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Hsp70 and Hsp90 of *E. coli* directly interact for collaboration in protein remodeling

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Abstract

Hsp90 is a highly conserved molecular chaperone that remodels hundreds of client proteins, many involved in the progression of cancer and other diseases. It functions with the Hsp70 chaperone and numerous cochaperones. The bacterial Hsp90 functions with an Hsp70 chaperone, DnaK, but is independent of Hsp90 cochaperones. We explored the collaboration between *E. coli* Hsp90 and DnaK and found that the two chaperones form a complex that is stabilized by client protein binding. A J-domain protein, CbpA, facilitates assembly of the Hsp90_{Ec}-DnaK-client complex. We identified *E. coli* Hsp90 mutants defective in DnaK interaction in vivo and show that the purified mutant proteins are defective in physical and functional interaction with DnaK. Understanding how Hsp90 and Hsp70 collaborate in protein remodeling will provide the groundwork for the development of new therapeutic strategies targeting multiple chaperones and cochaperones.

Graphical Abstract

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Keywords

Hsp40; CbpA; DnaJ; molecular chaperone; protein remodeling

Introduction

Proteins belonging to the Hsp90 family are present in nearly all organisms and comprise a highly conserved class of ATP-dependent molecular chaperones^{1–4}. In eukaryotes, Hsp90 is essential for cell viability. It is required for remodeling and activation of hundreds of client proteins involved in many crucial cell processes, such as cell signaling and response to stress. Protein remodeling by Hsp90 requires the assistance of the Hsp70 chaperone and numerous Hsp90 cochaperones.

Hsp90 is a homodimer with each protomer containing an N-terminal ATP binding domain (NBD), a middle domain (M-domain) that participates in client binding^{5–7} and a C-terminal domain (C-domain), which is involved in dimerization and client binding^{4,5}. Eukaryotic Hsp90 also contains a linker region of about 50 amino acids between the NBD and the M-domain and a C-terminal extension of 35 amino acids that interacts with several cochaperones. Hsp90 undergoes multiple conformational changes in response to ATP binding and hydrolysis^{8–14}. In the absence of ATP, the Hsp90 dimer adopts an open V-shaped structure with the protomers interacting via the C-terminal dimerization domain¹³. When ATP is bound, the protein adopts a closed conformation with the two N-domains of the dimer interacting and a portion of the N-domain, the “lid”, closing over the nucleotide in each protomer¹⁵. Additional conformational changes occur upon ATP hydrolysis^{8–12}, and after ADP release, Hsp90 reverts back to the open conformation^{2,11}. Additionally, client protein binding and cochaperone interactions cause changes in the conformation of Hsp90 and affect the residence time in the various conformations^{1,14,16,17}.

The Hsp70 chaperone and more than 20 cochaperones, including Hop/Sti1, Aha1/Hch1, p23/Sba1, Cdc37 and Sgt1, collaborate with Hsp90 to remodel and activate the diverse group of client proteins in the eukaryotic cytosol¹. The cochaperones regulate the Hsp90 ATPase activity and recruit specific client proteins. Some cochaperones direct the chaperone cycle by stabilizing specific conformations such as the open or the closed state of Hsp90. For example, Hop/Sti1 interacts simultaneously with Hsp70 and Hsp90 through its multiple

tetratricopeptide repeat (TPR) domains and facilitates substrate transfer from Hsp70 to Hsp90 by stabilizing the open conformation of Hsp90^{18,19}.

The bacterial homolog of Hsp90 in *Escherichia coli*, the product of *hspG* and referred to as Hsp90_{Ec}, is a very abundant protein in nonstress conditions and is further induced in stress conditions¹. It shares about 50% sequence similarity with human Hsp90. Hsp90_{Ec} is not an essential protein in laboratory conditions²⁰. However, when cells carry mutations in Hsp90_{Ec} they grow more slowly at high temperature²⁰, exhibit a slight increase in aggregated proteins at high temperature²¹, lose adaptive immunity conferred by the CRISPR system²² and show a subtle defect in motility²³. Additionally, when Hsp90_{Ec} is overexpressed in *E. coli*, cells filament and become sensitive to SDS⁵.

Both eukaryotic Hsp90 and Hsp90_{Ec} have been shown to remodel proteins in vitro. For example, eukaryotic Hsp90 reactivates denatured luciferase in conjunction with Hop/Sti1, Hsp70 and Hsp40^{19,24,25}. Similarly, Hsp90_{Ec} has the ability to reactivate heat-denatured luciferase in vitro²⁶. This reaction requires ATP hydrolysis by Hsp90_{Ec} and also requires DnaK, the *E. coli* homolog of Hsp70, and the DnaK cochaperone, DnaJ (or a DnaJ homolog, CbpA)²⁶. GrpE, the prokaryotic nucleotide exchange factor stimulates the rate of reactivation, although it is not essential²⁶. Hsp90_{Ec} and DnaK physically interact to mediate protein reactivation independent of a Hop/Sti1-like cochaperone²⁶. *E. coli* Hsp90 is not unique^{3,4,27}; recently it has been reported that Hsp90 and Hsp70 contact one another directly in complexes containing Hop and client protein^{28–30}. Moreover, Hsp90 from *Synechococcus elongates*, *Neurospora crassa* and *Plasmodium falciparum* have also been shown or suggested to interact with their cognate Hsp70 system^{31–34}. Biochemical experiments using *E. coli* proteins suggest that DnaK and DnaJ/CbpA act first on the client protein and then Hsp90_{Ec} and the DnaK system collaborate synergistically to complete remodeling of the client protein²⁶.

