Analysis of Hsp90 Cochaperone Interactions Reveals a Novel Mechanism for TPR Protein Recognition[†]

Ahmed Chadli,* Elizabeth S. Bruinsma, Bridget Stensgard, and David Toft

Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, Minnesota 55905

Received November 26, 2007; Revised Manuscript Received January 4, 2008

ABSTRACT: The chaperone Hsp90 is required for the appropriate regulation of numerous key signaling molecules, including the progesterone receptor (PR). Many important cochaperones bind Hsp90 through their tetratricopeptide repeat (TPR) domains. Two such proteins, GCUNC45 and FKBP52, assist PR chaperoning and are thought to interact sequentially with PR−Hsp90 complexes. TPR proteins bind to the C-terminal MEEVD sequence of Hsp90, but GCUNC45 has been shown also to bind to a novel site near the N-terminus. We now show that FKBP52 is also able to bind to this site, and that these two cochaperones act competitively, through Hsp90, to modulate PR activity. The N-terminal site involves noncontiguous amino acids within or near the ATP binding pocket of Hsp90. TPR interactions at this site are thus strongly regulated by nucleotide binding and Hsp90 conformation. We propose an expanded model for client chaperoning in which the coordinated use of TPR recognition sites at both N- and C-terminal ends of Hsp90 enhances its ability to coordinate interactions with multiple TPR partners.

Hsp90[1] is intimately connected to numerous cellular functions through an extended molecular network of cochaperones and client proteins (*1*). Many Hsp90 client proteins are key signal transduction molecules, and Hsp90 coordinates the actions of a variety of chaperones and cochaperones to bring these clients to their functional conformation (*2–4*). Chaperoning steroid receptors through the Hsp90 pathway has been studied extensively, and three main complexes have been described for the chaperoning of the progesterone receptor (PR) through this pathway (*5, 6*). An early complex is initiated by the binding of Hsp40 to PR followed by incorporation of Hsp70 and Hip. Recruitment of Hop and Hsp90 generates an intermediate PR complex that is still unable to bind hormone. Finally, the mature complex is characterized by the presence of the cochaperone p23 and one of the large immunophilins, FKBP52, FKBP51, or Cyp40. The receptor in this complex binds hormone with high affinity.

The majority of Hsp90 cochaperones in this pathway interact with Hsp90 through tetratricopepetide repeat (TPR) motifs (*7*). This repertoire of TPR cochaperones (GCUNC45, PP5, FKBP51, FKBP52, Cyp40, and others) is thought to provide a diversity of Hsp90 complexes needed to chaperone the wide variety of client proteins, yet in most cases, the individual roles of TPR cochaperones are not well understood even though their importance is clear. There is evidence that the large immunophilins facilitate nuclear import of the glucocorticoid receptor (GR) (*5, 8*). FKBP52 has been shown to enhance GR hormone binding and signaling in a yeast model (*9*). FKBP52 gene deletion in mice results in defective sexual development, caused by deficiencies in the action of PR and androgen receptors (*10–12*). The rapid exchange of immunophilins in client−Hsp90 complexes indicates a selection process for the most appropriate cochaperone for a given client at a given stage of its chaperoning (*13, 14*). Initial immunoprecipitation experiments showed, however, that PR−Hsp90 heterocomplexes contain several TPR proteins such as FKBP52, FKBP51, Cyp40, Hop, and possibly others (*15–18*). This has been interpreted to reflect an assortment of PR complexes at different stages of maturation, each complex containing only one type of TPR cochaperone. The recent identification of a second TPR binding site in the N-terminus of Hsp90 (*19*) opened the possibility of two simultaneous TPR interactions. The mechanism for this dynamic interchange and the possibility of simultaneous TPR interactions with Hsp90 are the subject of this study.

