ATP-dependent Hexameric Assembly of the Heat Shock Protein Hsp101 Involves Multiple Interaction Domains and a Functional C-proximal Nucleotide-binding Domain*  

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Members of the Hsp100 family of heat stress proteins are present in species throughout the bacterial, plant, and fungal kingdoms. Most Hsp100 proteins are composed of five domains that include two nucleotide-binding domains required for their ATP-dependent oligomerization. Mutations within the first but not the second nucleotide-binding site disrupt self-assembly of bacterial Hsp100, whereas the reverse is true for yeast Hsp104. We have examined the functional requirements for oligomerization of plant Hsp101 and have found that Hsp101 resembles Hsp104 in that it assembles into a hexameric complex in an ATP-dependent manner. Self-assembly of Hsp101 involves at least three distinct interaction domains located in the N-proximal domain and in the first and second nucleotide-binding domains. The interaction domain in the second nucleotide-binding domain included the Walker A motif, and mutations within this element disrupted self-assembly of Hsp101. In contrast, mutations affecting conserved residues of the Walker A motif within the first nucleotide-binding site did not affect self-assembly. No interaction between Hsp101 and Hsp104 was observed. These results suggest that plant Hsp101 self-assembly involves multiple evolutionarily diverged interaction domains as well as an evolutionarily conserved requirement for a functional C-proximal nucleotide-binding site.

A feature of the heat stress response in all of the species examined is the induction of heat stress proteins (Hsps) that serve to prevent protein denaturation or promote refolding of thermally denatured proteins (reviewed in Refs. 1–7). Several classes of Hsps have been described including Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and the small Hsps (reviewed in Refs. 5, 8, and 9), although how each class functions to ameliorate the deleterious effects of a thermal stress has not been thoroughly determined. Hsps are functionally similar to the extent that they act upon cellular proteins through protein-protein interactions. In addition to their extensive interaction with cellular proteins, interactions between classes of Hsps and the self-assembly of Hsps into complexes have been reported for several classes (reviewed in Refs. 1 and 10).

ClpB/Hsp104/Hsp101 members of the Hsp100 family are present in bacteria, yeast, and plants, respectively. Members of this family function as ATPases and contain Walker-type consensus sequences that are responsible for ATP binding (11). The bacterial ClpB is a member of a larger family of related proteins (reviewed in Refs. 3, 12, and 13) that differ as to whether they possess two ATP-binding domains (Class I, e.g. ClpA, ClpB, or ClpC) or one (Class II, e.g. ClpX or ClpY). Those members that possess two ATP-binding domains are further subdivided based on the length of the domain separating the nucleotide-binding domains. Members that contain two ATP-binding domains are larger than those that possess just one. Nevertheless, ClpA (containing two ATP-binding domains) and ClpX (containing one ATP-binding domain) both form hexamers in a symmetrical ring in the presence of ATP or its nonhydrolyzable analog, ATPγS (14–17). In contrast, ClpY (containing one ATP-binding domain) can form either a 6- or 7-fold ring (16, 18), suggesting that proteins with sequence homology do not necessarily adopt a similar ring structure. Mutations that affect ATP binding can inhibit oligomerization (19–25).

Although ClpA and ClpX assemble with ClpP peptidase, a structurally distinct protein that forms a tetradecamer organized as two 7-fold rings stacked back to back (14, 16, 26, 27), ClpB does not. Consequently, ClpA and ClpX serve as ATPases that not only regulate ATP-dependent, ClpP-mediated proteolysis but function as molecular chaperones (28, 29). In contrast, ClpB/Hsp104/Hsp101 family members possess molecular chaperone activity but do not regulate ClpP-mediated proteolysis. The ClpB/Hsp104/Hsp101 family also differs from other ClpP families in that expression of its members is heat-inducible and is essential to confer thermostolerance (30, 31). Mutations to either of the ATP-binding sites in ClpB abolish its thermostolerance activity in Escherichia coli, indicating that ATP is essential for ClpB function (32). In conjunction with DnaK/DnaJ/GrpE, the molecular chaperone activity of ClpB/Hsp104 serves to reactivate already aggregated protein (2, 33–35). Of the Clp family, yeast Hsp104 and plant Hsp101 share the greatest degree of sequence and structural similarity with ClpB, which correlates with the conservation of their heat stress function.

