refolding (Brodsky *et al.*, 1999). Others have found that both wild-type and the C-terminal halves of the mammalian Hsp110, which lacks the ATP-binding domain, exhibit holdase activity (Oh *et al.*, 1997, 1999), suggesting that the ATP-binding domain is dispensable. To determine whether our Sse1 mutant proteins were similarly holdase proficient, galactose-inducible, hexahistidine-tagged forms of wild-type and the mutant Sse1

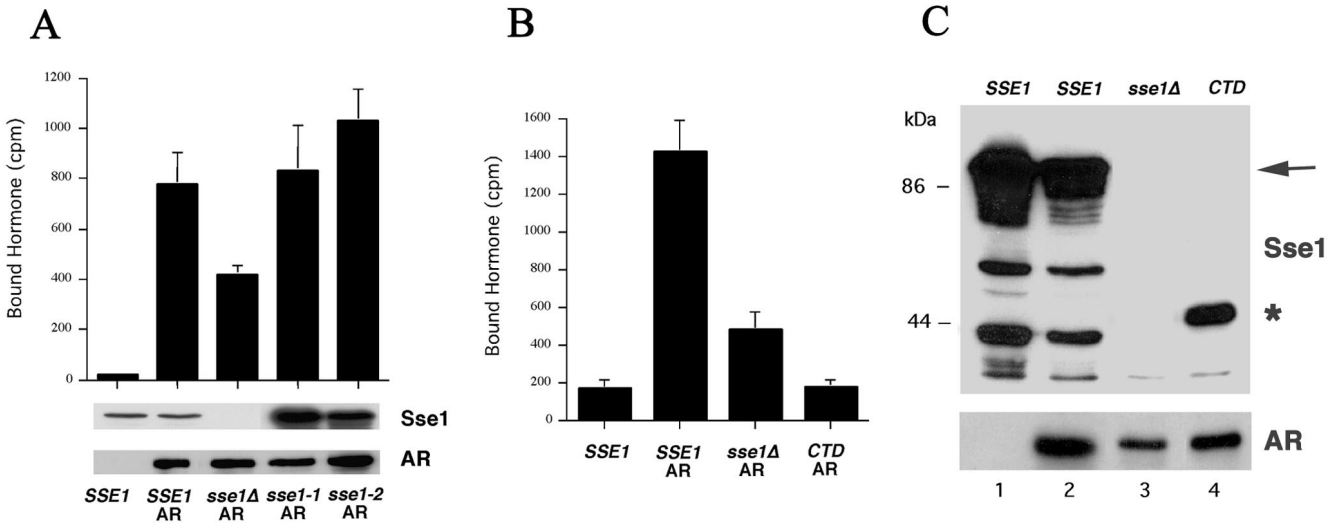

Figure 6. Sse1p mutants restore hormone binding to *sse1*Δ cells expressing androgen receptor. (A) Wild-type (*SSE1*) or *sse1*Δ mutant cells either lacking or containing the androgen receptor expression vector (AR) and/or galactose-inducible expression vectors for wild-type Sse1p (*SSE1*), or the Sse1-1 (*sse1-1*), Sse1-2 (*sse1-2*), or Sse1 CTD (*CTD*) proteins, or the vector lacking an insert (*sse1*Δ) were assayed for binding to synthetic hormone (R1881) as described in MATERIALS AND METHODS. Top, data represent the means of three independent determinations, ± SD. Bottom, immunoblot analyses using anti-Sse1p and anti-androgen receptor antisera. (B) In a separate experiment, the binding of hormone to *sse1*Δ cells expressing wild-type *SSE1* or the Sse1p CTD was examined. Here, the CTD exerted a negative effect on hormone binding (binding was lower than in yeast lacking Sse1p). (C) An immunoblot of extracts from the strains used in B probed with anti-Sse1p (top) and anti-AR (bottom) antisera. The arrow indicates the migration of full-length Sse1p, and the asterisk denotes the position of Sse1p CTD.

proteins were expressed in yeast and purified as described in the MATERIALS AND METHODS.

