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Interaction of *E. coli* Hsp90 with DnaK involves the DnaJ binding region of DnaK

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Abstract

Hsp90 is a widely conserved and ubiquitous molecular chaperone that participates in ATP-dependent protein remodeling in both eukaryotes and prokaryotes. It functions in conjunction with Hsp70 and the Hsp70 cochaperones, an Hsp40 (J-protein) and a nucleotide exchange factor. In *E. coli* the functional collaboration between Hsp90_{Ec} and Hsp70, DnaK, requires that the two chaperones directly interact. We used molecular docking to model the interaction of Hsp90_{Ec} and DnaK. The top-ranked docked model predicted that a region in the nucleotide-binding domain of DnaK interacted with a region in the middle domain of Hsp90_{Ec}. We then made substitution mutants in DnaK residues suggested by the model to interact with Hsp90_{Ec}. Eleven of the twelve mutants tested were defective or partially defective in their ability to interact with Hsp90_{Ec} in vivo in a bacterial two-hybrid assay and in vitro in a Bio-Layer Interferometry assay. These DnaK mutants were also defective in their ability to function collaboratively in protein remodeling with Hsp90_{Ec}, but retained the ability to act with DnaK cochaperones. Taken together these results suggest that a specific region in the nucleotide-binding domain of DnaK is involved in the interaction with Hsp90_{Ec} and this interaction is functionally important. Moreover, the region of DnaK that we found to be necessary for Hsp90_{Ec} binding includes residues that are also involved in J-protein binding, suggesting a functional interplay between DnaK, DnaK cochaperones and Hsp90_{Ec}.

Graphical abstract

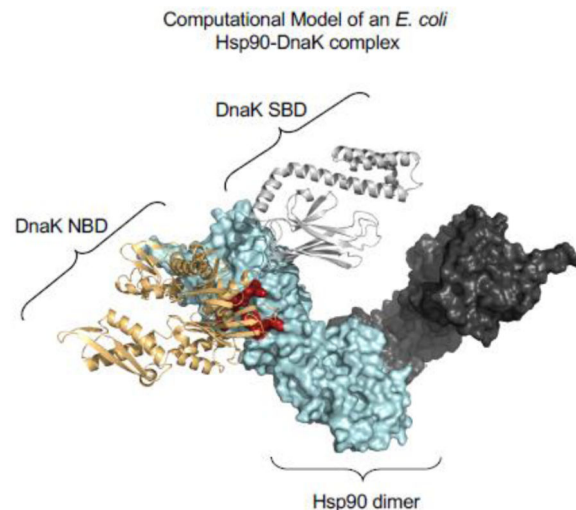
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Keywords

Hsp40; CbpA; HtpG; molecular chaperone; protein remodeling

Introduction

The 90-kDa heat shock protein (Hsp90) is a highly ubiquitous and evolutionarily conserved molecular chaperone [1–5]. It is essential in eukaryotes, where it is involved in the folding, stability and activation of more than 200 client proteins including many transcription factors, steroid hormone receptors and protein kinases [4, 6, 7]. In addition, Hsp90 stabilizes and activates oncoproteins and therefore is a potential target for drug discovery for the treatment of cancer [8]. *Escherichia coli* Hsp90, referred to as Hsp90_{Ec} and encoded by *hspG*, is an abundant protein and is further induced upon heat shock and other stress conditions [9]. Strains lacking Hsp90_{Ec} exhibit modest phenotypes, including slow growth at elevated temperature [9], accumulation of aggregated proteins at high temperature [10], loss of adaptive immunity conferred by the CRISPR system [11], decreased ability to form biofilms at elevated temperature [12] and decreased ability to swarm [13]. Overexpression of Hsp90_{Ec} causes defects in cell division that results in filamentous cells as well as SDS sensitivity [14].

Hsp90 is a homodimer with each monomer consisting of three domains: an N-terminal domain that possesses an ATP binding site [15, 16]; a middle domain containing residues that participate in binding client proteins [7, 14, 15, 17]; and a C-terminal domain that is essential for dimerization and is also involved in client binding [3, 14]. ATP binding and hydrolysis by Hsp90 triggers large conformational changes that are necessary for the cycle of client binding, remodeling and release [4, 15, 16, 18–21]. The Hsp90 dimer exists in a predominantly open V-shaped conformation in the absence of nucleotide with the protomers interacting via the C-terminal domain [21]. ATP binding triggers closing of the ATP lid on the ATP-binding site, followed by dimerization of the two N-terminal domains [4, 18, 21, 22]. ATP hydrolysis and ADP release leads to the dissociation of the N-domains [4, 18, 21]

and Hsp90 returns to the open conformation [18–20, 22]. Cochaperones and client protein binding bias the Hsp90 chaperone cycle and stabilize or destabilize various conformations of Hsp90 [1, 3, 4].

Hsp90 functions with the Hsp70 chaperone system in protein activation and remodeling [1, 5, 23]. Eukaryotic Hsp70 and its prokaryotic homolog, DnaK, are highly conserved proteins [24–27]. Hsp70/DnaK is comprised of an N-terminal nucleotide binding domain (NBD) and a C-terminal substrate-binding domain (SBD) that are connected by a flexible linker [25]. It collaborates with two cochaperones, an Hsp40 (J-domain protein) and a nucleotide exchange factor (NEF). The Hsp40 protein stimulates ATP hydrolysis by Hsp70/DnaK and presents substrate to Hsp70/DnaK while the NEF stimulates nucleotide exchange by Hsp70/DnaK [24, 27, 28].

In addition to collaborating with the Hsp70 chaperone system, eukaryotic Hsp90 also functions with numerous cochaperones, including Hop/Sti1, Aha1/Hch1, p23/Sba1, Cdc37 and Sgt1 [1, 15, 16]. Cochaperones regulate Hsp90 in various ways, such as imparting client protein specificity, modulating ATPase activity or stabilizing specific Hsp90 conformations [1, 4]. Moreover, Hop/Sti1 stabilizes the interaction between eukaryotic Hsp90 and Hsp70 by simultaneously interacting with tetratricopeptide repeat domains at the extreme C-terminus of each chaperone. In contrast to eukaryotes, bacterial Hsp90 functions with the DnaK chaperone system independently of other Hsp90 cochaperones.

Protein reactivation by bacterial Hsp90 in vitro is simpler in its requirements than its eukaryotic homolog, requiring only DnaK and a J-domain protein [23, 29]. GrpE, the bacterial NEF stimulates reactivation, but is not essential [23]. ATP hydrolysis and client binding by both chaperones is essential for reactivation [30]. Moreover, bacterial Hsp90 physically interacts with DnaK in vivo and in vitro [29, 30] through a region identified on the M-domain of Hsp90_{Ec} [30].

In the work presented here, we examined the collaboration between Hsp90_{Ec} and DnaK. We used molecular modeling to identify a region on DnaK that interacts with the middle domain of Hsp90_{Ec}. We then constructed DnaK mutants in some of the residues suggested by the docked model and tested the mutants for defects in interaction and functional collaboration with Hsp90_{Ec}. We found that the region of DnaK involved in the physical and functional interaction with Hsp90_{Ec} comprises residues in the DnaK NBD. This region of DnaK overlaps the region where DnaJ binds, suggesting a mechanism where Hsp90_{Ec} directly interacts with a client bound DnaK-DnaJ complex to displace DnaJ and promote the transfer of substrate to Hsp90_{Ec}.

Results

Identification of residues in DnaK potentially involved in protein interactions with Hsp90_{Ec}

We previously showed that *E. coli* DnaK collaborates with Hsp90_{Ec} in protein reactivation and that the two chaperones physically interact through a region in the middle domain of Hsp90_{Ec} [23, 30]. To elucidate the region of DnaK essential for binding Hsp90_{Ec}, we used molecular docking to predict potential interactions between the two chaperones. Four

combinations of Hsp90_{Ec} and DnaK molecules available in the protein data bank were tested [21, 31, 32]: 1) ADP-bound DnaK with apo Hsp90_{Ec}, 2) ADP-bound DnaK with ADP-bound Hsp90_{Ec}, 3) ATP-bound DnaK with apo Hsp90_{Ec} and 4) ATP-bound DnaK with ADP-bound Hsp90_{Ec} (see Materials and Methods). Without imposing restraints, the proteins were docked using ZDOCK [33, 34] and the top model for each combination was selected based on the lowest energy using ZRANK [35]. Three of the four models (2, 3 and 4, above) could be eliminated since they predicted interacting regions that were inconsistent with earlier work [30] or with results shown in Supplemental Information (Supplemental Results and Supplemental Fig. S1-S5 and Supplemental Tables S2-S4).