Hsp104 and Hsp101 are essential for thermostolerance in yeast and plants, respectively (36–38). Moreover, expression of Hsp101 can complement the thermostolerance defect in yeast caused by deletion of the Hsp104 gene (39–41), suggesting conservation of the molecular chaperone activity required for the acquisition of thermostolerance. Like class I bacterial Hsp100 proteins, Hsp104 and Hsp101 possess two nucleotide-binding domains (39–42). Electron micrographs of Hsp104 re-
revealed that the protein assembles as a hexamer that requires ATP for its assembly (21). As with Clp family members, hexamerization of Hsp104 can be inhibited by mutations that affect ATP binding (21–23). Although the domain organization is conserved between Clp proteins and Hsp104, they differ functionally in at least two important aspects. Mutations within the N-proximal but not the C-proximal nucleotide-binding site impaired self-assembly of ClpA and ClpB (19, 20, 24, 25), whereas mutations in the C-proximal nucleotide-binding site affected hexamerization of Hsp104 (21–23). Secondly, Hsp104 assembles into a hexamer (21), whereas ClpB has been reported to form either a heptamer or a hexamer (19, 35, 43). Little else is known about the requirements for self-assembly of either Hsp104 or ClpB. The difference in the requirement of the first or second nucleotide-binding domains of bacterial ClpB and yeast Hsp104 for their oligomerization is surprising given their conservation of protein organization and that they share 58% similarity. Plant Hsp101 shares 60% similarity with Hsp104 and is 64% similar to E. coli ClpB. As with all class I members, Hsp101 contains five domains, of which the two nucleotide-binding domains and the middle domain exhibit the greatest degree of conservation among plant, yeast, and bacterial orthologs. Expression of Hsp101 is induced by heat and is developmentally regulated (44). Although Hsp101 is required to confer thermotolerance in plants as well as in thermosensitive yeast (36, 37, 39, 40, 41, 44), little is known about its structure/functional requirements and whether they would be similar to those of Hsp104 or to those of ClpB.

In this study, we demonstrate that Hsp101 forms hexamers in an ATP-dependent process. Mutations of the C-proximal nucleotide-binding site that are known to inhibit ATP-binding reduced self-assembly, whereas the analogous mutations to the N-proximal nucleotide-binding site did not affect self-association. Three interaction domains were identified, one of which mapped to the second nucleotide-binding site. Mutation of the P-loop within this ATP-binding site inhibited self-interaction of the full-length protein but not the interaction of the isolated domain, suggesting that ATP binding regulates self-assembly through global changes in protein conformation. Despite the conservation between Hsp101 and Hsp104, no interaction between these proteins was detected, suggesting that the interaction domains have diverged sufficiently to prevent cross-species interaction or that Hsp104 utilizes entirely different domains for its self-assembly. Our findings indicate that self-assembly of Hsp101 requires multiple domains whose interaction is regulated by a functional C-proximal nucleotide-binding site.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Mutagenesis**—The tobacco Hsp101 was introduced into the BamHII/NotI sites of pGAL under the control of the GAL7 promoter or into the BamHI site of pTPI under the control of the triose-phosphate isomerase (TPI) promoter as described previously (41). The cDNA was also introduced into the BamHI site of pLexA, pGAD424, and pACT2. The Hsp101 cDNA was introduced into the BamHI site of pET19b for expression of recombinant His-tagged protein. Hsp101 deletion constructs were made by PCR amplification of the appropriate region using oligonucleotides that included restriction sites for introduction into the two-hybrid or yeast expression vectors. The fragments were sequenced to ensure that they were in frame with the activation or deletion domains of the two-hybrid vectors.

Point mutations of each ATP-binding site were made by designing oligonucleotides corresponding to an ATP-binding site that altered a specific amino acid. The GeneEditor in vitro site-directed mutagenesis system was used as described by the manufacturer (Promega Corp.) to incorporate the mutation into the Hsp101 sequence. Successful mutagenesis was confirmed by DNA sequencing. The fragment containing each mutant ATP-binding site was then used to replace the wild-type sequence in the full-length tobacco Hsp101 cDNA by using unique restriction sites. The single and double point mutants in the yeast vectors were confirmed by DNA sequencing.

**Oligomerization Assay**—Expression of recombinant His-tagged Hsp101 from pET19b was induced with 1 mM isopropyl-1-thio-
β-D-galactopopyranoside overnight in 375 ml of E. coli strain BL21 in LB medium. The harvested cells were washed with binding buffer (5 mM imidazole, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl) at 4 °C and resuspended in 4 ml of binding buffer supplemented with lysozyme (1 mg/ml), protease inhibitor mixture (Sigma), pepstatin A (1 μg/ml), leupeptin (10 μg/ml), and phenylmethylsulfonyl fluoride. The cells were sonicated for 2 min in a dry iced/ETOH bath, and the cell debris was pelleted for 20 min at 4 °C. Protein from the supernatant was allowed to bind to 700 μl of Talon metal affinity resin (CLONTECH Laboratories, Inc.) with gentle shaking for 20 min at 4 °C. The collected resin was washed three times with binding buffer, and the protein was eluted with 300 μl and then 150 μl of elution buffer (1 mM imidazole, 500 mM NaCl, 2.5 mM Tris-HCl, pH 7.9) for 15 min each at 4 °C. The eluant was then run over a 750-μl Sephadex G50 column to remove the imidazol. 100 μl of the protein was incubated in oligomerization buffer (20 mM HEPES, pH 7.5, 140 mM KC1, 15 mM NaCl, 10 mM MgCl2, and 2 mM dithiothreitol) for 15 min at room temperature and cross-linked with glutaraldehyde. 100 μl of 1 M glycine was added to quench the cross-linking, and the protein was precipitated at ~20 °C with 1.3 ml of acetone and 5% trichloroacetic acid. The precipitated protein was collected by centrifugation, washed with ethanol, resolubilized in SDS running buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol, and 0.1% bromphenol blue) and resolved on a 3.5% acrylamide gel. The gel was fixed overnight in 40% ethanol and 7% acetic acid, and the protein bands were revealed by silver staining.