Interactions of Hsp90 with TPR proteins are mediated by the TPR motifs (*20–23*). The immunophilins and the TPR cochaperone Hop compete for binding to the C-terminus of Hsp90 (*24, 25*), and a cocrystal structure of the C-terminal MEEVD of Hsp90 with a TPR domain of Hop is available (*21*). Mutagenesis studies have shown that basic and hydrophobic residues in the TPR groove are involved in binding to Hsp90 (*26, 27*). However, sequences outside of these motifs significantly impact their binding as well (*27, 28*).

GCUNC45 is an Hsp90 cochaperone that has been studied for its activity in chaperoning myosin (*29*); however, it also appears to be involved in PR chaperoning (*19*). GCUNC45 interacts with the N-terminal domain of Hsp90 in a TPR-dependent manner (*19*). A primary function of this Hsp90 N-terminal domain is to bind ATP, which induces interaction

[†] This research was supported by NIH Grant RO1 DK46249.

* To whom correspondence should be addressed: Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905. Phone: (507) 284-2918. Fax: (507) 284-2053. E-mail: chadli.ahmed@mayo.edu.

[1] Abbreviations: GCUNC45, general cell UNC45; TPR, tetratricopeptide repeat; Hsp90, heat shock protein 90; FKBP52, FK506 binding protein 52; PR, progesterone receptor.

10.1021/bi7023332 CCC: $40.75
Published on Web 01/23/2008

between the N-terminal domains of the Hsp90 dimer (*30*). Therefore, this domain is a major site for conformational regulation of Hsp90 structure and function. GCUNC45 appears to act upstream of FKBP52 during PR chaperoning (*19*). Interestingly, in a purified system containing Hsp90, Hsp70, Hop, Hsp40, and p23, GCUNC45 inhibits PR chaperoning. However, FKBP52 reverses this repression and promotes the progression of the cycle toward the hormone binding state.

We investigated the molecular mechanism of interplay between GCUNC45 and FKBP52 and found that, like GCUNC45, FKBP52 can also bind at the N-terminal domain of Hsp90. The two cochaperones compete for one another for this site. We also show that this TPR binding site is near the ATP binding pocket of Hsp90. Mutational analysis suggests a spatial positioning of noncontiguous residues in the ATP binding domain to generate an acidic motif for binding the TPR domains of GCUNC45 and FKBP52.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. GCUNC45 with a thrombin cleavage site followed by a six-His tag at its C-terminus was cloned into the pET-23a vector using *Nde*I and *Not*I restriction sites. The cDNA was transformed into BL21-CodonPlus (DE3)-RIL cells, and protein production was induced with 0.5 mM IPTG for 3 h at 30 °C. Cell pellets were suspended in 20 mL of buffer A (10 mM Tris, 50 mM KCl, and 2.5% glycerol) supplemented with protease inhibitors [0.1 mM leupeptin, 0.1 mg/mL bacitracin, 77 μg/mL aprotinin, 1.5 μM pepstatin, and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride]. After sonication, the cell lysate was supplemented with 5 mM imidazole and 400 mM KCl and clarified by ultracentrifugation (100000*g*) for 45 min. The supernatant was loaded onto a TALON metal affinity column (BD Bioscience) equilibrated with buffer A supplemented with 5 mM imidazole and 400 mM KCl. Unbound protein was removed with extensive washing, and GCUNC45 was eluted with buffer A containing 100 mM imidazole. GCUNC45 was further purified using a UnoQ column (Bio-Rad), a heparin-Sepharose column where GCUNC45 was collected in the unbound protein fraction, and, finally, a Superdex-200 column (Pharmacia).

Human Hsp90β was expressed in SF9 cells and purified as described previously (*31*). Hsp70, Ydj1, Hop, and p23 were expressed and purified as described previously (*32*).

FKBP52 was expressed in Sf9 cells, and FKBP51 and Cyp40 were expressed in *Escherichia coli*. The proteins were purified from cell lysates by gradient elution from columns of DEAE-cellulose, hydroxyapatite, and UnoQ. The isolated proteins were stored at −70 °C.