The model of the complex of ADP-bound DnaK and apo Hsp90_{Ec} predicted that DnaK interacted exclusively with residues on the Hsp90_{Ec} middle domain, many of which had been shown experimentally to be involved in binding DnaK [30] (Fig. 1a and Table 1). To gain additional evidence for this model, we constructed two Hsp90_{Ec} mutants with substitutions in residues predicted by the model to be important in interaction with DnaK (Supplemental Fig. S6a). Hsp90_{Ec}(Q302A, R303A) was defective in functional and physical interaction with DnaK in vitro; Hsp90_{Ec}(D329A, L330A, P331A) was slightly defective (Supplemental Fig. S6b and S6c).

To further test our model, we used the PRISM webserver [36, 37] to predict the interface between DnaK in the ADP-bound conformation and apo Hsp90_{Ec} by structural matching. The model we obtained was very similar to our top docking result obtained with ZDOCK and ZRANK (Supplemental Fig. S7a and S7b and Supplemental Table S1).

The model of ADP-bound DnaK and apo Hsp90_{Ec} predicted that residues in the DnaK nucleotide-binding domain (NBD) interacted with Hsp90_{Ec} (Fig. 1a and 1b, Table 1 and Supplemental Table S1). Interestingly, of the 35 DnaK residues predicted from this docked model to interact with Hsp90_{Ec}, 20 were previously identified as important for interacting with DnaJ [38–40], suggesting that Hsp90_{Ec} and DnaJ share a common binding interface on DnaK (Fig. 1c, Table 1). Additionally, 26 of the 35 predicted residues are also involved in the association between the DnaK NBD and substrate-binding domain (SBD) that occurs when DnaK is in the ATP conformation [32, 41] (Fig. 1d). The NBD and SBD of DnaK are not in contact with one another when DnaK is in the ADP-bound conformation [31], indicating that Hsp90_{Ec} likely interacts with the ADP-bound conformation of DnaK. Importantly, the docked model of ADP-bound DnaK and apo Hsp90_{Ec}, would allow a client protein bound to the SBD of DnaK to readily interact with the client-binding site of Hsp90_{Ec}, making this model mechanistically plausible (Fig. 1a).

Mutations in the potential Hsp90_{Ec} binding region of DnaK cause defective interaction with Hsp90_{Ec} in vivo

We wanted to explore the validity of the docked model of the apo Hsp90_{Ec} structure with DnaK in the ADP-bound form. To do this we constructed site-directed amino acid substitutions in surface exposed residues of DnaK in and near the residues suggested by the model to interact with Hsp90_{Ec} and screened the mutants in a bacterial two-hybrid assay (Fig. 2a). Some substitutions were previously described mutants, including DnaK_{Y145A,N147A,D148A} [40], DnaK_{R167H} [39], DnaK_{N170A,T173A} [39] and

DnaK_{E217A,V218A} [40], while other substitutions were in residues that had previously been substituted with alternate amino acids, including DnaK_{D208R}, DnaK_{E209C} and DnaK_{V210R} [38]. The remaining mutants were alanine substitutions or charge changes, including DnaK_{R84E}, DnaK_{D211R}, DnaK_{E213Q,K214A}, DnaK_{D224K,T225A} and DnaK_{S234A,I237A,N238A}.

We had previously observed that DnaK wild-type and Hsp90_{Ec} interact in the bacterial two-hybrid assay [23, 30]. For this assay one domain of the *Bordetella pertussis* adenylate cyclase protein, T18, was fused to Hsp90_{Ec} and the other domain, T25, was fused to DnaK wild-type or to a DnaK mutant. If the two fusion proteins interact when they are coexpressed in an *E. coli cya*-strain, cyclic AMP is synthesized and the cAMP reporter gene, β -galactosidase, is expressed [42]. As we saw previously, the coexpression of T25-DnaK wild-type and T18-Hsp90_{Ec} wild-type resulted in colonies that appeared red on indicator plates and expressed ~12-fold higher levels of β -galactosidase compared to coexpression of T25-DnaK wild-type and T18-vector [30] (Fig. 2b and 2c).

When plasmids expressing T25-DnaK mutant proteins were coexpressed with T18-Hsp90_{Ec} in an *E. coli cya*-strain, we observed that 11 of the 12 colonies appeared white or pale pink on indicator plates and had lower levels of β -galactosidase activity than cells coexpressing T25-DnaK wild-type and T18-Hsp90_{Ec}, suggesting that the DnaK mutant fusion proteins were defective in interaction with Hsp90_{Ec} (Figure 2b and 2c). These mutants included DnaK_{Y145A,N147A,D148A}, DnaK_{R167H}, DnaK_{N170A,T173A}, DnaK_{D208R}, DnaK_{V210R}, DnaK_{E217A,V218A}, DnaK_{S234A,I237A,N238A}, DnaK_{E209C}, DnaK_{D224K,T225A}, DnaK_{D211R} and DnaK_{R84E}. One mutant, DnaK_{E213Q,K214A}, produced red colonies on indicator plates and had β -galactosidase activity similar to T25-DnaK wild-type (Fig. 2b and 2c) and was therefore not studied further. Control experiments showed that all of the mutant fusion proteins were expressed at levels similar to the T25-DnaK wild-type protein (Supplemental Fig. S8). Thus, all but one of the DnaK substitution mutants constructed in residues that were predicted by the model to interact with Hsp90_{Ec} or were in nearby surface exposed residues were defective in interaction in vivo. These results suggest the DnaK mutants may be defective in direct interaction with Hsp90_{Ec}. However, in vivo the interaction may be affected by other cellular components [42].

DnaK mutant proteins defective in Hsp90_{Ec} interaction in vivo are defective in direct complex formation with Hsp90_{Ec} in vitro

We next tested if the DnaK mutants that were defective in the two-hybrid screen were defective in direct protein-protein interaction in vitro. We first cloned, purified and characterized the mutant proteins (Supplemental Results and Supplemental Fig. S9 and S10). DnaK and Hsp90_{Ec} proteins have been shown to form a binary complex in vitro in both the presence and absence of hydrolyzable ATP, although the interaction is very weak [29, 30]. We tested our DnaK mutant proteins for direct interaction with Hsp90_{Ec} by Bio-Layer Interferometry (BLI). Hsp90_{Ec(E584C)} was specifically labeled with biotin and immobilized on a streptavidin-coated biosensor (see Material and Methods). Binding was monitored using buffer conditions that allowed an optimal signal to noise ratio (see Material and Methods). We first monitored the binding of DnaK wild-type and Hsp90_{Ec} (Fig. 3a and 3b). Since the binding curves at high DnaK concentrations were complex, we used a steady-

state analysis and calculated the K_d to be $\sim 13 \mu\text{M}$ under the conditions used in this assay (Fig. 3a and 3b). The observed K_d is consistent with the weak interaction seen previously between Hsp90_{Ec} and DnaK wild-type [23, 30] as well as between *Synechococcus elongates* Hsp90 and DnaK [29] and mitochondrial Hsp90 (TRAP) and Hsp70 (Mortalin) [43].

We then used BLI to monitor the direct interaction between the DnaK mutant proteins and Hsp90_{Ec}, at a concentration of DnaK (50 μM) that was saturating for the interaction of DnaK wild-type with Hsp90_{Ec} (Fig. 3c and 3d). The results indicated that all of the DnaK mutants were defective to varying degrees in interaction with Hsp90_{Ec}. The most defective were DnaK_{E217A,V218A}, DnaK_{V210R} and DnaK_{R167H} (Fig. 3c and 3d). Others, including DnaK_{Y145A,N147A,D148A}, DnaK_{R84E}, DnaK_{D208R}, DnaK_{D224K,T225A}, DnaK_{N170A,T173A}, DnaK_{D211R} and DnaK_{E209C} were partially defective in Hsp90_{Ec} binding (Fig. 3c and 3d). One mutant, DnaK_{Y145A,N147A,D148A}, was further evaluated and found to have ~ 3 -fold lower affinity for Hsp90_{Ec} than wild-type DnaK; the K_d was $\sim 44 \mu\text{M}$ (Fig. 3e). DnaK_{S234A,I237A,N238A} was the least defective mutant protein tested (Fig. 3c and 3d). These results show that the DnaK residues identified by molecular docking are important for the direct interaction with Hsp90_{Ec}.