**Thermotolerance Assay**—Constructs were introduced into SL304A (leu2-3, 112 trpl-1 uro3-1 ade2-1 his3-11,15 lys2-1 can1-100 hisp104::LEU2) (40), a Δhsp104 yeast mutant (generously provided by Dr. Susan Lindquist, University of Chicago), using polyethylene glycol/LiCl as described (45, 46) and grown in synthetic dextrose medium containing the appropriate supplements. The yeast were diluted to the indicated O.D., and complementation of Hsp104 function was determined in a thermotolerance assay in which survival of the yeast at 51 °C was measured (42, 48).

**Western Analysis**—Anti-Hsp101 antiserum raised against wheat Hsp101 was described previously (47). For Western analysis, a membrane containing the protein of interest was blocked for 30 min in TBPS (0.1% Tween 20, 13.7 mM NaCl, 0.27 mM KCl, 1 mM Na2HPO4, 0.14 mM KH2PO4) with 5% reconstituted dry milk and incubated with anti-Hsp101 antiserum (from 1:500 to 1:2000) in TPBS with milk for 1.5 h. The blots were then washed with TPBS and incubated with goat anti-rabbit-horseradish peroxidase antibody (Southern Biotechnology) diluted 1:20,000 for 1 h, and Hsp101 was detected using chemiluminescence (Amersham Biosciences). Yeast extracts were prepared by boiling 6 × 106 cells in 2× SDS loading buffer, the protein was resolved on a 10% SDS-PAGE gel, and Hsp101 was measured by Western analysis as described above.

**Two-hybrid Analysis**—Yeast strain L40 was transformed simultaneously with a HIS pLexA (DB fusion) construct and a LEU pGAD424 or pACT2 (AD fusion) construct. LacZ reporter gene activity was monitored visually by the X-gal filter assay (48) and was quantified by measuring β-galactosidase activity in mid-log phase liquid cultures as described (49) using chlorophenol red-β-D-galactopyranoside as the substrate. For the filter assay, yeast on supporting Whatman filters were cracked open by immersing the filters in liquid nitrogen twice. The filters were placed on a second Whatman filter soaked in Z buffer (60 mM NaHPO4, 40 mM KH2PO4, 10 mM KCl, 1 mM MgSO4, pH 7.0) with 0.1% β-mercaptoethanol and 3 mg/ml X-gal and incubated at room temperature. For the quantitative assays, mild phenolization of cells grown in SD medium lacking leucine and histidine were harvested and resuspended in buffer 1 (10 mM HEPES, 15 mM NaCl, 0.45 mM aspartate, 10 mM bovine serum albumin, 0.05% Tween 20), cracked open by immersing the tubes in liquid nitrogen three times, and the reaction was started by addition of 2× chloroform and phenylmethylsulfonyl fluoride in buffer 1. Following color development, 3 mM ZnCl2 was added to stop the reaction. The samples were centrifuged prior to determining the absorbency at 578 nm. A unit of β-galactosidase activity is defined as the amount that hydrolyzes 1 mmol of chlorophenol red-β-D-galactopyranoside/min/cell.

**RESULTS**

**Plant Hsp101 Assembles into Hexamers in an ATP-dependent Manner**—To examine whether plant Hsp101 is similar to yeast...
Hsp104 in assembling into hexameric complexes, N-terminal histidine-tagged tobacco Hsp101 expressed in E. coli was purified using affinity chromatography. Assembly of Hsp104 into hexameric complexes was demonstrated previously using SDS-PAGE of protein cross-linked with glutaraldehyde (21). Using the same approach, purified Hsp101 was cross-linked in solution with glutaraldehyde and resolved on a SDS-PAGE gel, and the complexes were detected by silver staining. Self-assembly up to a hexamer complex was observed over a range of cross-linking times (Fig. 1A) and concentrations of glutaraldehyde (Fig. 1B). The molecular mass of the Hsp101 hexameric complex determined from this analysis was 671 kDa, which is within 7.5% of the predicted molecular mass of the complex based on the cDNA-derived monomer mass of 104 kDa and is within 2.5% of the predicted molecular mass of the complex based on an apparent monomer molecular mass of 109 kDa. Hsp101 self-assembly was ATP-dependent as the monomer or dimer in the absence of ATP (Fig. 1C). Self-assembly also required Mg2+ (Fig. 1D, lane 1).