In this paper we explored the mechanism of collaboration between Hsp90_{Ec} and DnaK both in vivo and in vitro. We show that Hsp90_{Ec} and DnaK form a binary complex and Hsp90_{Ec}, DnaK and client protein form a ternary complex. CbpA promotes assembly of the ternary complex. We identified Hsp90_{Ec} mutants defective in DnaK interaction in vivo and show that the purified mutant proteins are defective in DnaK interaction in vitro and impaired in protein reactivation with DnaK and its cochaperones. Together, these findings provide a better understanding of how these two important chaperones collaborate in client remodeling.

Results

Formation of an Hsp90_{Ec}-DnaK-client protein complex is facilitated by CbpA

We previously showed that Hsp90_{Ec} functions synergistically with DnaK and its cochaperones, a J-domain protein (CbpA or DnaJ) and GrpE in client protein reactivation in vitro²⁶. In addition we showed that Hsp90_{Ec} and DnaK interact in vivo in a bacterial two-hybrid assay and in vitro using purified proteins²⁶. To shed light on the mechanism of protein remodeling by Hsp90_{Ec} and DnaK, we sought to dissect the multiprotein reaction pathway into intermediates and partial reactions.

We explored the interaction between Hsp90_{Ec} and DnaK by testing whether binding of client protein or DnaK cochaperones affects the stability of the previously observed Hsp90_{Ec}-DnaK complex²⁶. We used an in vitro protein-protein interaction assay (pull-down assay) in which DnaK was labeled with biotin, DnaK D45C-biotin, and incubated with various pure proteins in the presence of ATP (Fig. 1 and Supplemental Fig. S1A). Biotinylated DnaK, along with DnaK-associated proteins were then captured on neutravidin agarose beads, the beads were washed, proteins were eluted and the eluted proteins were analyzed by SDS-PAGE. When biotinylated DnaK and Hsp90_{Ec} were incubated together, Hsp90_{Ec} weakly associated with DnaK (Fig. 1A, lane 2), as observed previously by an ultrafiltration assay²⁶. When the two chaperones were incubated with ribosomal protein L2, a client protein known to interact with Hsp90_{Ec}^{5,16}, we observed significantly more Hsp90_{Ec} associated with biotinylated DnaK as well as L2, suggesting formation of a more stable ternary Hsp90_{Ec}-DnaK-L2 complex (Fig. 1A, lane 3). CbpA further stimulated assembly or stabilization of the DnaK-Hsp90_{Ec}-L2 complex (Fig. 1A, lane 4, and Supplemental Fig. S1B) and the stimulatory effect of CbpA required L2 (Fig. 1A, lane 6). Additional experiments indicated that DnaK could associate with L2 and CbpA alone, as well as L2 and CbpA together (Supplemental Fig. S1C). In contrast, DnaJ did not affect assembly of the DnaK-Hsp90_{Ec}-L2 complex (Supplemental Fig. S1B). We do not understand why CbpA and DnaJ behaved differently in these experiments and in protein reactivation²⁶. However, they differ in that DnaJ contains a cysteine-rich Zn²⁺ binding region (Type I J-domain protein) and CbpA lacks this region (Type II J-domain protein). GrpE had no detectable effect on the association of Hsp90_{Ec} with DnaK and L2 in the presence of CbpA and was not detected in association with the complex (Fig. 1A, lane 5). In a control experiment, Hsp90_{Ec}, L2, CbpA and GrpE did not bind detectably to the neutravidin agarose (Fig. 1A, lane 8). Together these results suggest that the DnaK-Hsp90_{Ec} complex is strengthened upon binding of a client protein and CbpA promotes formation of the ternary complex.

Since L2 independently binds Hsp90_{Ec}¹⁶ and DnaK (Supplemental Fig. S1C) and also stabilizes the Hsp90_{Ec}-DnaK complex, we tested if client binding by Hsp90_{Ec}, DnaK or both chaperones was necessary for complex stabilization. When we mixed Hsp90_{Ec} W467R, a client binding defective mutant⁵ with DnaK D45C-biotin in the presence of L2 and CbpA, we were unable to detect the mutant Hsp90_{Ec} in association with DnaK (Fig. 1B, lane 3). We next tested a DnaK mutant defective in client binding, V436F^{35,36}, in pull-down experiments using biotinylated V436F. Hsp90_{Ec} was not detected in association with DnaK V436F in the presence of L2 and CbpA (Fig. 1B, lane 6). Together these results demonstrate that the ternary DnaK-Hsp90_{Ec}-L2 complex requires client binding by both Hsp90_{Ec} and DnaK.