Based on our previous observation that a model client protein, ribosomal protein L2, stabilizes the DnaK-Hsp90_{Ec} interaction [30], we next tested if L2 similarly stabilizes the physical interaction between the DnaK mutant proteins and Hsp90_{Ec}. We used an in vitro protein-protein interaction assay (pull down assay) in which DnaK wild-type or mutant protein was incubated with biotin labeled Hsp90_{Ec} in the presence of L2. Biotinylated Hsp90_{Ec} and associated proteins were then captured on neutravidin agarose beads and analyzed by SDS-PAGE. As shown previously [30], in the absence of L2 the interaction between Hsp90_{Ec} and DnaK wild-type was not detectable by Coomassie staining of the gels (Fig. 4a, compare lanes 1 and 2), although the weak interaction could be detected by Western blot analysis [30]. Similarly, interaction between Hsp90_{Ec} and the DnaK mutant proteins in the absence of L2 was not observed by Coomassie staining (Fig. 4a, lanes 4-14). In the presence of L2, the three DnaK mutants that were most defective in binary interaction with Hsp90_{Ec} (Fig. 3c and 3d), including DnaK_{E217A,V218A}, DnaK_{R167H} and DnaK_{V210R}, were most defective in interaction with Hsp90_{Ec} in the pull-down assay (Fig. 4b and 4c). Other mutants were partially defective, including DnaK_{R84E}, DnaK_{N170A,T173A}, DnaK_{D211R}, DnaK_{Y145A,N147A,D148A}, DnaK_{D208R} and DnaK_{E209C} (Fig. 4b and 4c). DnaK_{D224K,T225A}, which was partially defective in binary interaction with Hsp90_{Ec}, bound Hsp90_{Ec} in the presence of L2 to a similar extent as DnaK wild-type (Fig. 4b and 4c). This observation suggests that L2 rescues the defective Hsp90_{Ec}-DnaK_{D224K,T225A} interaction. The mutant that was least defective in the BLI assay, DnaK_{S234A,I237A,N238A}, bound Hsp90_{Ec} in the presence of L2 similarly to DnaK wild-type (Fig. 4b and 4c). In control experiments, all of the DnaK mutants were able to bind L2 like DnaK wild-type, showing that the differences in complex formation in the presence of L2 were not due to defects in client binding by the DnaK mutant proteins (Supplemental Fig. S10c). Together these results indicate that many of the DnaK mutant proteins are defective in direct physical interactions with Hsp90_{Ec} and show decreased ability to interact with Hsp90_{Ec} in the presence of L2.

DnaK mutants are defective in functioning synergistically with Hsp90_{Ec}

We next wanted to determine whether DnaK mutants that are defective in their ability to physically interact with Hsp90_{Ec} are also defective in collaborating synergistically with Hsp90_{Ec} in functional assays. It has previously been shown that ATP hydrolysis is stimulated ~2-fold above additive by the combined action of Hsp90_{Ec} and DnaK in the presence of ribosomal protein L2, demonstrating a functional collaboration between the two chaperones [30] (Fig. 5). This synergy requires client binding by DnaK and Hsp90_{Ec} as well as ATP hydrolysis by both chaperones [30]. Although all of our DnaK mutant proteins bound L2 similarly to wild-type (Supplemental Fig. S10c) and hydrolyzed ATP (Supplemental Fig. S10d), we observed that several of the DnaK mutants showed no synergy in ATP hydrolysis in combination with Hsp90_{Ec} and L2, including DnaK_{E217A,V218A}, DnaK_{V210R} and DnaK_{D208R} (Fig. 5). Others stimulated ATP hydrolysis 1.1 to 1.3-fold above additive compared to the 1.9-fold stimulation seen with wild-type DnaK, including DnaK_{R84E}, DnaK_{N170A,T173A}, DnaK_{R167H}, DnaK_{Y145A,N147A,D148A}, DnaK_{D211R} and DnaK_{E209C} (Fig. 5). DnaK_{S234A,I237A,N238A} was slightly defective in stimulating ATP hydrolysis, consistent with its slight defect in the BLI assay. In combination with Hsp90_{Ec} and L2, DnaK_{D224K,T225A} stimulated ATP hydrolysis 2.7-fold above additive (Fig. 5). The stabilization of the Hsp90_{Ec}-DnaK_{D224K,T225A} interaction by L2 (Fig. 4b and 4c) may explain the increased synergy in ATP hydrolysis. Taken together, the results indicate that the DnaK mutants that are defective or partially defective in physical interaction with Hsp90_{Ec} are defective or partially defective in functional interaction as well, with the exception of DnaK_{D224K,T225A}.

We next tested if the DnaK mutants were also defective in functional collaboration with Hsp90_{Ec} in client protein remodeling. We assessed remodeling ability by monitoring reactivation of heat-inactivated luciferase by the combination of the DnaK chaperone system and Hsp90_{Ec}. In this assay, the chaperone concentrations were optimized in order to observe the largest dependence on Hsp90_{Ec}. Under these conditions, DnaK and two cochaperones, CbpA (a DnaJ homolog in *E. coli*) and GrpE (a nucleotide exchange factor), catalyze luciferase reactivation at a slow rate and Hsp90_{Ec} stimulates the rate ~6-fold [30] (Fig. 6a). The percentage of luciferase reactivated is low; however, only ~20% of the total heat-inactivated luciferase is soluble and available for reactivation [23]. When we tested our DnaK mutant proteins, all were defective or partially defective in functioning synergistically with Hsp90_{Ec} (Fig. 6). DnaK_{R167H}, DnaK_{E217A,V218A} and DnaK_{V210R}, reactivated luciferase at rates similar to DnaK wild-type in the absence of Hsp90_{Ec}, but were not stimulated or only slightly stimulated by Hsp90_{Ec} (Fig. 6b-6d). These three DnaK mutants were also the most defective in binary complex formation with Hsp90_{Ec} (Fig. 3) and in complex formation with Hsp90_{Ec} and L2 (Fig. 4). The remainder of the mutants with the exception of DnaK_{D208R} were also defective or partially defective in synergistic reactivation of luciferase with Hsp90_{Ec}. However, in the absence of Hsp90_{Ec} several of these mutants were similar to DnaK wild-type in the ability to reactivate luciferase with CbpA and GrpE, including DnaK_{D211R}, DnaK_{E209C} and DnaK_{D224K,T225A} (Fig. 6e-6g), while others, including DnaK_{N170A,T173A}, DnaK_{S234A,I237A,N238A}, DnaK_{Y145A,N147A,D148A} and DnaK_{R84E}, were more active than DnaK wild-type (Fig. 6h-6k). The reason for the increased activity of these mutants with CbpA and GrpE is not understood. One mutant, DnaK_{D208R},

was defective in the absence and presence of Hsp90_{Ec} (Fig. 6l), consistent with previous results showing that this mutant is defective in J-domain stimulated ATPase activity [38]. Altogether, the results indicate that many of the DnaK mutants are defective or partially defective in their ability to function collaboratively with Hsp90_{Ec} in protein reactivation. They suggest that the functional defects of the DnaK mutants are a consequence of defects in the physical interaction between the mutants and Hsp90_{Ec}.

In summary, we have identified a region of DnaK that is required for the interaction of DnaK with Hsp90_{Ec}. The DnaK mutants define a site located in the nucleotide-binding domain of DnaK that is essential for Hsp90_{Ec} interaction. This region overlaps the DnaK site known to bind DnaJ, suggesting an interplay between DnaK, DnaJ and Hsp90_{Ec} during protein remodeling.

Discussion

In this work, we identified a region in the nucleotide-binding domain of DnaK that is important for the physical and functional interaction with a region in the middle domain of Hsp90_{Ec}. Direct interactions between eukaryotic Hsp90 and Hsp70 have been observed previously. A recent study showed a direct interaction between the mitochondrial Hsp90 and Hsp70 homologs, TRAP1 and Mortalin [43]. Like bacteria, mitochondria lack Hop/Sti1, a cochaperone known to interact simultaneously with both Hsp90 and Hsp70 and facilitate the Hsp90-Hsp70 interaction [1, 44]. Therefore, the mechanism of action of TRAP1 may be more similar to that of bacterial Hsp90 than eukaryotic cytoplasmic Hsp90. However, in studies using crude lysates from rabbit reticulocytes or using purified cytoplasmic Hsp90 and Hsp70 from *Neurospora crassa* [45–47], direct interactions were also observed. In both systems, Hop/Sti1 increased the amount of Hsp90 and Hsp70 in the complex [45–47]. Thus, the functional significance of the observed direct interaction between cytoplasmic chaperones is not clear, and current models suggest that eukaryotic Hsp90 and Hsp70 need not contact one another directly, but rather Hop/Sti1 forms a bridge between the two chaperones [3, 4, 15, 16, 44].

Our finding that many residues in the DnaK NBD that are involved in Hsp90_{Ec} interaction are also involved in binding DnaJ/CbpA [38–40, 48], suggests the possibility of a functional interplay of DnaK with Hsp90_{Ec} and DnaJ/CbpA. One hypothesis that invokes a DnaK binding site shared by DnaJ/CbpA and Hsp90_{Ec} is that Hsp90_{Ec} participates in displacing DnaJ/CbpA from DnaK once DnaJ/CbpA has promoted ATP hydrolysis by DnaK. This is consistent with our previous studies that showed substoichiometric concentrations of CbpA (~1:100, CbpA:Hsp90_{Ec}) facilitated formation of the DnaK-Hsp90_{Ec}-L2 ternary complex while CbpA was not detected in the complex [30]. Previous studies have also suggested a similar role for Hsp40, where Hsp40 stimulated formation of Hsp90-Hop-Hsp70 complexes but was not seen in the final ternary complex [49–51]. Together, these results suggest that the overlapping binding site on DnaK should lead to competition between DnaJ/CbpA and Hsp90_{Ec}. However, in the assays used above, we have not seen competition, suggesting the reaction pathway may be more complex.