Yeast Hsp104 is a hexamer in the presence of ATP but is a monomer or dimer in the absence of ATP (21). However, ADP, like ATP, can stabilize the hexameric form of Hsp104 (21). Although ClpB assembles into an ATP-dependent hexamer and remains a monomer without ATP like Hsp104 (50), full oligomerization of ClpB is inhibited by ADP (50). In contrast to the bacterial ortholog, ADP supported at least partial hexamerization of Hsp101 (Fig. 1D, lane 2), data suggesting that Hsp101 is more similar to Hsp104 than it is to ClpB in that it can form a hexamer in a Mg2+-ATP or ADP-dependent manner.

**Hsp101 Self-assembly Requires the C-proximal but Not the N-proximal ATP-binding Site**—To determine whether Hsp101 self-assembly required ATP in vivo and, if so, to identify which nucleotide-binding site is required, we examined oligomerization of Hsp101 using the yeast two-hybrid assay. Like all Hsp100 family members, Hsp101 has two domains that contain a Walker A motif (i.e., GXGGXGKT, where X is any amino acid) (11). These two ATP-binding domains are separated by a middle domain, the length of which is characteristic of the class I family (12). Mutations within the first but not the second Walker A motif of ClpA or ClpB affected their oligomerization (19, 20, 24, 25). In contrast, mutations within the second but not the first ATP-binding site affected hexamerization of Hsp104 (21, 22, 23). To examine which nucleotide-binding domain (NBD) is required for self-assembly of Hsp101, mutations within each Walker A motif were introduced, and their effect on self-interaction was determined. Three sets of matching mutations were introduced individually into each ATP-binding site. The mutations were chosen based on the crystal structure and previous mutational analyses of Walker type ATP-binding mo-
tifs, which were shown to inhibit ATP-binding without altering domain structure. The first was a threonine substitution of the conserved lysine (i.e. K214T and K612T) in the P-loop of NBD1 and NBD2 that interacts with the β- and γ-phosphates of the bound nucleotide (11) (Fig. 2). A second substitution at this site was a more conserved mutation that changed the lysine to an arginine, i.e. K214R and K612R. A third set was a valine substitution of the conserved glycine (i.e. G213V and G611V) that is required to form a flexible loop within the NBD (11).

To establish that self-interaction can be detected, wild-type Hsp101 was tested using the two-hybrid vectors, pLexA and pGAD424. Strong self-interaction was observed that was specific because no signal was observed between pLexA-Hsp101 and pGAD424 or between pLexA-lamin and pGAD424-Hsp101 (Fig. 3A and Table I). Therefore, the two-hybrid assay confirms the self-interaction observed with the physical interaction data in Fig. 1, which suggests that results with the two-hybrid approach are reliable. Relative to the strength of self-interaction of the wild-type protein, no reduction was observed between the G213V mutant and wild-type protein, and only a small decrease in interaction was observed when the G213V mutant was tested against itself (Fig. 3A and Table I). Muta-

dition of the lysine at position 214 in NBD1 to a threonine (i.e. K214T) or arginine (i.e. K214R) had little to no effect on self-interaction and on interaction with wild-type Hsp101. In contrast, these same mutations in NBD2 abolished or substantially reduced self-interaction; no self-interaction or interaction with wild-type protein was observed for G611V and K612R, whereas self-interaction was just detectable for the K612T mutant (Fig. 3A and Table I). Interestingly, the K612T mutant was observed to interact with wild-type Hsp101 to a moderate level, suggesting that changing lysine 612 to threonine is not as disruptive as changing it to arginine. No interaction between any of the mutant Hsp101 proteins with lamin or the activation domain within the pGAD424 vector was observed (Fig. 3A). Similar effects on self-interaction of yeast Hsp104 was observed following changes at the conserved lysine or glycine in NBD2 (21, 22). Interestingly, altering the conserved lysine to threonine of the NBD2 in Hsp104 disrupted self-assembly at low but not at high protein concentration, suggesting that the presence of threonine at this position does permit hexamerization of Hsp104 under certain conditions (23). Moreover, consistent with the observations made with Hsp101, mutations in NBD1 of Hsp104 did not affect its self-interaction (21–23). One notable difference between Hsp101 and Hsp104, however, was that alteration of the conserved lysine of the Walker A motif in NBD2 to arginine abolished self-interaction of Hsp101 but had little effect on Hsp104 self-interaction.

The combination of mutations at both NBDs was also examined. The G213V/G611V, G213VK612R, and G213VK612T double mutants failed to exhibit self-interaction, although interaction with wild-type Hsp101 was just detectable in some cases (Table I). Similar results were obtained with double mutants that combined mutations in NBD2 with K214R or K214T. Interestingly, introduction of the K214T mutation to the K612T mutant abolished its interaction with wild-type Hsp101 when the mutant was fused to the Gal4 activation domain but not when fused to the LexA DNA-binding domain (Table I). It
Hsp101 increased survival at 51°C thermotolerance. In this experiment, expression of wild-type Hsp101 demonstrated what effect mutations introduced at either nucleotide-binding sites had on the thermotolerance activity of Hsp101, each mutant Hsp101 was expressed in SL304A and tested for thermotolerance. In this experiment, expression of wild-type Hsp101 increased survival at 51°C by ~25-fold (Fig. 4). In contrast, single amino acid substitutions in the conserved residues of NBD1 or NBD2 substantially reduced the thermotolerance function of Hsp101 (Fig. 4). Although a slightly lower level of expression from the mutant Hsp101 proteins (with the exception of K214T) may account for some reduction in thermotolerance, only a very small quantity of this class of protein is required for thermotolerance (51), suggesting that both functional nucleotide-binding sites are required for thermotolerance activity.