To assess whether ATP hydrolysis by Hsp90_{Ec} and/or DnaK was required for formation of the ternary complex, we tested ATP hydrolysis defective variants of the two chaperones. We found that Hsp90_{Ec} E34A, a mutant that binds but does not hydrolyze ATP⁸, associated much more weakly with DnaK D45C-biotin than wild type Hsp90_{Ec} in the presence of L2 and CbpA (Fig. 1B, lane 4). Similarly, a DnaK mutant, T199A, which can bind but not hydrolyze ATP³⁷, was biotinylated and found to bind Hsp90_{Ec} more weakly than a similarly biotinylated wild type DnaK in the presence of L2 and CbpA (Fig. 1B, lane 7). Together

these results indicate that ATP hydrolysis and/or ATP-driven conformational changes by both chaperones are important for formation of a stable Hsp90_{Ec}-DnaK-L2 complex.

ATP hydrolysis by Hsp90_{Ec} and DnaK is synergistically stimulated

The demonstration of an Hsp90_{Ec}-DnaK-client complex prompted us to examine the functional significance of the complex by monitoring the effect of a client protein, L2, on ATP hydrolysis by the combined action of Hsp90_{Ec} and DnaK. In the absence of L2, the rate of hydrolysis by the mixture of Hsp90_{Ec} and DnaK was only slightly greater than the sum of hydrolysis by each chaperone separately, suggesting the possibility of a synergistic stimulation of ATP hydrolysis (Fig. 2A). As previously shown, ATP hydrolysis by Hsp90_{Ec} alone was stimulated by L2^{5,16} (Fig. 2B–2D), while ATP hydrolysis by DnaK was unaffected (Fig. 2B–2D). In the presence of 1 μ M L2 the rate of hydrolysis by the pair of chaperones was ~1.6-fold higher than the additive rates of hydrolysis of each chaperone separately in the presence of L2 (Fig. 2B–2D). These observations suggest that the physical interaction between Hsp90_{Ec} and DnaK is reflected in a functional collaboration between the two chaperones in stimulating ATP hydrolysis.

To determine if Hsp90_{Ec} and DnaK act synergistically or if one chaperone activates the ATPase of the other in the presence of L2, we tested ATP hydrolysis-defective Hsp90_{Ec} and DnaK mutant proteins. The synergistic activity was prevented when Hsp90_{Ec} E34A²⁶ was substituted for wild type (Fig. 2C). Geldanamycin, a specific inhibitor of Hsp90³⁸, also blocked the synergistic stimulation of ATPase activity (Fig. 2C). In addition, DnaK T199A, was unable to stimulate ATPase activity with Hsp90_{Ec} in the presence of L2 (Fig. 2D). Thus ATP hydrolysis and/or the associated ATP-dependent conformational changes by both Hsp90_{Ec} and DnaK are essential for the collaborative activity of the two chaperones.

As seen for Hsp90_{Ec}-DnaK-L2 complex formation, client binding by both chaperones was essential for synergistic ATPase stimulation in the presence of L2. ATP hydrolysis was not stimulated above additive when a client binding defective mutant of Hsp90_{Ec}, W467R⁵, or DnaK, V436F^{35,36}, was substituted for wild type in the ATPase assay with L2 (Fig. 2C and 2D). These results show that both chaperones must have functional client binding sites to collaborate in synergistic ATP hydrolysis. In contrast to ternary complex formation, we saw no detectable effect of CbpA and GrpE on the synergistic stimulation of ATP hydrolysis by the two chaperones in the presence of L2 (Supplemental Fig. S2A). DnaJ and GrpE slightly inhibited the stimulation of ATP hydrolysis by DnaK and Hsp90_{Ec} in the presence of L2 (Supplemental Fig. S2B).

Together these results demonstrate that Hsp90_{Ec} and DnaK form a physical and functional complex in the presence or absence of a client protein.

Mutations in the M-domain of Hsp90_{Ec} cause defective interaction with DnaK in vivo

We developed a screen for the isolation of Hsp90_{Ec} mutants potentially defective in DnaK interaction with the aim of determining the region of Hsp90_{Ec} that interacts with DnaK. The screen took advantage of our previous observation that Hsp90_{Ec} and DnaK interact in vivo in a bacterial two-hybrid assay²⁶. In our earlier work, plasmids carrying fusions between the

gene coding for Hsp90_{Ec} and one fragment of the *Bordetella pertussis* adenylate cyclase gene, T18, and between DnaK and the other fragment of the cyclase gene, T25, were constructed. When the two plasmids were coexpressed in an *E. coli* strain carrying a mutation in the gene encoding adenylate cyclase (*cya*[−]) and a deletion of the gene encoding Hsp90_{Ec} (*htpG*), the cAMP reporter gene, β -galactosidase, was expressed and colonies appeared red on indicator plates²⁶ (Fig. 3A and 3B). These results indicate that DnaK and Hsp90_{Ec} interact in vivo, but do not exclude the possibility that other cellular proteins are involved in the interaction, such as client proteins. However, Hsp90_{Ec} W467R and other client-binding defective mutants interacted with DnaK similarly to wild type Hsp90_{Ec} in the two-hybrid assay (Supplemental Fig. S3A).