In *E. coli*, the concentrations of molecular chaperones and cochaperones varies under different conditions (i.e. cellular stress or starvation) and growth phase. At 30 °C in log phase growth, DnaK is highly abundant with a concentration ~5-fold higher than Hsp90_{Ec} or GrpE, ~25-fold higher than DnaJ and >25-fold higher than CbpA [52, 53]. However, CbpA expression increases as cells enter stationary phase and DnaK may only be ~2-fold higher in concentration than CbpA in late stationary phase [54]. This suggests that the interplay between DnaK and DnaJ/CbpA or Hsp90_{Ec} likely varies depending on the growth phase or stress condition.

Our current working model for the mechanism of protein remodeling by Hsp90_{Ec} and DnaK is speculative, but consistent with the current knowledge of the bacterial chaperones (Fig. 7). First, the client protein is bound by DnaK, in a reaction requiring ATP hydrolysis and facilitated by DnaJ/CbpA and GrpE (Fig. 7, step 1). This is in keeping with our previous work showing the DnaK chaperone system functions prior to Hsp90_{Ec} in protein remodeling [23]. Then, client-bound DnaK recruits Hsp90_{Ec}, likely through a direct interaction between the DnaK NBD and the middle domain of Hsp90_{Ec}. Based on our molecular docking results, a client protein bound to the SBD of DnaK would be poised for interaction with the client binding site of Hsp90_{Ec} (Fig. 7, step 2). The binding of Hsp90_{Ec} to DnaK may displace DnaJ/CbpA, since our results suggest DnaJ/CbpA and Hsp90_{Ec} bind overlapping regions on DnaK. Next, ATP binding and hydrolysis by Hsp90_{Ec} leads to conformational changes in Hsp90_{Ec} that promote client transfer from DnaK, stabilize client binding by Hsp90_{Ec} and may release DnaK (Fig. 7, step 3). Then, nucleotide release from Hsp90_{Ec} likely causes the release of an active client (Fig. 7, step 4). However, in cases where the client does not attain its active conformation it may enter another cycle of remodeling. Taken together, these studies are providing insight into the interactions and collaboration between Hsp90 and Hsp70.

Materials and Methods

Plasmids and Strains

Substitution mutations of Hsp90_{Ec} and DnaK were made with the QuikChange Lightning mutagenesis kit (Agilent) using pET-HtpG [23], pRE-DnaK [55], pET-DnaK [56] or pT25-DnaK [23]. All mutations were verified by DNA sequencing.

Proteins

Hsp90_{Ec} wild-type and mutants [23], DnaK wild-type and mutants [55], CbpA [57], GrpE [55] and His-tagged L2 [58] were isolated as described. All proteins were >95% pure as determined by SDS-PAGE. All DnaK substitution mutants exhibited similar physical properties as the wild-type, including partial proteolysis patterns with and without ATP (Supplemental Fig. S9a) and CD spectra (Supplemental Fig. S9b). All exhibited some DnaK functional activities, including: reactivation of GFP with DnaJ and GrpE (Supplemental Fig. S10a) and further stimulation of GFP reactivation by ClpB (Supplemental Fig. S10b), client binding (L2) (Supplemental Fig. S10c) and basal ATPase activity (Supplemental Fig. S10d). The Hsp90_{Ec} E584C-biotin mutant was similar to Hsp90_{Ec} wild-type in luciferase reactivation activity (Supplemental Fig. S11a). Luciferase and luciferin were from Promega.

Concentrations given are for Hsp90_{Ec}, CbpA and GrpE dimers and DnaK, L2 and luciferase monomers. Hsp90_{Ec} E584C was labeled using a 20-fold excess of Maleimide-PEG₁₁-Biotin (Thermo, Life Technologies) as recommended by the manufacturer. CbpA was labeled using a 1.5-fold excess of NHS-PEG4-Biotin (Thermo, Life Technologies). Excess biotin reagent was removed by extensive dialysis.

Luciferase reactivation

Luciferase reactivation was performed as previously described [23, 30]. 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 wild-type or mutant, 0.15 µM CbpA, 0.05 µM GrpE and 0.5 µM Hsp90_{Ec}. 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. To test for potential defects in binding between the DnaK mutants and CbpA that could lead to defects in luciferase reactivation, we performed pull-down assays with our DnaK mutants using biotinylated-CbpA (Supplemental Fig. S11b).

ATPase activity

Steady state ATP hydrolysis was measured at 37 °C in 25 mM Hepes, pH 7.5, 50 mM KCl, 5 mM DTT, 5 mM MgCl₂ and 2 mM ATP using a pyruvate kinase/lactate dehydrogenase enzyme-coupled assay as described [18] and using 1 µM Hsp90_{Ec}, 1 µM L2 and 1 µM DnaK wild-type or mutant.

Bio-Layer Interferometry (BLI) Assay

BLI was used to monitor the interaction between Hsp90_{Ec} and DnaK using a FortéBio (Menlo Park, CA) Octet RED96 instrument and streptavidin (SA) biosensors at 30 °C. Each assay step was 200 µL containing BLI buffer (20 mM Tris, pH 7.5, 25 mM NaCl, 0.01% Triton X-100 (vol/vol), 0.02% Tween-20 (vol/vol), 1 mg/ml BSA, 1 mM ATP, and 10 mM MgCl₂). Hsp90_{Ec} E584C-biotin was loaded on the biosensors to a BLI response signal of 1 nm. The biosensors were blocked in BLI buffer containing 10 µg/ml biocytin (Sigma) for 1 min, and a baseline was established in BLI buffer alone. Association of DnaK wild-type or mutant (5 to 100 µM) to the Hsp90-biotin bound sensor tip in BLI buffer was monitored over time. Lastly, dissociation was monitored in BLI buffer alone. For each experiment, nonspecific binding was monitored using a reference biosensor subjected to each of the above steps in the absence of biotinylated Hsp90_{Ec} and non-specific binding signal was subtracted from the corresponding experiment. For steady state analysis of kinetic association data, the association curve at each DnaK concentration was fit using a single exponential equation without constraints in Prism (GraphPad Software, La Jolla California USA, www.graphpad.com) and the plateau value determined from the fit was plotted versus the concentration of DnaK. The resulting binding curve was analyzed using a one-site specific binding model in Prism to determine the K_d and B_{max} values.

Protein-protein interaction assay

Interaction of Hsp90_{Ec} with DnaK in the presence of L2 was measured using a pull down assay. Hsp90_{Ec} E584C-biotin (1.5 μ M) was incubated for 5 minutes at 23 °C in reaction mixtures (50 μ L) containing GPD 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) with 2 μ M DnaK wild-type or mutant, and 2.3 μ M L2. Neutravidin agarose (40 μ L 1:1 slurry) (Thermo, Pierce) was then added and incubated 5 min at 23 °C with mixing. The reactions were diluted with 0.4 mL GPD buffer, centrifuged 1 min at $1000 \times g$ and the recovered agarose beads were washed twice with 0.4 mL GPD buffer. Bound proteins were eluted with buffer containing 2 M NaCl and analyzed by Coomassie blue staining following SDS-PAGE. Where indicated, protein band intensities from replicate gels were quantified using ImageJ (<http://imagej.nih.gov/ij>). The results were normalized to Hsp90_{Ec} E584C-biotin and the ratio of DnaK mutant relative to DnaK wild-type was calculated and plotted.

Bacterial two-hybrid assay

Bacterial two-hybrid assays were performed as previously described [23, 42].

Modeling DnaK-Hsp90_{Ec} interaction

Starting with the ADP-bound conformation of DnaK, PDB ID 2KHO [31], or separately, the ATP-bound form of DnaK, PDB ID 4BQ9 [32], the CHARMM molecular modeling program [59] was used to build in missing atoms as described previously [60]. Similarly, the dimeric structure of ADP-bound Hsp90_{Ec} was constructed from biological assembly 1 of PDB ID 2IOP [21] using CHARMM to build in missing atoms. The dimeric structure of apo Hsp90_{Ec} had several missing regions at positions 1-15, 98-114, 493-501 and 544-565 [21]. Therefore, we constructed a full length monomer using the I-TASSER structure prediction software [61–63] by threading the sequence of *E. coli* Hsp90 along the apo conformation of Hsp90_{Ec} (PDB ID 2IOQ, chain A). Two copies of the resulting full-length monomer were then oriented as to minimize the RMSD with each monomer of the dimer to create a dimer of apo Hsp90_{Ec}.