Assembly of the Complex of the Progesterone Receptor with Purified Proteins. PR expressed in Sf9 cells was adsorbed onto PR22 antibody−protein A-Sepharose and was assembled into complexes as described previously (*32, 33*) using ~0.05 μM PR with 1.4 μM hsp70, 0.8 μM Hsp90 dimer, 0.2 μM Ydj-1, 0.08 μM Hop, and 2.6 μM p23 in reaction buffer [20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.01% Nonidet P-40, 50 mM KCl, and 5 mM ATP]. After incubation for 7 min at 37 °C or 30 min at 30 °C, 0.1 μM [^{3}H]progesterone (American Radiolabeled Chemicals, Inc., St. Louis, MO) was added for incubation on ice for 3 h. The complexes were then washed with reaction buffer and assessed for bound progesterone and protein composition.

Site-Directed Mutagenesis. Mutations in the N-terminal domain of Hsp90β were generated in pET23a-Hsp90$_{1–212}$ using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutant clones were confirmed by DNA sequencing.

Protein Binding Assays. GCUNC-45, FKBP52, and Hop were covalently linked to Sepharose 4B as described previously (*19*). BSA was immobilized as a control. To test the binding of Hsp90 and its fragments to these cochaperones, 15 μL of resin and 10 μg of protein were used in a final reaction volume of 200 μL of incubation buffer [10 mM Tris, 100 mM KCl, 0.02% NP40, 2.5% glycerol, and 2 mM DTT (pH 7.5)]. Samples were incubated for 40 min at 30 °C or 15 min at 37 °C and washed four times with 1 mL of buffer. Bound proteins were extracted with sample buffer, resolved by SDS−PAGE, and stained with Coomassie Blue. For pull-down experiments with the Hsp90 N-terminal domain, 10 μL of nickel resin was used to immobilize 2 μg of His-tagged Hsp90$_{1–212}$, washed with incubation buffer without DTT, and incubated with 10 μg of GCUNC45, FKBP52, or Hop. Alternatively, Hsp90 was bound to antibody H9010 on protein A-Sepharose.

Quantification of Protein Levels. Arbitrary densitometric values (pixel AU) for the indicated electrophoretic bands of protein were obtained by using NIH image 1.62. For evaluation of the binding of Hsp90 fragments [1–212 and 616−C-terminus (Ct)] to GCUNC45 and FKBP52, AU values were converted to micromolar equivalent by normalizing them against 1 μg samples of fragments run on the same gel. To obtain the values of the free N- and C-terminal domain of Hsp90, the amount of bound fragment was subtracted from the initial amount added to the sample. The Scatchard plots were linear and lead to the following constants: GCUNC45−Hsp90.1–212, K_d = 11 μM; GCUNC45−Hsp90.616−Ct, K_d = 4 μM; FKBP52−Hsp90.1–212, K_d = 21 μM; FKBP52−Hsp90.6–616, K_d = 4 μM.

RESULTS

FKBP52 Displaces GCUNC45 Bound to Hsp90 but Not That Bound to PR. We have previously reported that GCUNC45 interacts directly with Hsp90 and also with PR and that FKBP52 displaces GCUNC45 from the PR−Hsp90 complex (*19*). This competitive action of FKBP52 on GCUNC45 binding could focus on the interaction with PR, Hsp90, or both. These possibilities were tested in binding assays in which immobilized Hsp90 or PR (purified or in chaperone complexes) was used to test for the binding of GCUNC45 in the presence of increasing concentrations of FKBP52 (Figure 1A). These data show that FKBP52 does not compete with GCUNC45 binding to purified PR [Figure 1A (▲)]. However, FKBP52 competes with GCUNC45 bound to purified Hsp90 [Figure 1A (■)]. This competition is concentration-dependent, and a 5-fold molar excess of the immunophilin blocks 50–60% of GCUNC45 binding. These findings were confirmed in reverse, using immobilized GCUNC45 to pull down Hsp90 in the presence of increasing amounts of FKBP52 (Figure 1B). The presence of FKBP52 at an 30-fold excess is needed to prevent Hsp90 from binding