We constructed a T18-Hsp90_{Ec} plasmid library containing randomly mutagenized *htpG*. We then coexpressed the T18-Hsp90_{Ec} mutagenized plasmid library with the T25-DnaK plasmid in *E. coli cya*[−] *htpG* and screened for white colonies on indicator plates (see Supplemental Methods). Among the Hsp90_{Ec} mutants obtained, two contained R355 substitutions; one had a substitution to Cys and the other to Leu. We reconstructed the R355C and R355L mutations in the T18-Hsp90_{Ec} plasmid, since the mutated genes also coded for several additional amino acid changes. When T18-Hsp90_{Ec} R355C or T18-Hsp90_{Ec} R355L was coexpressed with T25-DnaK in the *cya*[−] *htpG* strain, we observed that the colonies were very pale pink on indicator plates (Fig. 3A) and the β -galactosidase level was ~10% that of wild type (Fig. 3B). These results suggest residue R355 of Hsp90_{Ec} is important for the in vivo interaction with DnaK.

Hsp90_{Ec} R355 is located in a long α -helix in the M-domain and is surface exposed in both the open and closed conformation of Hsp90_{Ec}¹³ (Fig. 3C and Supplemental Fig. S3B and S3C). To identify additional Hsp90_{Ec} residues important for DnaK interaction in vivo, we substituted amino acids in other surface exposed residues in the vicinity of R355 using site-directed mutagenesis. Three mutants were constructed in the same α -helix as R355, including K354C, Q358C and D366C (Fig. 3C and Supplemental Fig. S3B and S3C). Other mutants in the region, including K238C, R267C, E269C and G270A/K271A were also constructed (Fig. 3C and Supplemental Fig. S3B and S3C). The mutant proteins were then tested for the ability to interact with DnaK in the bacterial two-hybrid assay. We observed that strains expressing T25-DnaK and T18-Hsp90_{Ec} K238C, E269C or G270A/K271A were pink on indicator plates (Fig. 3A) while those expressing T25-DnaK and T18-Hsp90_{Ec} R267C, K354C, Q358C, or D366C were red (Fig. 3A). The level of β -galactosidase produced by the cells reflected the color of the colonies: β -galactosidase levels in cells expressing T25-DnaK and T18-Hsp90_{Ec} K238C, G270A/K271A or E269C were ~16% that of wild type (Fig. 3B). Cells expressing the other four mutants, T18-Hsp90_{Ec} R267C, K354C, Q358C, or D366C with T25-DnaK, produced levels of β -galactosidase slightly lower than wild type (~80% of wild type) (Fig. 3B). In control experiments the steady state levels of all of the mutant fusion proteins were similar to wild type (Supplemental Fig. S3D).

Thus, these mutants define a region in the Hsp90_{Ec} M-domain that is important for interaction with DnaK in vivo. However, the two-hybrid assay does not distinguish between a binary interaction and an interaction involving other cellular components.

DnaK interaction-defective Hsp90_{Ec} mutant proteins identified in vivo are defective in DnaK binding in vitro

To determine if the Hsp90_{Ec} mutant proteins we identified were defective in direct interaction with DnaK, we purified the mutant proteins and tested them for their ability to bind DnaK in vitro in the absence of other proteins. Using an ultrafiltration assay, we previously showed that labeled DnaK was specifically retained on 100 kDa MWCO filters in the presence of wild type Hsp90_{Ec}²⁶ (Fig. 4A). When we tested the Hsp90_{Ec} mutant proteins in this assay, we observed that Hsp90_{Ec} R355L, R355C, G270A/K271A, E269C, R267C and K354C were partially defective in DnaK binding compared to wild type (Fig. 4A and 4B). Hsp90_{Ec} Q358C and D366C were like the wild type (Fig. 4B). These results show that the Hsp90_{Ec} mutants that were most defective in DnaK interaction in vivo are defective in DnaK interaction in vitro.

We next tested the mutant proteins in the pull-down assay and again found that the mutants that were very defective in the two-hybrid assay, Hsp90_{Ec} R355C, R355L, K238C, G270A/K271A and E269C, were also defective in the ability to associate with biotinylated DnaK in the presence of L2 with or without CbpA (Fig. 4C and Supplemental Fig. S4A). The mutants that were partially defective in the two-hybrid assay, R267C and K354C showed decreased ability to interact with DnaK in the presence of L2 (Fig. 4C). Altogether our results suggest that a region of the Hsp90_{Ec} M-domain near R355 is important for a binary interaction with DnaK and for a ternary interaction with DnaK and L2, in the presence or absence of CbpA.