Hsp90_{Ec}-DnaK complexes were generated using ZDOCK [33, 34] (version 2.3), which employs rigid body docking and utilizes a scoring function based on pairwise shape complementarity, desolvation, and electrostatic energies. Docking complexes were created without restraining the interaction between DnaK and Hsp90_{Ec}. The top 2000 docked complexes from ZDOCK were then reranked using ZRANK [35]; a detailed scoring function that takes into account desolvation energy and both attractive and repulsive van der Waals and electrostatic energies that are calculated using the CHARMM19 polar hydrogen force-field [64]. The docked complex with the best score was selected for each combination: 1) ADP-bound DnaK with apo Hsp90_{Ec}, 2) ADP-bound DnaK with ADP-bound Hsp90_{Ec}, 3) ATP-bound DnaK with apo Hsp90_{Ec} and 4) ATP-bound DnaK with ADP-bound Hsp90_{Ec}.

Contacts were computed by calculating distances from the center of geometry of residue pairs within an 8 Å distance measured from alpha carbons. This larger distance cutoff value was used to define a general region of DnaK that may participate in binding Hsp90_{Ec}, which aided in the selection of residues for DnaK substitution mutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

NBD	nucleotide-binding domain
SBD	substrate-binding domain
NEF	nucleotide exchange factor

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Research Highlights

1. Molecular docking predicts Hsp90_{Ec} interacts with the ADP conformation of DnaK
2. The predicted Hsp90_{Ec} interacting region is in the DnaK nucleotide-binding domain
3. Many predicted Hsp90_{Ec} interacting residues on DnaK are involved in DnaJ binding
4. Substitutions in predicted DnaK residues cause defects in interaction with Hsp90_{Ec}
5. The DnaK mutants are defective in collaboration with Hsp90_{Ec} in protein remodeling

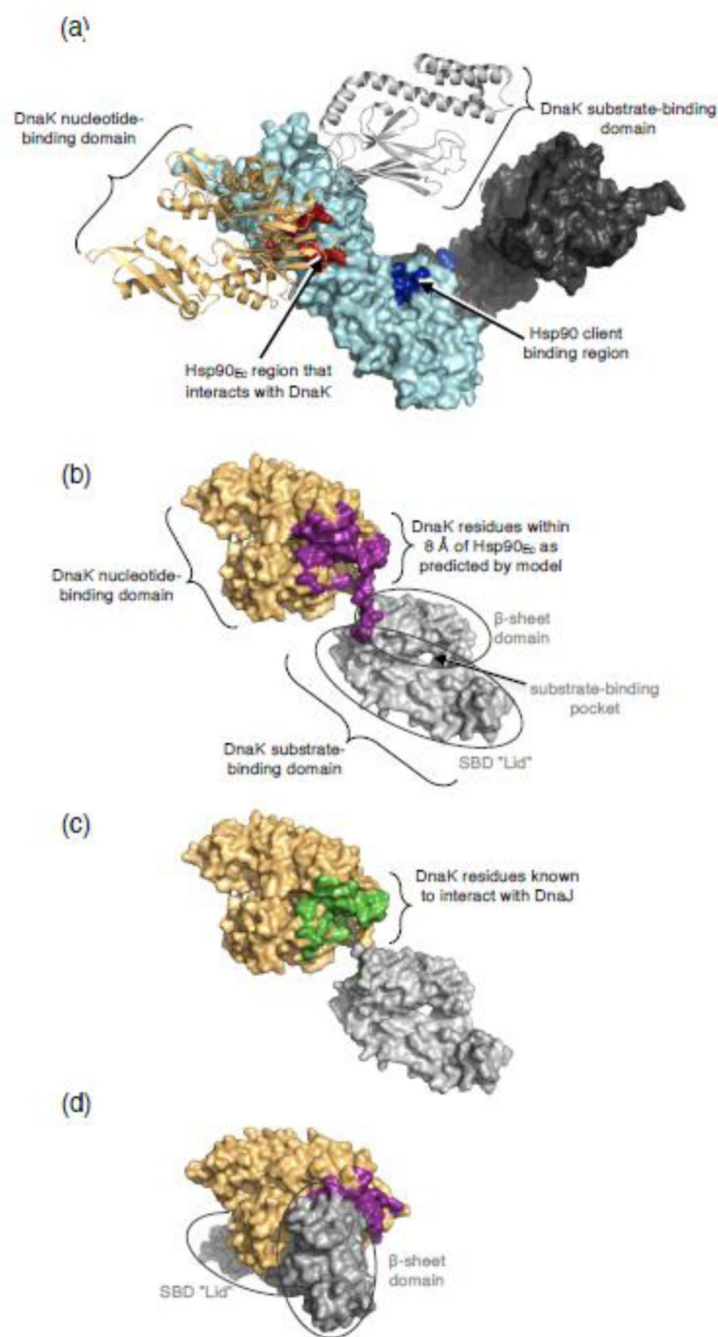
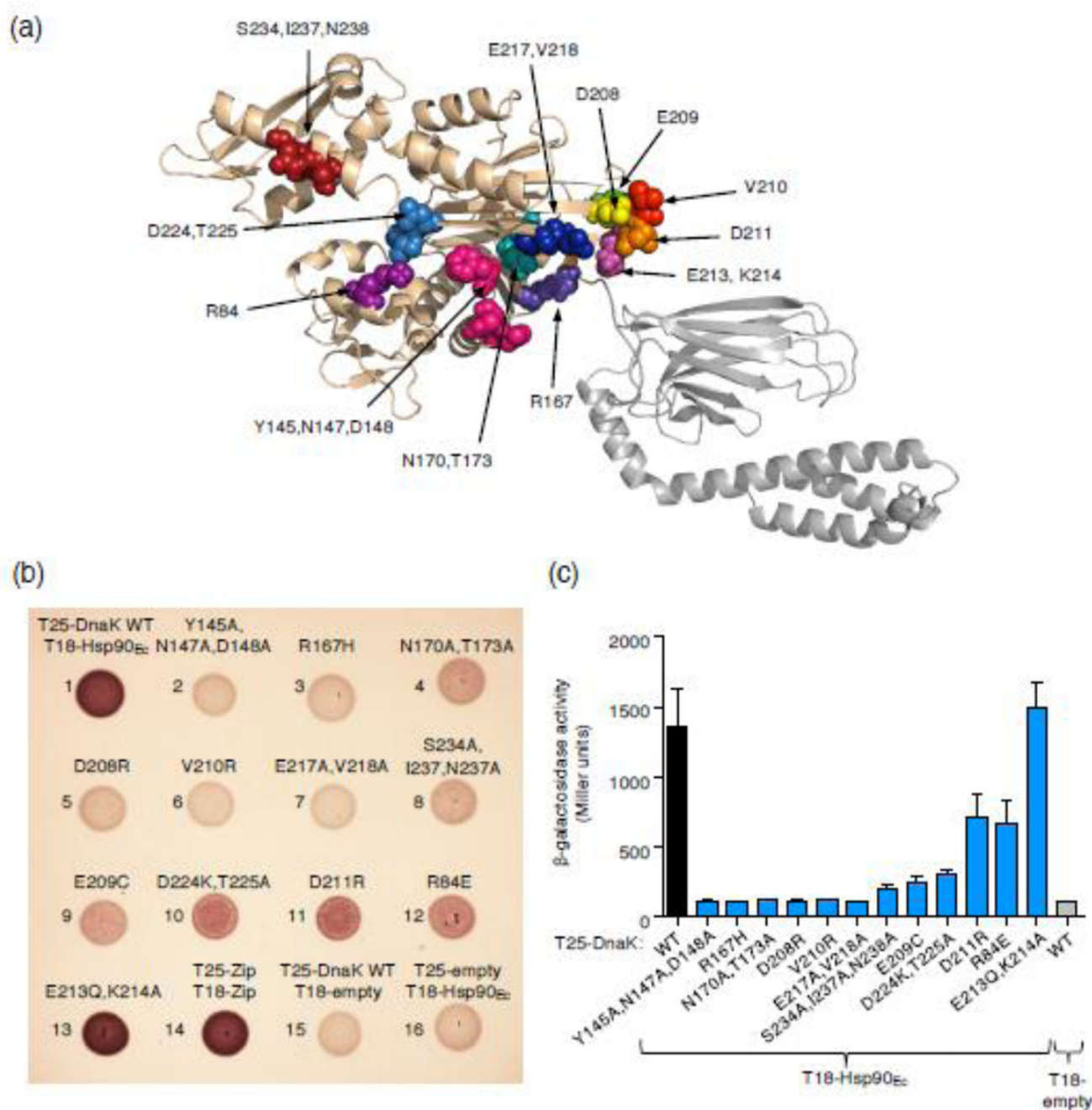


Fig. 1. Regions of interaction on DnaK and Hsp90_{Ec}. (a) Docked model of the apo structure of Hsp90_{Ec} [21] and ADP-bound DnaK [31] as determined using ZDOCK and ZRANK and described in Materials and Methods. Hsp90_{Ec} is shown as a surface rendering with one protomer in dark gray and one protomer in light cyan. The DnaK interacting region of Hsp90_{Ec} [30] is shown in red while the client binding region is in blue [14]. DnaK in the ADP-bound conformation is shown as a ribbon model with the NBD in light orange and the SBD in light gray. (b) DnaK in the ADP-bound conformation [31] showing residues (purple)

on DnaK within 8 Å of Hsp90_{Ec} as predicted by the docked model in (a). In (b-d) DnaK is shown as a surface rendering with the NBD in light orange and the SBD in light gray. (c) DnaK in the ADP-bound conformation [31] showing residues (green) experimentally identified as interacting with DnaJ [38–40]. (d) DnaK in the ATP-bound conformation [32] showing residues (purple) on DnaK within 8 Å of Hsp90_{Ec} as predicted by the docked model in (a). In the ATP-bound conformation, only some of the DnaK residues within 8 Å of Hsp90_{Ec} in the model are surface exposed. Images in (a-d) were made using PyMOL (Schrodinger, LLC; www.pymol.org).