**Hsp101 Self-assembly Involves Three Interaction Domains**

Because many Clp/Hsp104/Hsp101 members form hexameric complexes that have an end-on appearance of a two-layered structure with an equal distribution of mass across the equatorial plane of the ring (14–16, 19), multiple domains involved in their self-assembly would be predicted. To map the interaction domains for Hsp101, deletion constructs were examined for their ability to interact with each other and with full-length Hsp101. Of the five domains present in Hsp101, three were found to contain regions required for self-interaction: the first and second nucleotide-binding domains (Table II, reactions 11 and 12), and a third in regions that Table II, reactions 29 and 31, respectively). The loss of interaction in the two-hybrid assay of Hsp101 ATP-binding site point mutants agrees well with the requirement for ATP in hexameric assembly (Fig. 1).

Mutations of NBD1 or NBD2 that reduced or abolished ATP hydrolysis of Hsp104 also reduced thermotolerance (42). To examine what effect mutations introduced at either nucleotide-binding site have on the thermotolerance activity of Hsp101, each mutant Hsp101 was expressed in SL304A and tested for thermotolerance. In this experiment, expression of wild-type Hsp101 increased survival at 51°C by ~25-fold (Fig. 4).
The Walker A motif within this domain was not required as an interaction was observed between Hsp101\(^{318-399}\) (in which the nucleotide-binding site was absent) and full-length Hsp101 (Fig. 3B and Table II, reaction 15). Hsp101\(^{318-399}\) was sufficient to interact with Hsp101\(^{386-909}\) (Fig. 3B and Table II, reaction 17). The importance of the region between 386 and 399 was supported by the observation that Hsp101\(^{319-399}\) and Hsp101\(^{318-399}\) interacted with Hsp101\(^{386-909}\) (Fig. 3B and Table II, reaction 17). Also no interaction was observed between Hsp101\(^{386-422}\) and either Hsp101\(^{386-909}\) or Hsp101\(^{399-909}\) (Table II, reactions 23 or 24, respectively). Moreover, interaction was just detectable between Hsp101\(^{386-422}\) and full-length Hsp101 (Table II, reaction 27). Together, these data suggest that the region between 319 and at least 399 is required for self-interaction.

The middle domain (i.e. Hsp101\(^{399-570}\)) is notable because it is predicted to contain a 100-amino acid-long region that forms a coiled-coil structure (52). Such structures are thought to be important in mediating protein-protein interactions (53). Because the coiled-coil structure has been conserved among ClpA, ClpB, and ClpC family members, including the yeast and plant orthologs, it has been suggested that this structural feature may be important for self-assembly (52). However, no interaction was observed between Hsp101\(^{386-399}\) and Hsp101\(^{399-909}\) or Hsp101\(^{386-909}\) (Table II, reactions 23 or 24, respectively). Moreover, interaction was just detectable between Hsp101\(^{386-422}\) and full-length Hsp101 (Table II, reaction 27). Together, these data suggest that the region between 319 and at least 399 is required for self-interaction.

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Hsp101 (data not shown), suggesting that if the middle domain is a protein interaction domain, it is likely to be involved in nonhomologous interactions.

As noted above, the second nucleotide-binding domain (i.e., Hsp101_{570–726}) was observed to interact with full-length Hsp101 (Table II, reaction 31). Strong interaction was also observed between Hsp101_{399–626} and Hsp101_{477–570} (Table II, reactions 37, 38, and 40–42, respectively). No interaction was observed between Hsp101_{399–626} and Hsp101_{477–570} (Table II, reaction 39). Similar results were observed with Hsp101_{399–626} and Hsp101_{570–626} with Hsp101_{399–626} and Hsp101_{477–570} (Table II, reactions 44–47 and 49–53). Together, these data suggest that the region between 570 and 626 contains a self-interaction domain.