Although we had determined that Sse1p holds thermally denatured firefly luciferase in a folding-competent conformation (Brodsky *et al.*, 1999), the ATP dependence of refolding and at what molar ratio maximal holdase activity was evident were not explored. As shown in Figure 7A, we found that addition of yeast cytosol and an ATP-regenerating system permitted ~60% of the luciferase to refold that had first been denatured at 42°C in the presence of a 10-fold molar excess of Sse1p. In contrast, minimal refolding was apparent either if Sse1p or ATP was absent from the thermal denaturation or during the refolding reaction, respectively. The requirement for ATP is consistent with our previous data indicating that *ssa1* mutant cytosol is defective for luciferase refolding (Brodsky *et al.*, 1999) and that mammalian Hsc70 (an Ssa1p homolog) and Hdj-1 (a DnaJ homolog) in the presence of ATP are sufficient to refold Hsp110-held luciferase (Oh *et al.*, 1997).

Next, we titrated the molar ratio of Sse1p to luciferase during the 42°C denaturation before cytosol and ATP were added and found that maximal refolding occurred at an Sse1p:luciferase ratio of 5–10:1 (Figure 7B). We then compared the activities of the Sse1-1p (K69Q), Sse1-2p (G233D), CTD Sse1p mutant, to wild-type Sse1p in this assay. As shown in Figure 7C, we found that the activities of the mutant proteins were indistinguishable from wild-type Sse1p. Finally, we assayed whether wild-type Sse1p, Sse1-1p, Sse1-2p, and the CTD of Sse1p reduced firefly luciferase aggregation upon thermal denaturation. Addition of Sse1p to the cuvette at a 5:1 molar ratio to luciferase resulted in approximately a 41% reduction relative to a reaction lacking Sse1p, and the Sse1-1 and Sse1-2 mutant proteins yielded values of 35 and 48%, respectively; surprisingly, the Sse1p CTD was less efficient at preventing luciferase aggregation than the full-length proteins (17%; Figure 7D), even though it acted as an efficient holdase (Figure 7C). An explanation for this phenomenon is provided in DISCUSSION. As a negative control, we found that bovine serum albumin was without effect in this assay (our unpublished data). These combined data indicate that the ATP-binding domain of the yeast Hsp110 homolog is dispensable for in vitro holdase activity (Figure 7), although this domain is required to suppress *ydj1-151* thermosensitivity (Figure 5). These data suggest that the rescue of Hsp90-dependent activity in the *ydj1-151* mutant is more elaborate than simply increasing the cell's capacity to prevent protein aggregation.

DISCUSSION

We report herein that overexpression of Sse1p, the more abundant of two yeast Hsp110s, rescues the temperature-sensitive growth defect of the *ydj1-151* mutant. One established biochemical function of the Hsp110s is that they exhibit holdase activity (Oh *et al.*, 1997, 1999; Brodsky *et al.*, 1999), i.e., they can retain a thermally or chemically denatured polypeptide in a refolding-competent conformation. Our in vitro and in vivo analyses of wild-type and mutant derivatives of yeast Sse1p suggest that holdase activity may be insufficient to restore growth of the *ydj1-151* strain at elevated temperatures. This result was initially somewhat surprising as overexpression of Hsp110 in mammalian cells confers thermotolerance (Oh *et al.*, 1997). However, we also