**Fig. 2.**

Identification of DnaK amino acid residues involved in Hsp90_{Ec} interaction in vivo. (a) Model of *E. coli* DnaK in the ADP-bound conformation [31] with the mutated residues used in this study shown as CPK models. The NBD is colored light orange and the SBD is gray. DnaK was rendered using PyMOL. (b, c) Interaction between DnaK wild-type or mutant and Hsp90_{Ec} in a bacterial two-hybrid system in vivo, as described in Materials and Methods. Interaction was measured by monitoring β -galactosidase activity on MacConkey indicator plates (b) and in liquid assays (c). In (b), a representative plate from three independent

experiments is shown with colonies labeled 1 through 16. For colonies 2 through 13, all DnaK substitution mutants, named by substituted residues, have been constructed in T25-DnaK and are present in reactions with the T18-Hsp90_{Ec} construct. In (c), β -galactosidase activity is shown as mean \pm SEM ($n = 3$) and are also presented in Supplemental Table S5.

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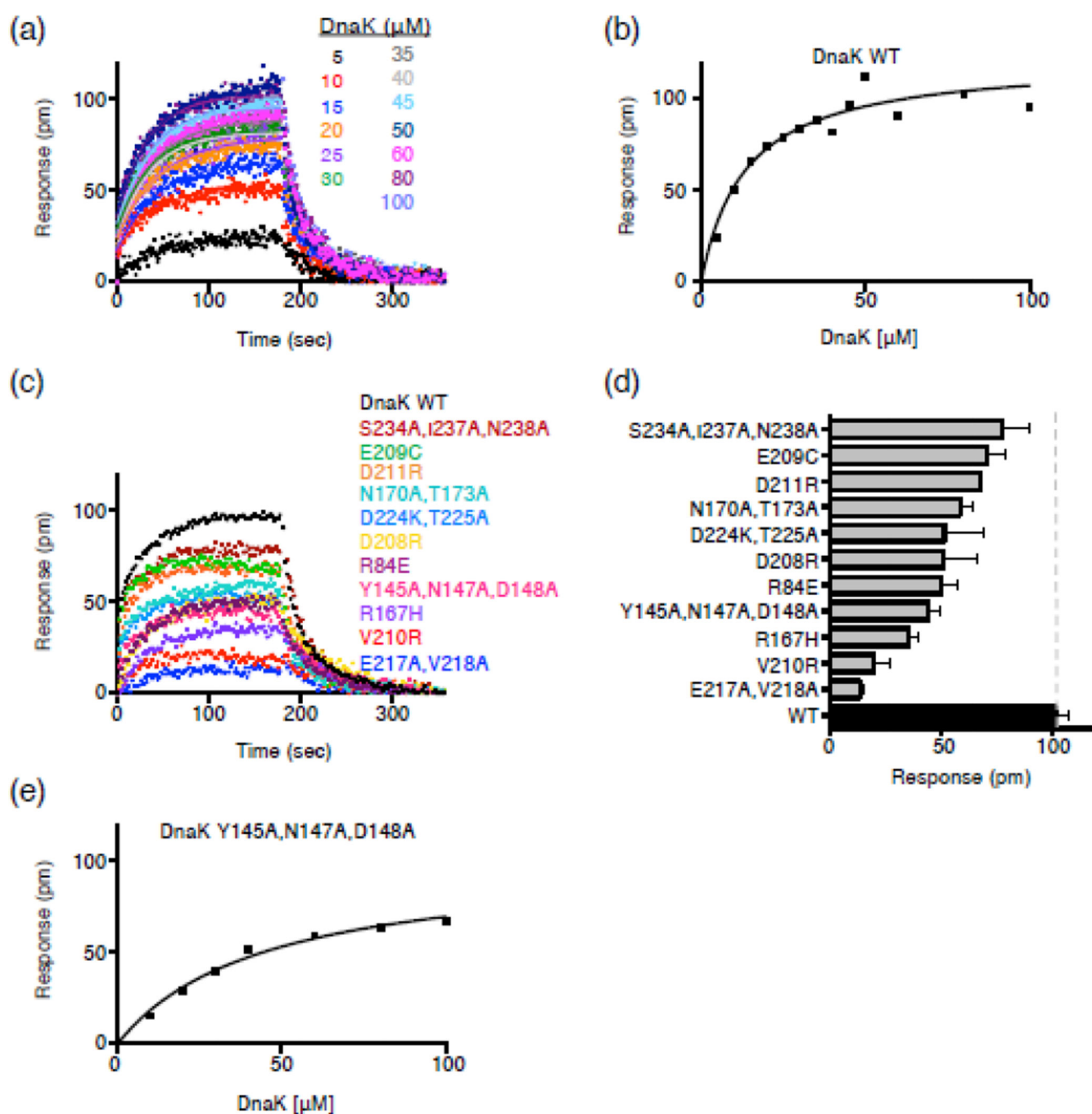
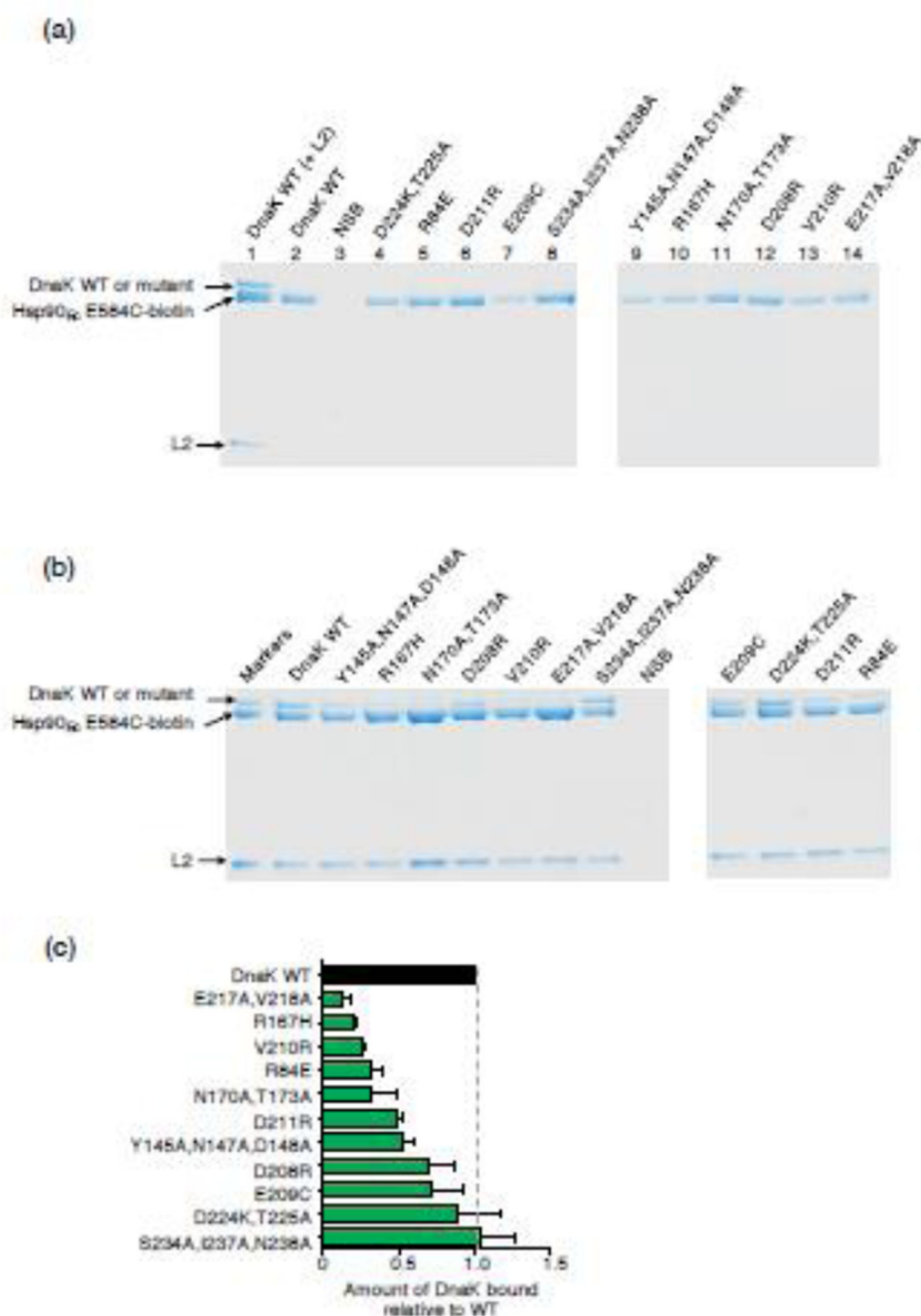