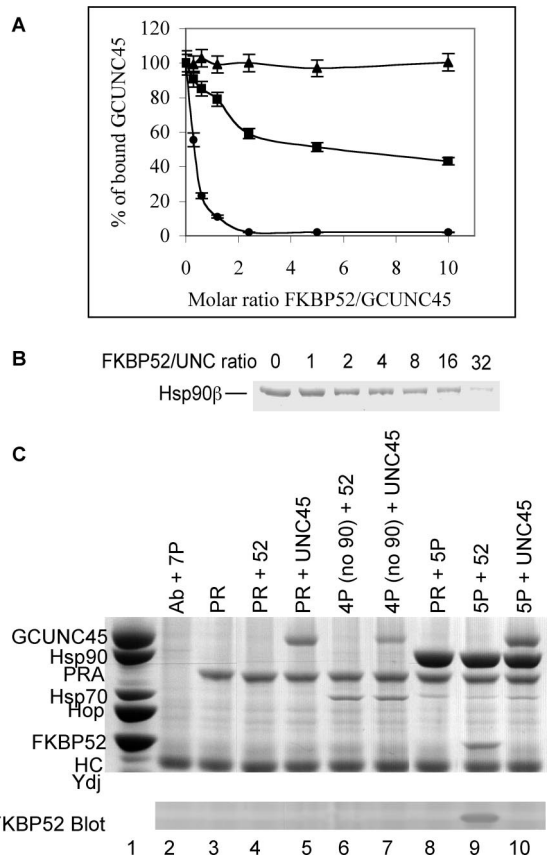

FIGURE 1: (A) FKBP52 competes with binding of GCUNC45 to Hsp90, but not that to PR. The effects of FKBP52 on GCUNC45 binding to purified PR (▲), purified Hsp90 (■), or Hsp90 in PR−chaperone complexes (●) were tested using 0.5 μM GCUNC45 and increasing amounts of FKBP52, as indicated. The complexes immobilized on antibody resin were isolated, and GCUNC45 binding was assessed by densitometry scans of SDS−PAGE gels stained with Coomassie Blue. (B) GCUNC45 covalently immobilized to Sepharose beads was incubated with 0.5 μM Hsp90 in the presence of an increasing amount of FKBP52. GCUNC45 complexes were isolated and assessed for bound Hsp90. (C) Incorporation of FKBP52 into the PR complex is Hsp90-dependent. Immobilized PR was incubated with FKBP52 or GCUNC45 alone or in the presence of additional proteins as indicated: lane 1, loading control of all seven proteins (p23 not shown); lane 2, resin control plus the seven proteins; lane 3, PR alone; lane 4, PR with FKBP52; lane 5, PR with GCUNC45 (UNC45); lanes 6 and 7, PR with four proteins (no Hsp90) with FKBP52 or GCUNC45 as indicated; lane 8, PR with the five-protein chaperoning system; and lanes 9 and 10, PR with the five-protein system and FKBP52 or GCUNC45 as indicated. In the top panel, protein complexes were separated by SDS−PAGE and stained with Coomassie Blue. The the bottom panel, one-tenth of each sample was resolved on a separate gel, transferred, and blotted for FKBP52.

to GCUNC45. FKBP52 most effectively competes for GCUNC45 binding to Hsp90 that is bound to PR in the five-protein chaperoning system [Figure 1A (●)]. The displacement potency of FKBP52 for GCUNC45 in the PR−chaperone complex indicates that FKBP52 has a much higher affinity for Hsp90 when in the context of the PR complex.