Another region identified as important in self-interaction of Hsp101 is present in the N-terminal domain (i.e., Hsp101_{1–126}; see Table II, reaction 1). Hsp101_{1–126} remained competent to interact with full-length Hsp101 (Table II, reaction 3). Although Hsp101_{1–34} was unable to interact with full-length Hsp101 (Table II, reaction 8), Hsp101_{1–126} was able to interact with full-length Hsp101 (Fig. 3B and Table II, reaction 5), indicating that this region contains the interaction domain. Although no interaction between Hsp101_{1–126} and Hsp101_{399–570} was observed (Table III, reaction 4), an interaction between Hsp101_{1–126} and Hsp101_{399–570}, Hsp101_{399–626}, Hsp101_{477–570}, Hsp101_{570–626}, or Hsp101_{570–726} was observed (see Table III, reactions 5–9). An interaction between Hsp101_{1–126} and itself was also observed (Table III, reaction 2), suggesting that

Hsp101_{1–126} may make contact both with the N-terminal domain and NBD2. Although all other regions of Hsp101 not included in these three interaction domains did not exhibit any involvement in self-interaction, it is possible that they may participate in Hsp101 self-assembly but were not detected with the current methodology.

A Functional ATP-binding Site Is Required for the Interaction between NBD2 and the N-terminal Domain—As noted above, mutations within the nucleotide-binding site of NBD2 abolished the self-interaction of full-length Hsp101, raising the possibility that the ATP-binding site may be necessary to induce localized or global changes in protein folding to promote self-assembly. To examine whether the NBD2 ATP-binding site is required for any localized alterations needed for the self-interaction of this domain, the Walker A motif mutation, i.e., G611V, that abolished the self-interaction with full-length, wild-type Hsp101 (Fig. 3A) was introduced into the Hsp101_{399–626} fragment that in the wild-type state interacted strongly with full-length Hsp101. The G611V_{399–626} fragment interacted with full-length Hsp101 as strongly as did Hsp101_{399–626} (Table III, compare reactions 11 and 10). Moreover, G611V_{399–626} and Hsp101_{399–626} interacted equally well with Hsp101_{399–626} (Table III, compare reactions 13 and 12); with Hsp101_{570–726} (Table III, compare reactions 15 and 14); and with Hsp101_{570–726} (Table III, compare reactions 17 and 16). These data suggest that the nucleotide-binding site of NBD2 is not involved in the self-interaction of the region contained within NBD2.

We next investigated whether the ATP-binding site of NBD2 that is required for Hsp101 self-interaction is required for the interaction with the Hsp101_{1–126} interaction domain. An interaction between Hsp101_{1–126} and full-length Hsp101 containing a mutation within the Walker A motif of NBD1 (i.e., G213V) was observed (Table III, reaction 22) but not with Hsp101 containing mutations within the Walker A motif of NBD2 (i.e., G611V, K612R, or K612T) (Table III, reactions 19–21). In contrast, Hsp101_{399–626} interacted with full-length Hsp101 containing mutations within the ATP-binding site of NBD1 or NBD2 as it did with wild-type Hsp101 (Table III, reactions 24–26).
The wild-type protein (23).

The inhibition effect of truncated proteins is consistent with the deleterious effect on thermostability observed for mutant Hsp104 proteins (which re-cated proteins is indicated above the schematic. The consensus sequence for each signature sequence element is indicated below the schematic. The two Walker A ATP-binding sites, the predicted coiled-coil regions, and the interaction domains identified with this work are also indicated. The amino acid positions referred to in the text are indicated immediately below the schematic.

| Domain          | Amino Acid Positions |
|-----------------|----------------------|
| N-terminal      | 33 126 177           |
| N-proximal      | 319 396 399          |
| Middle          | 438 477 517          |
| C-proximal      | 570 626 653          |
| C-terminal      | 686 726 736          |

= Walker A ATP-binding site

= Predicted Coiled-coil structure

= Interaction domain

**Fig. 6. Schematic structure of Hsp101 used for the delineation of the region involved in self-interaction.** The schematic structure of Hsp101 is shown. The five domains and the conserved signature sequence elements are indicated above the schematic. The consensus sequence for each signature sequence element is indicated below the schematic. The two Walker A ATP-binding sites, the predicted coiled-coil regions, and the interaction domains identified with this work are also indicated. The amino acid positions referred to in the text are indicated immediately below the schematic.

23–27). These data suggest that the loss of interaction between molecules of full-length Hsp101 containing mutations within the ATP-binding site of NBD2 result from a loss in the interaction with the N-terminal interaction domain.

**Hsp101 Truncated Proteins Act as Dominant Negative Inhibitors of Hsp101 Thermotolerance Activity**—The results presented above suggest that Hsp101 contains multiple interaction domains. Although domain deletions of Hsp101 abolished its thermostability activity (data not shown), we examined whether Hsp101 truncated proteins that retain the ability to interact with full-length Hsp101 might alter Hsp101 function when expressed with wild-type Hsp101. Consequently, Hsp1011–570, Hsp1011–899, Hsp1011–726, Hsp1011–609, Hsp101177–570, and Hsp101177–899 were co-expressed with full-length Hsp101 in SL304A yeast, which was then examined for its thermostability. Expression of Hsp101 deletion proteins reduced the thermostolerance conferred by Hsp101 (Fig. 5). The extent of their inhibitory effect varied, which may have been a function of the fragment expressed as well as their expression level (which could not be determined by Western analysis because not all fragments are recognized equally well by the polyclonal anti-Hsp101 antiserum). However, partial inhibition of Hsp101 function was the common outcome of expression of Hsp101 deletion proteins. The inhibitory effect of the truncated proteins is consistent with the deleterious effect on thermostolerance observed for mutant Hsp104 proteins (which retain the ability to assemble with Hsp104) when co-expressed with the wild-type protein (23).