DnaK binding defective Hsp90_{Ec} mutants are impaired in functional collaboration with DnaK in vitro

Based on the in vivo and in vitro protein-protein interaction results, we predicted that the Hsp90 mutants defective in DnaK interaction would also be defective in the synergistic stimulation of ATP hydrolysis with DnaK. In control experiments, we observed that the mutant Hsp90_{Ec} proteins had basal rates of ATP hydrolysis similar to wild type showing that the mutations do not cause defects in ATP hydrolysis (Fig. 5A). Moreover, in the presence of the client protein, L2, ATPase activity of the Hsp90_{Ec} mutants was stimulated similar to wild type (Fig. 5A). Since ATP hydrolysis by client binding defective Hsp90_{Ec} mutants is not stimulated by L2⁵ (Fig. 2C), these observations suggest that Hsp90_{Ec} mutants defective in DnaK interaction are not defective in client binding.

We then tested the mutants for their ability to synergistically stimulate ATP hydrolysis in combination with DnaK in the presence of L2. When Hsp90_{Ec} R355C, R355L, K238C, E269C or K354C was substituted for wild type, synergistic stimulation of ATPase was not observed; instead, hydrolysis was similar to the sum of hydrolysis by each chaperone alone with L2 (Fig. 5B). ATP hydrolysis by Hsp90_{Ec} G270A/K271A or R267C and DnaK in the presence of L2 was greater than additive, but the stimulation was less than wild type Hsp90_{Ec} (Fig. 5B). ATP hydrolysis by Hsp90_{Ec} Q358C or D366C with DnaK and L2 was similar to or greater than the synergistic stimulation seen with wild type Hsp90_{Ec} with DnaK and L2 (Fig. 5B). These results provide in vitro evidence to suggest that the region surrounding R355 in the M-domain is important for the functional collaboration between Hsp90_{Ec} and DnaK.

Hsp90_{Ec} mutant proteins defective in DnaK interaction are defective in protein reactivation in collaboration with the DnaK system in vitro

To determine if the Hsp90_{Ec} residues important for interaction with DnaK are also important for protein reactivation by Hsp90_{Ec} we tested the mutant proteins in an in vitro protein reactivation assay. We previously showed that the combination of wild type Hsp90_{Ec} and the DnaK system, comprised either of DnaK, CbpA and GrpE or DnaK, DnaJ and GrpE, function together to reactivate heat-inactivated luciferase²⁶ (Fig. 5C). Hsp90_{Ec} alone is unable to reactivate luciferase and the DnaK system reactivates luciferase poorly²⁶ (Fig. 5C). Four mutants, Hsp90_{Ec} R355C, R355L, K238C and G270A/K271A were defective in luciferase reactivation and exhibited rates of reactivation indistinguishable from the DnaK system alone (Fig. 5C). Hsp90_{Ec} E269C, K354C and R267C were partially defective in luciferase reactivation, displaying rates of reactivation ~50% of wild type Hsp90_{Ec} with the DnaK system (Fig. 5C). Hsp90_{Ec} Q358C and D366C reactivated luciferase at rates comparable to wild type Hsp90_{Ec} (Supplemental Fig. S5). Thus the Hsp90_{Ec} residues that are important for the interaction with DnaK both in vivo and in vitro and for the synergistic stimulation of ATPase with DnaK are also important in client remodeling with the DnaK system.

Altogether these results suggest that an Hsp90_{Ec}-DnaK complex and a larger complex of Hsp90_{Ec}-DnaK-client protein are intermediates in the pathway of protein remodeling. Additionally, they suggest that DnaK interacts directly with Hsp90_{Ec} through residues in the middle domain of Hsp90_{Ec}. Collectively, these results provide insight into the mechanism of collaboration during protein remodeling between two important molecular chaperones.

Discussion

In this work we explored the mechanism of collaboration between Hsp90 of *E. coli* and the DnaK chaperone system. Unlike client activation and remodeling by eukaryotic Hsp90 and the Hsp70 system, the *E. coli* system is independent of Hsp90 cochaperones and thus provides a much simpler system to study. It is possible that cochaperones, which have not yet been identified in *E. coli*, are involved in organizing a complex of Hsp90_{Ec} and the DnaK chaperone system. However, it is also possible that the DnaK system by interacting with Hsp90_{Ec} directly bypasses the need for additional cochaperones and provides some of the regulatory roles of the eukaryotic Hsp90 cochaperones.

Our results show Hsp90_{Ec} and DnaK form a weak complex that is stabilized by client binding. Ternary complex formation is facilitated by a J-protein, CbpA. Whether CbpA is associated with the DnaK-Hsp90_{Ec}-client complex remains to be unequivocally determined. ATP hydrolysis or conformational changes associated with hydrolysis by both Hsp90_{Ec} and DnaK is required for this collaboration. Moreover ATP hydrolysis is synergistically stimulated by the two chaperones in the presence of client protein, suggesting the functionality of the Hsp90_{Ec}-DnaK complex. However, further study is necessary to clarify the synergistic action of the two chaperones and determine whether DnaK stimulates the activity of Hsp90_{Ec}, Hsp90_{Ec} stimulates the activity of DnaK or they stimulate each other.