Fig. 3. DnaK NBD substitution mutants are defective in direct interaction with Hsp90_{Ec} in vitro. (a) Bio-Layer Interferometry (BLI) was used to monitor the kinetics of association and dissociation between biotinylated Hsp90_{Ec} and DnaK wild-type as described in Materials and Methods. Representative curves are shown for numerous concentrations of DnaK as indicated. The single-exponential fit of the association step was used to obtain the response value (pm) for the plateau plotted in (b). (b) Steady-state analysis of the DnaK wild-type-Hsp90_{Ec} interaction. The response value (pm) for the plateau obtained in (a) is plotted vs.

each DnaK concentration and fit as described in Materials and Methods. The K_d and B_{max} for the Hsp90_{Ec} interaction with DnaK wild-type are $13.4 \pm 3.3 \mu\text{M}$ and $0.12 \pm 0.01 \text{ nm}$, respectively. (c) Curves showing association and dissociation of 50 μM DnaK wild-type (black) or mutant (colored) and biotinylated Hsp90_{Ec} using BLI. (d) Average plateau response value for the interaction between DnaK wild-type or mutant and biotinylated Hsp90_{Ec}. Data are plotted as mean \pm SEM (n=2 or more) and are also presented in Supplemental Table S5. (e) Steady-state analysis of the DnaK_{Y145A,N147A,D148A}-Hsp90_{Ec} interaction as described in (b). The K_d and B_{max} for the Hsp90_{Ec} interaction with DnaK_{Y145A,N147A,D148A} are $44.4 \pm 7.5 \mu\text{M}$ and $0.10 \pm 0.01 \text{ nm}$, respectively.

**Fig. 4.**

DnaK mutants exhibit defective interaction with Hsp90_{Ec} in the presence of L2. Hsp90_{Ec}-biotin was incubated with DnaK wild-type or mutant, without or with L2. Hsp90_{Ec}-biotin associated proteins were monitored using a pull-down assay and analyzed by Coomassie blue staining following SDS-PAGE as described in Materials and Methods. (a) In control experiments, Hsp90_{Ec}-biotin was incubated with DnaK wild-type with L2 (lane 1) or without L2 (lane 2) or with DnaK mutant proteins without L2 (lanes 4-14). DnaK wild-type is seen in association with Hsp90_{Ec}-biotin in the presence of L2 (lane 1), but not in the

absence of L2 by this analysis (lane 2); however, the weak association between Hsp90_{Ec} and DnaK is seen by Western blot analysis [30]. NSB indicates non-specific binding of DnaK wild-type in the absence of Hsp90_{Ec}-biotin. (b) Interaction between Hsp90_{Ec}-biotin and DnaK wild-type or mutant in the presence of L2 was determined as in a. Pure proteins are shown in the first lane as markers. NSB indicates non-specific binding of DnaK wild-type and L2 in the absence of Hsp90_{Ec}-biotin. The gels shown are representative of at least three independent experiments. (c) Quantification of DnaK wild-type or mutant associated with Hsp90_{Ec}-biotin in the presence of L2. The results were normalized to Hsp90_{Ec}-biotin and the ratio of DnaK mutant to wild-type was plotted. Data from three or more replicates are presented as mean \pm SEM and are also presented in Supplemental Table S5. The gray dashed line indicates DnaK wild-type binding and is meant to aid the eye.

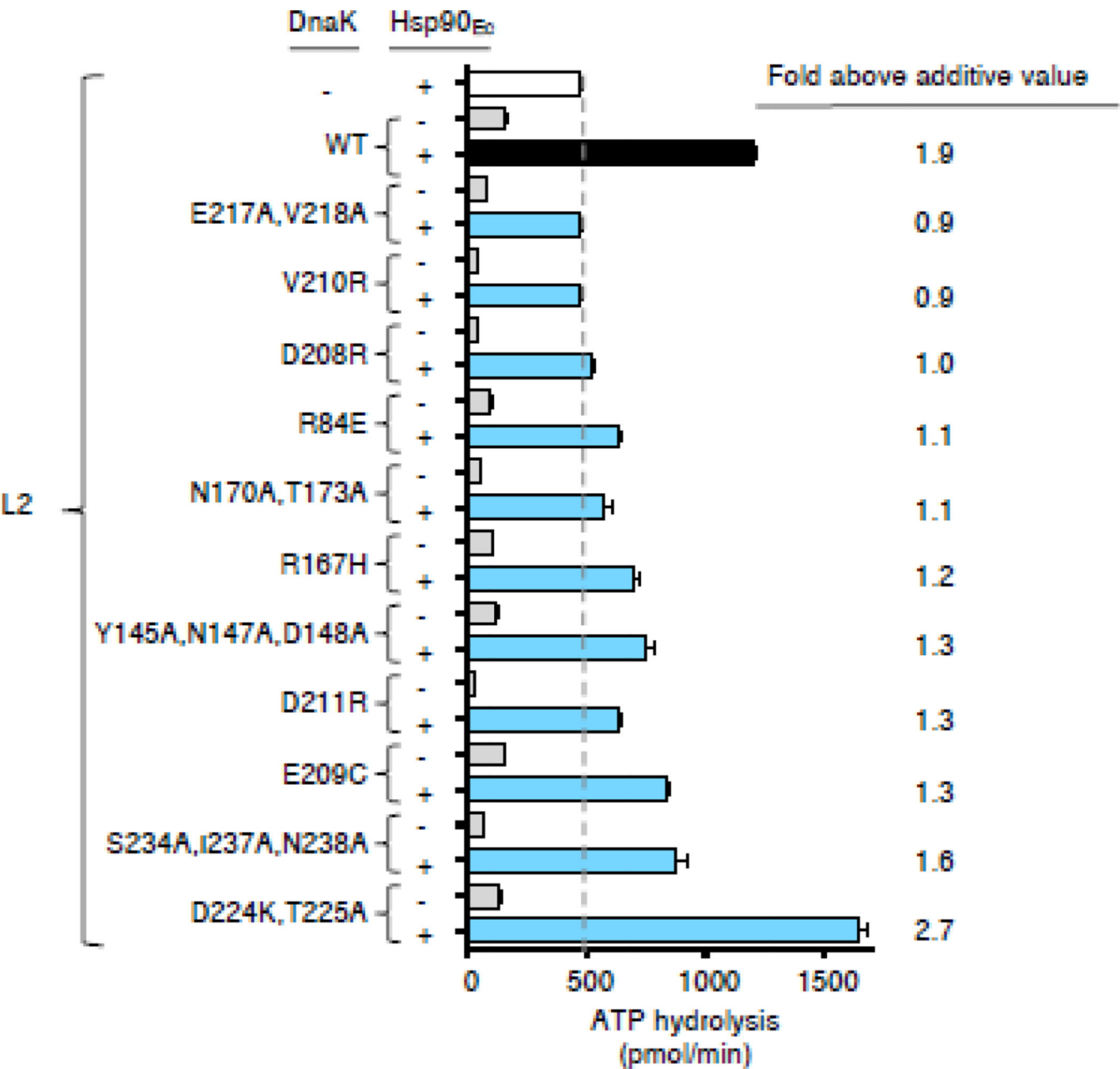


Fig. 5. DnaK mutants defective in Hsp90_{Ec} interaction are defective in synergistic stimulation of ATP hydrolysis with Hsp90_{Ec} and a client protein, L2. ATP hydrolysis by DnaK wild-type or mutant in the presence of L2 was determined in the absence and presence of Hsp90_{Ec} as described in Materials and Methods. The fold above additive is calculated by dividing the rate of ATP hydrolysis by Hsp90_{Ec} and DnaK in the presence of L2 by the sum of the rate for Hsp90_{Ec} in the presence of L2 and the rate for DnaK in the presence of L2. Data from three or more replicates are presented as mean ± SEM and are also presented in Supplemental Table S5. The dashed line indicates the rate of ATP hydrolysis by Hsp90_{Ec} with L2 and is meant to aid the eye.