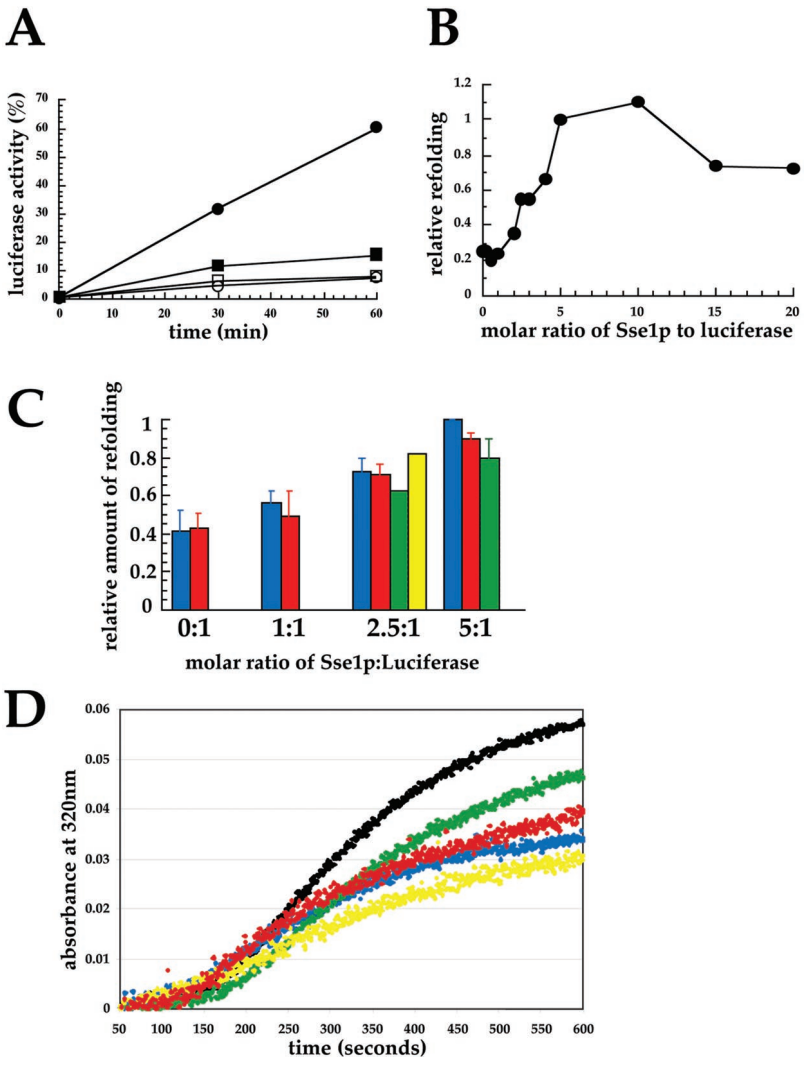

Figure 7. Sse1p ATP-binding domain mutants are proficient for in vitro holdase activity. (A) In vitro Sse1p holdase activity was assayed as described in MATERIALS AND METHODS. ●, Reactions containing Sse1p at a 10:1 molar ratio of chaperone to luciferase during luciferase denaturation and chased with cytosol and ATP; ■, reactions lacking Sse1p but chased with cytosol and ATP; ○, reactions containing Sse1p and chased with cytosol but no ATP; □, reactions lacking Sse1p and chased with cytosol but no ATP. Refolding activity (100%) represents the amount of luminescence when samples containing luciferase were mock denatured and then incubated with cytosol and ATP. (B) Reactions containing the indicated molar ratio of Sse1p to luciferase during thermal denaturation were chased with cytosol and ATP and luciferase refolding was assayed. The amount of refolding at a 5:1 ratio of Sse1p to luciferase was set to 1 in each experiment. Data represent the means of at least three independent experiments. (C) Relative amounts of refolding at the indicated molar ratios of wild-type Sse1p (blue), Sse1-1p (red), Sse1-2p (yellow), and the Sse1p CTD (green) to luciferase were assayed after the addition of cytosol and ATP. When error bars are present, the data represent the means of at least three independent experiments, ± SD. The amount of luciferase refolding in reactions containing a 5:1 M ratio of wild-type Sse1p to luciferase was always set to 1. (D) Luciferase aggregation over time was assayed upon thermal denaturation in the presence of a 5:1 M ratio of wild-type or the mutant Sse1p derivatives as described in MATERIALS AND METHODS. The black line indicates luciferase denatured in the absence of Sse1p, and the colored lines correspond to the protein preparations described in C.