In Eukaryotes, recent studies have identified multiprotein assemblies of Hsp90, Hsp70 and Hop with client protein^{28–30}. Although Hsp40 was not observed in the chaperone complexes, it was included in the reaction mixtures for formation of the complexes. The presence of dimeric Hsp90, Hop and a client (a fragment of glucocorticoid receptor) was common to all of the ternary complexes. The quaternary form of Hsp70 observed differed between the studies. In one study, Hsp70 formed an antiparallel dimer in the complex³⁰; in the other studies it was a monomer^{28,29}. In all three studies, an interaction was observed between Hsp90 and Hsp70, although the regions of interaction suggested by the various studies differed^{28–30}. Moreover, post-translational modifications have been shown to affect formation of the complexes³⁰. Together these observations suggest the existence of multiple conformations for the Hsp90-Hop-Hsp70-substrate complex, but the details of the interactions between the proteins in the complexes remain to be elucidated.

The recent visualization of the eukaryotic Hsp90-Hsp70-Hop-client complex as well as earlier biochemical work from many groups suggests a mechanism for substrate transfer from Hsp70 to Hsp90^{19,28–30,39,40}. In the current model, the Hsp70 cochaperone, Hsp40, likely presents the client to Hsp70 and stabilizes the interaction between the client and Hsp70 prior to the binding of Hsp70 to Hop and Hop to Hsp90^{28–30}. The formation of a quaternary Hsp90-Hsp70-Hop-client complex then positions the Hsp70-bound client near the Hsp90 client-binding region^{28–30}. Coordination of the ATP hydrolysis cycles by the two chaperones, in ways that are not fully understood, promotes conformational changes in the chaperones and leads to substrate transfer and maturation^{28–30}.

Our previous work²⁶ and that presented here to elucidate the mechanism of protein remodeling by the combined action of DnaK and Hsp90_{Ec} suggests a similar but simpler mechanism of client remodeling by the prokaryotic chaperones than by the eukaryotic chaperones²⁶. First, like remodeling by the DnaK system alone^{35,41}, DnaJ/CbpA targets the client for recognition by DnaK and some initial protein remodeling is performed by DnaK and DnaJ/CbpA alone. This step requires ATP hydrolysis. Next DnaK recruits Hsp90_{Ec} to the client via a direct interaction between DnaK and Hsp90_{Ec}. This interaction is further stabilized by an additional interaction between the client and Hsp90_{Ec} and is facilitated by a J-domain protein. Then Hsp90_{Ec} and DnaK act synergistically in a reaction requiring ATP hydrolysis by both chaperones. Likely coordinated, ATP hydrolysis-driven conformational changes in Hsp90_{Ec} and DnaK promote remodeling and release of the client²⁶.

In an analogous reaction to the collaboration between DnaK and Hsp90_{Ec} in prokaryotes and between Hsp70, Hsp90 and Hop in eukaryotes, DnaK and Hsp70 have been shown to function collaboratively with the ClpB and Hsp104 AAA+ disaggregases, respectively, in protein disaggregation in vivo and in vitro^{42–44}. The collaboration between Hsp104/ClpB by Hsp70/DnaK involves a direct protein-protein interaction between the two chaperones^{45–49}. Additionally, ATP hydrolysis by the DnaK system and ClpB is synergistically stimulated in the presence of aggregated substrate⁴³. The similarities in these two bichaperone protein remodeling reactions suggest a new role for DnaK/Hsp70 as a regulator of the activity of multiple ATP dependent molecular chaperones.

In summary, this study illuminates how *E. coli* Hsp90 and Hsp70 interact and how they collaborate in protein remodeling. Understanding this interaction is of universal importance and is critical to developing cancer therapies, possibly drug combinations that simultaneously target Hsp90 and Hsp70 or a cochaperone.

Materials and Methods

Plasmids and Strains

Single substitution mutations of Hsp90_{Ec} and DnaK were made with the QuikChange mutagenesis system (Stratagene) using pET-HtpG²⁶, pRE-DnaK⁵⁰, pET-DnaK⁵¹ or pT18-Hsp90_{Ec}²⁶. All mutations were verified by DNA sequencing. The Hsp90_{Ec} random mutagenesis library and Bth101 *htpG* were constructed as described in Supplementary Methods.

Proteins

Hsp90_{Ec} wild type and mutants²⁶, DnaK wild-type and mutants⁵⁰, DnaJ⁵⁰, CbpA⁵², GrpE⁵⁰ and His-tagged L2¹⁶ were isolated as described. All proteins were >95% pure as determined by SDS-PAGE. Mutant Hsp90_{Ec} proteins exhibited ATPase activity and gel filtration chromatograms similar to wild type (Fig. 5A and Supplemental Fig. S4B). Luciferase and luciferin were from Promega and Roche, respectively. Concentrations given are for Hsp90_{Ec}, DnaJ, CbpA and GrpE dimers and DnaK, L2 and luciferase monomers. DnaK D45C was labeled using a 20-fold excess of Maleimide-PEG₁₁-Biotin (Thermo, Life Technologies) and DnaK wild type, T199A, and V436F were labeled using a 1.5-fold excess of NHS-PEG₄-Biotin (Thermo, Life Technologies) as recommended by the manufacturer. Excess biotin reagent was removed by dialysis. DnaK D45C-biotin was similar to wild type in luciferase reactivation (Supplemental Fig. S1A). DnaK was labeled with ³H as described (740 cpm/pmol)⁵³.