**DISCUSSION**

Despite their functional and structural conservation, bacterial ClpB and yeast Hsp104 differ significantly in their functional requirements for oligomerization and in the reported size of the assembled complexes. In this study, we have demonstrated that Hsp101 is similar to Hsp104 in that it assembles into hexamers in an ATP-dependent process for which the NBD2 is essential. Nonconservative mutations of the lysine within the P-loop of the second Walker A motif, i.e. GX4GXGKT, that interacts with the β- and γ-phosphates of the bound nucleotide, inhibit ATP binding and abolish thermosterol tolerance activity in Hsp104 (21–23, 42). Hsp101 was similar to Hsp104 in that nonconserved mutations within the second but not the first nucleotide-binding site-impaired hexamerization. An exception to this was altering the conserved lysine of the NBD2 to a threonine. This had only a moderate effect when interacted with wild-type Hsp101 but was most evident during self-interaction (Fig. 3A). The same mutation in Hsp104 was observed to disrupt self-assembly at low but not at high protein concentration (23). The data from the plant and yeast proteins suggest that threonine at this position weakens self-assembly but can permit hexamerization under certain conditions. The effect of threonine substitution at this position did not prevent cooperativity of ATP hydrolysis under conditions that promoted complex formation (23), data suggesting that the threo-nine substitution may affect the affinity of the mutant mono-meric protein for ATP at this site. Self-assembly of Hsp101 differed from that of Hsp104 in that a conservative mutation at the lysine in the NBD2 of Hsp101 inhibited self-interaction or interaction with wild-type Hsp101, whereas the same mutation was not inhibitory to Hsp104 hexamerization, data suggesting that self-assembly of Hsp101 may be more sensitive to changes in the NBD2 than is Hsp104.

A nonconservative mutation of the third glycine of the GX4GXGKT Walker A motif within the NBD2 of Hsp101 also inhibited its self-assembly. This is in good agreement with the loss in self-interaction for this same mutation in Hsp104, which also reduced ATP binding (22, 23). These data suggest that the flexibility of this loop within the nucleotide-binding site is important to the ability of eukaryotic Hsp100 proteins to self-assemble. These data also support the conclusion that plant Hsp101 and yeast Hsp104 proteins differ from bacterial ClpB and ClpA proteins in the function of the nucleotide-binding domains; a functional NBD2 is required for eukaryotic Hsp104/ Hsp101 self-assembly, whereas NBD1 is required for bacterial ClpB/ClpA self-assembly.
Analysis of the domains within Hsp101 identified three regions involved in its self-interaction. Consistent with the inhibitory effect that mutations of the NBD2 ATP-binding site have on self-interaction, the interaction domain within NBD2 included the Walker A motif (Fig. 6). Although ATP is required for oligomerization of all members of the Clp/Hsp104/Hsp101 family and has been proposed to induce a conformational change in the protein (21, 54), our observation that the ATP-binding site serves as a component of an interaction domain suggests that ATP binding may play a direct role in protein assembly for this family. Because mutation of the ATP-binding site abolished interaction of the full-length protein but did not substantially affect the self-interaction of the isolated NBD2 domain, any conformational change induced by ATP binding would be expected to affect the protein beyond this interaction domain. This conclusion is consistent with the effect that point mutations in the second nucleotide-binding region had on the self-interaction of the full-length protein; self-interaction was inhibited despite the fact that the remaining two interaction domains were present in the mutant Hsp101. The facts that ATP was required for hexameric assembly of Hsp101 in vitro and that mutations within the nucleotide-binding site of NBD2 abolish self-interaction support the conclusion that ATP binding to NBD2 may serve to regulate Hsp101 self-assembly. The observation that the interaction domain in NBD2 interacted with itself is consistent with the prediction made from electron micrographs of the yeast and bacterial orthologs that the hexameric ring structure is maintained through contacts within each nucleotide-binding domain. Such an arrangement of partner domains would permit a near co-linear alignment of the NBD2 in subunits of the hexamer.

Because of the homology between NBD1 and NBD2, it might be expected that the Walker A motif of NBD1 might also be part of an interaction site. Although the NBD1 does indeed contain an interaction domain, the interaction domain does not require the Walker A motif. This observation is consistent with the observation that mutations to the Walker A motif of NBD1 do not affect self-interaction (Fig. 3A). Instead, the interaction domain mapped to the C-terminal portion of NBD1 (Fig. 6). Interestingly, a region within this interaction domain that is required for the interaction mapped precisely to the conserved Walker B2 motif (i.e., XXXXXXXIDLhD, where h is a hydrophobic residue) (12). This conserved element is not present in NBD2, which may explain why this region of NBD1 but not NBD2 is involved in protein interaction. Like the interaction domain in NBD2, that of NBD1 interacted with itself, which would again permit a nearly co-linear alignment of NBD1 domains within the hexamer.