show that overexpression of Sse1p rescues an Hsp90-mediated defect in the *ydj1-151* strain (Figure 3). Because Hsp90 itself, and other components of the Hsp90 complex, exhibit holdase activity (Bose *et al.*, 1996; Freeman and Morimoto, 1996; Freeman *et al.*, 1996) and are required for the maturation and folding of many cellular substrates (reviewed in Caplan, 1999), our data suggest instead that Sse1p repairs a *ydj1-151*–induced defect in the Hsp90 complex. Consistent with this hypothesis, Sse1p associates with the Hsp90 complex, and *sse1*Δ cells exhibit defects ascribed to Hsp90-dependent phenomena (Liu *et al.*, 1999; Figure 6). Furthermore, when we precipitated overexpressed wild-type or mutant Sse1p from transformed *ydj1-151* cells, we found that the amount of coprecipitated Ydj1-151p was lower when the Sse1p mutant proteins were overexpressed (our unpublished data). Clearly, a more detailed mapping and examination of the physical interactions and communications between these and other chaperones associated with the Hsp90 complex is warranted.

In contrast to the pronounced rescue of v-Src activity upon Sse1p overexpression in the *ydj1-151* mutant (Figure 3), the ability of *SSE1* to rescue the *ydj1-151* translocation defect was subtle (Figure 2). We suggest that this modest level of rescue is independent of Hsp90 action for the following reasons. First, Hsp90 mutant cells do not exhibit a defect in ppαF translocation (our unpublished data), and second, based on models for Hsp70/Ssa1p-promotion of posttranslational translocation (reviewed in Fewell *et al.*, 2001), increasing the concentration and thus the extent of Sse1p-mediated holdase activity in the cell might help retain ppαF in a translocation-competent conformation. Because an Ssa1p-Ydj1p interaction is required for translocation (Caplan *et al.*, 1992; Becker *et al.*, 1996; McClellan and Brodsky, 2000), and because overexpression of Sse1p fails to rescue the ppαF translocation defect in an *ssa1* mutant, it will be interesting in the future to explore whether Sse1p affects the interaction of Ssa1p and/or Ydj1p with posttranslationally translocated precursor proteins (Chirico, 1992).

Sse1p is one of two hsp110s in yeast, and it is curious that we only uncovered *SSE1* as a multicopy suppressor of *ydj1-151*. One trivial explanation for this result is that the library lacked *SSE2*, which may result from the fact that *SSE2*

mRNAs are rare, although the transcription of *SSE2* is induced upon heat shock (Mukai *et al.*, 1993; Shirayama *et al.*, 1993). However, it is important to note that cells deleted for *SSE2* grow as well as wild-type yeast, even at high temperature, but that the *sse1*Δ strain grows poorly. This observation may arise from the fact that Sse1p, but not Sse2p, interacts with the Hsp90 complex (Liu *et al.*, 1999), and is consistent with the isolation of only *SSE1* from the screen described in this study.

When examining the ability of the Sse1p mutants to exhibit in vitro holdase activity and the suppression of luciferase aggregation (Figure 7) we found that the CTD of Sse1p, although being almost as active as wild-type Sse1p for its ability to retain luciferase in a folding-competent conformation, exhibited a reduced ability to prevent luciferase aggregation during thermal denaturation. One explanation for these data is that not all luciferase "aggregates" that form during thermal denaturation will be unable to fold upon the subsequent addition of cytosol and ATP, and indeed, we observe some luciferase refolding in reactions lacking Sse1p. Another possibility is that the aggregates observed in the spectrophotometer represent both luciferase and the Sse1p-CTD, which may be aggregation prone, but the luciferase "held" by the CTD can still refold when cytosol and ATP are present; this scenario is also consistent with our observations that the Sse1p CTD acts as a dominant negative mutant for growth and for restoration of hormone binding (see RESULTS). More generally, the data presented in this report caution that the use of in vitro assays comparing wild-type and mutant chaperones might neglect more subtle, genetic consequences of the mutants in the crowded cellular milieu. This issue is particularly important since most chaperones exist in multimeric protein complexes, such as the Hsp90 complex.

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