Luciferase reactivation

Luciferase reactivation was performed as previously described with modifications²⁶. 40 nM heat-denatured luciferase was incubated at 24 °C in reaction mixtures (75 µl) containing 25 mM Hepes, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 2 mM DTT, 10 mM MgCl₂, 50 µg/ml bovine serum albumin (BSA), 3 mM ATP, an ATP regenerating system (25 mM creatine phosphate, 6 µg creatine kinase), 0.95 µM DnaK, 0.15 µM CbpA, 0.05 µM GrpE and 0.5 µM Hsp90_{Ec} wild type or mutant. Aliquots were removed at the indicated times and light output was measured using a Tecan Infinite M200Pro in luminescence mode with an integration time of 1000 ms. Reactivation was determined compared to a non-denatured luciferase control.

ATPase activity

Steady state ATP hydrolysis was measured as previously described using 1 µM Hsp90_{Ec} wild type or mutant^{5,8} with L2 (1 µM), DnaK wild type or mutant (1 µM) and geldanamycin (30 µM) where indicated.

Ultrafiltration assay

Association of Hsp90_{Ec} and DnaK was measured using an ultrafiltration assay as previously described with modifications²⁶. 0.13 μ M [³H]DnaK was incubated at 24° for 5 min in reaction mixtures (100 μ L) containing 20 mM Tris-HCl, pH 7.5, 75 mM KCl, 10% glycerol (vol/vol), 0.05% Triton X-100 (vol/vol), 5 mM DTT and Hsp90_{Ec} wild-type or mutant or BSA as indicated. Reactions were filtered through Microcon DNA Fast Flow filters (Millipore) by centrifugation at 3200 $\times$ g for 10 min. Retained proteins were recovered with 10% SDS and radioactivity was measured. Background corrections were made by subtracting the percentage of [³H]DnaK retained in the absence of Hsp90_{Ec} (<10%).

Protein-protein interaction assay

A pull-down assay was used to measure interaction of Hsp90_{Ec} with DnaK. 2.4 μ M DnaK D45C-biotin was incubated for 5 minutes at 23 °C in reaction mixtures (50 μ L) containing PD buffer (20 mM Tris-HCl pH 7.5, 75 mM KCl, 10% glycerol (vol/vol), 0.01% Triton X-100 (vol/vol), 2 mM DTT, 10 mM MgCl₂, 2 mM ATP) with 3.6 μ M Hsp90_{Ec} wild-type or mutant, 2.3 μ M L2, 0.4 μ M CbpA and 0.13 μ M GrpE. 20 μ L of neutravidin agarose (Thermo, Pierce) was then added and incubated 5 minutes at 23 °C with mixing. The reactions were diluted with 0.4 mL PD buffer, centrifuged 1 minute at 1000 $\times$ g and the recovered agarose beads were washed twice with 0.4 mL PD buffer. Bound proteins were eluted with buffer containing 2 M NaCl and analyzed by immunoblot analysis or coomassie blue staining following SDS-PAGE. Where indicated, biotinylated DnaK wild type, DnaK T199A or DnaK V436F were used.

Bacterial two-hybrid assay

Bacterial two-hybrid assays were performed as previously described^{26,54}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NBD	nucleotide-binding domain
NEF	nucleotide exchange factor
<i>E. coli</i> Hsp90	Hsp90 _{Ec}

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HIGHLIGHTS

- Mutations in the middle domain of Hsp90 define a DnaK interaction region
- Hsp90 and DnaK form a complex that is stabilized by client protein binding
- Hsp90-DnaK-client complex formation is facilitated by Hsp40
- Hsp90 and DnaK ATPase is synergistically stimulated in the presence of client

Fig. 1.

Stabilization of DnaK-Hsp90_{Ec} complex in the presence of client protein and cochaperone. **(A,B)** Interaction between DnaK and Hsp90_{Ec} in the presence or absence of L2, CbpA and GrpE. Pull-down assays were carried out as described in Methods using biotinylated DnaK. Proteins associated with DnaK-biotin were analyzed by SDS-PAGE. Proteins were visualized by coomassie blue staining; Hsp90_{Ec} was monitored by immunoblot analysis using Hsp90_{Ec} antiserum. **(A)** Pull down assays contained biotinylated DnaK D45C, Hsp90_{Ec}, L2, CbpA and GrpE where indicated. **(B)** Assays contained biotinylated DnaK D45C, biotinylated DnaK wild type or biotinylated DnaK mutant as indicated, Hsp90_{Ec} wild type or mutant as indicated, CbpA and L2. In A–B, 2.4 μ M DnaK, 3.6 μ M Hsp90_{Ec}, 2.3 μ M L2, 0.4 μ M CbpA and 0.13 μ M GrpE were used. M indicates DnaK, Hsp90_{Ec}, L2, CbpA and GrpE as markers. In **A,B** representative gels from three or more independent experiments are shown.