A distinctive feature of the ring structure of ClpA and ClpB is that each contains two major structural domains of equal size, resulting in a two-layered structure with an equal distribution of mass across the equatorial plane of the ring that is clearly observed in end-on views (14–16, 19). Because ClpX contains only one ATP-binding domain and the hexameric ring structure that it forms consists of a single tier, it has been postulated that the two tiers or structural domains observed in the ring structures of ClpA and ClpB each contain an ATP-binding domain (14). Our observation that one self-interacting domain is present in NBD1 and a second present in NBD2 supports the idea that each tier of the ring assembly contains a NBD.

Hsp101 mutated at either NBD exhibited a reduced ability to confer thermotolerance, data suggesting that both NBDs are required for thermotolerance. These results agree well with observations made with the yeast ortholog, in which loss of ATP binding to either NBD of Hsp104 resulted in loss of its thermotolerance function. Because self-assembly of Hsp101 and Hsp104 requires NBD2 but not NBD1, both are required for thermotolerance, the importance of ATP binding to the NBD1 in thermotolerance activity may be for reasons other than self-assembly.

The third region involved in self-interaction mapped to a N-proximal 90-amino acid region (Fig. 6). Notably, the ability of this domain to interact with full-length Hsp101 required a wild-type nucleotide-binding site within NBD2. This observation supports the notion that ATP-binding to NBD2 alters the global conformation of Hsp101 in such a way that allows at least the N-proximal interaction domain to function. Of the three interaction domains identified, the N-proximal interaction domain is the least conserved among plant Hsp101 proteins. Whereas the interaction domains present in each nucleotide-binding domain are highly conserved among plant Hsp101 proteins, it is possible that the relative divergence of the N-proximal interaction domain determines specificity of its self-interaction. Interestingly, the C-terminal domain of ClpB is required for self-association, whereas the N-terminal domain is not (55). Because the N-terminal domain of Hsp101 is involved in self-association, whereas the C-terminal domain is not, Hsp101 and ClpB may differ in the regions required for their self-assembly.

The middle domain contains a 100-amino acid-long region (i.e., Hsp101401–501) that is predicted to adopt a coiled-coil structure that is highly conserved among Hsp101 proteins (52). Despite a predicted role in protein association, the middle domain was not involved in self-interaction. A second region (i.e., Hsp101522–729) present in NBD1 that was also predicted to adopt a coiled-coil structure was not part of the interaction domain present within NBD1. These observations suggest that if each domain is involved in protein interaction, it may be for interaction with client proteins.

Our observations that the C-terminal domain does not appear to be involved in self-assembly allows for the possibility that this domain, which shares similarity to PDZ domains (56–59), may be available for interaction with partner proteins. The acidic C terminus of Hsp104 is a feature conserved with the plant orthologs, is required for its interaction with the Hsp90 co-chaperone Sti1 (60), supporting the notion that the C-terminal domain may be involved in protein interaction. No interaction between full-length Hsp101 and Hsp104 or between any of the three individual Hsp101 interaction domains with Hsp104 was detected (data not shown), suggesting that these domains may have diverged or that the yeast protein uses different domains altogether. The fact that the conservation between the domains is low is consistent with the lack of their cross-species interaction.

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REFERENCES
1. Becker, J., and Craig, E. A. (1994) Eur. J. Biochem. 219, 11–23
2. Glover, J. R., and Lindquist, S. (1998) Cell 94, 73–82
3. Gottesman, S., Wickner, S., and Maurizi, M. R. (1997) Genes Dev. 11, 815–823
4. Hendrick, J. P., and Hartl, F. U. (1993) Annu. Rev. Biochem. 62, 349–384
5. Miernyk, J. A. (1999) Plant Physiol. 121, 695–703
6. Parsell, D. A., Kowal, A. S., Singer, M. A., and Lindquist, S. (1994) Nature 372, 476–478
7. Parsell, D. A., and Lindquist, S. (1995) Annu. Rev. Genet. 29, 437–496
8. Winter, J., and R. Simonds (1991) in Results and Problems in Cell Differentiation: Heat Shock and Development (Nowak, L., and Hightower, L. E., eds) Vol. 17, pp. 85–105, Springer-Verlag, Berlin, Germany
9. Vierling, E. (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 579–620
10. Hendrich, A. L., Weber Ean, E. U., and Finley, D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11033–11040
11. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) EMBO J. 1, 945–951
12. Schirmer, R. C., Glover, J. R., Singer, M. A., and Lindquist, S. (1996) Trends Biochem. Sci. 21, 289–296
13. Suzuki, C. K., Rep, M., van Dijl, J. M., Suda, K., Grivell, L. A., and Schatz, G.