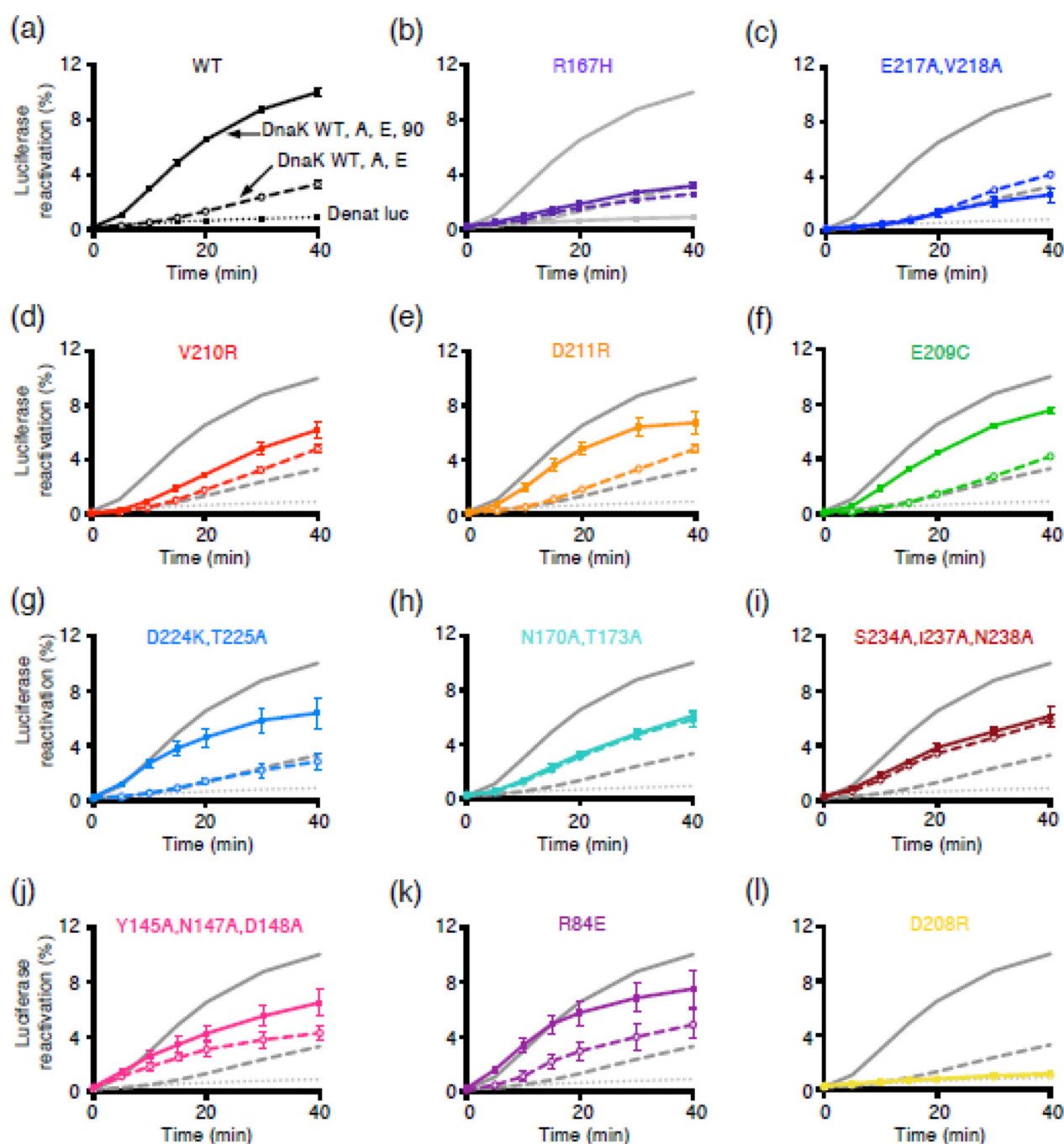


Fig. 6. DnaK NBD mutants defective in Hsp90_{Ec} interaction are defective in functional collaboration with Hsp90_{Ec} in luciferase reactivation. Heat-inactivated luciferase was reactivated as described in Materials and Methods using DnaK wild-type or mutant, CbpA, GrpE and Hsp90_{Ec} as indicated. (a-l) Luciferase reactivation by DnaK wild-type (black/gray) or mutant (color), as indicated in panels a-l in combination with CbpA and GrpE (open symbols and dashed lines) or CbpA, GrpE and Hsp90_{Ec} (filled symbols and solid lines) as a function of time. Dotted lines indicate luciferase alone control. In a-l, data from three or

more replicates are presented as mean \pm SEM. For some points, the symbols obscure the error bars.

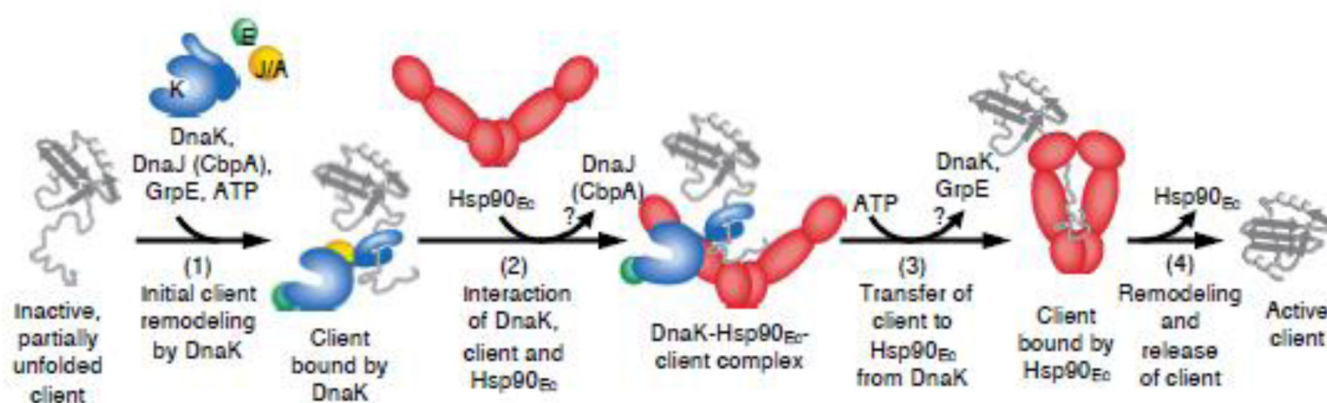


Fig. 7.

Working model for the mechanism of action of the DnaK system in collaboration with Hsp90_{Ec}. First, the client protein is bound by DnaK for initial ATP-dependent remodeling, a process that requires DnaJ/CbpA and GrpE (step 1). Next, DnaK, through a direct interaction of the DnaK NBD with the Hsp90_{Ec} middle domain, recruits Hsp90_{Ec} to the client, which further stabilizes the interaction between DnaK and Hsp90_{Ec} (step 2). DnaJ/CbpA may be released at this time. Binding and hydrolysis of ATP by Hsp90_{Ec} triggers conformational changes in the chaperone that lead to client transfer and stabilization of client binding to Hsp90_{Ec} (step 3). DnaK and GrpE may be released at this step. Hsp90_{Ec} promotes further client remodeling and releases the active native client (step 4). Client proteins that do not attain the active conformation may reenter the chaperone cycle.

Table 1

DnaK residues within 8 Å of Hsp90_{Ec} residues in the model of the ADP-bound DnaK-apo Hsp90_{Ec} complex.

DnaK Residue	Hsp90 _{Ec} Residue
144	237, 267
145	237
147	303
148	303
167	270
170	269
173	269
206	355
208	351, 354, 355
210	354, 391
211	347, 350, 351, 354
212	347, 350
214	268, 270, 271, 272, 347, 348, 351
215	268, 269, 270, 271, 272, 274, 351
216	268, 269, 270, 271
217	266, 267, 268, 269, 355
218	266, 267, 268, 269
219	265, 266, 355
220	238
221	237, 239, 265
222	237, 239
223	239
322	239
326	238, 239
327	238
328	362
329	362
386	271
387	271
388	271, 272, 329, 344
389	272, 329, 330, 331, 344
390	329, 330, 331, 338, 339, 342, 344
391	331, 334, 335, 338, 339, 342
392	331, 333, 334, 338
393	333

Red indicates DnaK residues shown in this study to be important for interaction with Hsp90_{Ec}. Blue indicates Hsp90_{Ec} residues previously shown to be important for the interaction with DnaK [30]. Green indicates Hsp90_{Ec} residues identified in this study to be important for interaction with DnaK. Black indicates residues not tested in this study, except for DnaK residue 214, which when mutated was similar to DnaK wild-type (Fig. 2). Bold, italics indicates DnaK residues interacting with DnaJ as identified from previous studies [38–40].