Fig. 2.

Hsp90_{Ec} and DnaK function synergistically in ATP hydrolysis. **(A)** ATP hydrolysis by DnaK, Hsp90_{Ec} or the combination of DnaK and Hsp90_{Ec}. Data from 7 replicates are presented as mean $\pm$ SEM. The additive value of hydrolysis by DnaK alone and Hsp90_{Ec} alone is shown as a black hatched bar and is meant to aid the reader. **(B)** ATP hydrolysis by Hsp90_{Ec}, DnaK or Hsp90_{Ec} and DnaK in the presence of increasing concentrations of L2. **(C)** ATPase activity by the combination of wild type or mutant Hsp90_{Ec} and DnaK in the presence or absence of 1 μ M L2. Geldanamycin (30 μ M) was added where indicated. **(D)** ATPase activity by the combination of wild type or mutant DnaK and Hsp90_{Ec} in the presence or absence of L2. In **A–D**, ATPase was measured as described in Methods using 1 μ M wild type or mutant DnaK, 1 μ M wild type or mutant Hsp90_{Ec} and 1 μ M L2. In **B–C**, data from 3 or more replicates are presented as mean $\pm$ SEM. In **C** and **D**, dashed lines indicate the additive ATPase activity of wild type Hsp90_{Ec} with L2 and wild type DnaK with L2 and are meant to aid the eye.

Fig. 3.

Identification of Hsp90_{Ec} amino acid residues involved in DnaK interaction in vivo. (**A,B**) Interaction between DnaK and Hsp90_{Ec} wild type or mutant in a bacterial two-hybrid system in vivo, as described in Methods. DnaK was fused to one domain of *B. pertussis* adenylate cyclase, T25. Hsp90_{Ec} wild type and mutants were each fused to the other domain, T18. T25-DnaK was coexpressed with each of the T18-Hsp90_{Ec} mutants separately in *cya- htpG* cells and the interaction between DnaK and Hsp90_{Ec} wild type or mutant was monitored by the expression of a reporter gene, β-galactosidase, on MacConkey indicator plates (**A**) and in liquid assays (**B**). In **A**, a representative plate from three independent experiments is shown. In **B**, β-galactosidase activity is shown as means ± SEM (n=3). (**C**) Surface rendered model of the crystal structure of the Hsp90_{Ec} dimer in the apo form (pdb: 2ioq)¹³ with the C-terminal domains aligned to the crystal structure of the isolated C-terminal domain (pdb: 1sf8)⁵⁵ using PYMOL (www.pymol.org). One protomer is gray. The NBD, M-domain and C-terminal domain of the other protomer are colored pale blue, wheat and pale green, respectively. The mutated residues are labeled and colored.

Fig. 4.

Hsp90_{E_C} M-domain mutants are defective in DnaK interactions in vitro. **(A)** Interaction between DnaK and Hsp90_{E_C} was monitored by measuring retention of [³H]DnaK on cellulose filters with a 100-kDa exclusion limit in the presence of increasing concentrations of wild type or mutant Hsp90_{E_C}, as described in Methods. **(B)** Interaction between DnaK and Hsp90_{E_C} wild type or mutant or BSA was measured by ultrafiltration as in **A** using 1 μM Hsp90_{E_C}. **(C)** Interaction between DnaK D45C-biotin and wild type or mutant Hsp90_{E_C} in the presence of L2 and CbpA was determined as described in Methods. DnaK-associated proteins were analyzed by immunoblot analysis and coomassie blue staining following SDS-PAGE. In **A,B**, data from at least 3 replicates are presented as mean ± SEM. In **A**, the apparent K_d values for the wild type, R355C, R355L, G270A/K271A, and E269C were 0.42, 1.49, 1.79, 1.53 and 0.85 μM, respectively. In **C**, 2.4 μM DnaK, 3.6 μM Hsp90_{E_C}, 2.3 μM L2 and 0.4 μM CbpA were used and a representative gel from three independent experiments is shown.

Fig. 5.

Hsp90_{Ec} mutants defective in DnaK interaction are defective in functional collaboration with DnaK. **(A)** ATPase activity of wild type or mutant Hsp90_{Ec} in the absence or presence of L2 was measured as described in Methods. **(B)** ATPase activity was determined in the presence of wild type or mutant Hsp90_{Ec} DnaK and L2. The additive value of ATP hydrolysis by DnaK in the presence of L2 and Hsp90_{Ec} WT or mutant in the presence of L2 is shown by gray bars to aid the reader. **(C)** Reactivation of heat-denatured luciferase was monitored over time as described in Methods using Hsp90_{Ec} wild type or mutant in combination with DnaK, CbpA and GrpE. Wild type, black; R267C, orange; K354C, purple; E269C, blue; G270A/K271A, pink; R355L, red; R355C, green; K238C, light blue; DnaK, CbpA, GrpE alone, black with open circles; heat-denatured luciferase alone, gray. In **A–C**, data from at least 3 replicates are presented as mean $\pm$ SEM.