We compared the requirements for the binding of FKBP52 and GCUNC45 to PR complexes (Figure 1C). As previously shown (*19*), GCUNC45 binds readily to immobilized PR alone, but no FKBP52 binding to PR was detected by staining or by Western blotting (compare lanes 4 and 5 in Figure 1C). These results were unchanged by the presence of Hsp40, Hsp70, Hop, or p23 in the binding assay (4P, lanes 6 and 7). The binding of FKBP52 to PR complexes was only observed when Hsp90 was present along with the other chaperone proteins, and this condition also enhanced the binding of GCUNC45 (5P, lanes 9 and 10). Thus, the modes of interaction of the two TPR proteins with PR clearly differ. GCUNC45 can bind PR at relatively early steps of maturation. FKBP52 binding is totally dependent upon the presence of Hsp90 and indeed favors a particular conformation of Hsp90 that exists in later more mature PR complexes.

GCUNC45 and FKBP52 Bind to Both the C-Terminal and the N-Terminal Domains of Hsp90. Since the major site of GCUNC45 versus FKBP52 competition in PR chaperoning appeared to be through Hsp90, we focused on dissecting the mechanism of this competition. FKBP51, FKBP52, Cyp40, Hop, and the TPR protein phosphatase PP5 bind competitively to the C-terminal domain of Hsp90 through their TPR domains (*24, 25, 28*), mediated by contacts with the C-terminal MEEVD sequence of Hsp90 (*20–23, 34*). We have shown that Hsp90 has a second TPR binding site in the N-terminal domain that recognizes GCUNC45, but not Hop (*19*).

We tested whether purified FKBP52 could bind the N-terminal fragment of Hsp90 (Figure 2A). The N-terminal domain of Hsp90 ($Hsp90_{1–212}$) binds to both GCUNC45 and FKBP52. The experiment in Figure 2B confirmed these data and compared the binding of FKBP52, GCUNC45, and Hop to the Hsp90 N-terminal and C-terminal domains, and to wild-type Hsp90. In this case, the cochaperones were immobilized and used to bind soluble Hsp90. Although, as shown by others, FKBP52 binds to the C-terminal domain, it also binds substantially to $Hsp90_{1–212}$ (Figure 2B, lanes 4–6). GCUNC45 binds the Hsp90 N-terminal binding site, but it can also bind to the Hsp90 C-terminal domain (Figure 2B, lanes 1–3) as reported previously for the related *Caenorhabditis elegans* protein UNC-45 (*19*). Hop, however, binds only to wild-type Hsp90 and to the C-terminal domain (Figure 2B, lanes 7–9). Further analysis of these interactions showed that the cochaperones bind more strongly to the C-terminal domain of Hsp90 (616−Ct) which binds FKBP52 and GCUNC45 with very similar affinities (K_d) of 4 μM (Figure 2C). However, the affinity of GCUNC45 for $Hsp90_{1–212}$ is 2 times greater than that for FKBP52, 11 and 21 μM, respectively (Figure 2D).

We next asked whether GCUNC45 and FKBP52 compete for binding to the N-terminal fragment $Hsp90_{1–212}$. The experiment summarized in Figure 3A clearly demonstrates that GCUNC45 displaces FKBP52 from $Hsp90_{1–212}$ in a concentration-dependent manner. This interaction requires the TPR domain of GCUNC45 as shown by comparison of $Hsp90_{1–212}$ binding by the N-terminal TPR domain and the C-terminal domain of GCUNC45 (Figure 3B). To determine whether the mechanism of interaction of GCUNC45 and FKBP52 with the N-terminal domain of Hsp90 shares characteristics with the classical binding to the Hsp90 C-terminus, we tested the effect of the synthetic peptide MEEVD used previously in competition experiments as an effective and specific competitor of interaction of *C. elegans* UNC45 with Hsp90 (*35*). The MEEVD peptide inhibited the binding of GCUNC45 [Figure 3C (■)] and FKBP52 [Figure 3C (◆)] to the N-terminus of Hsp90. Furthermore, the C-terminal domain of Hsp90 (616−Ct) also competes off $Hsp90_{1–212}$ from GCUNC45 (Figure 3D) and FKBP52 (not

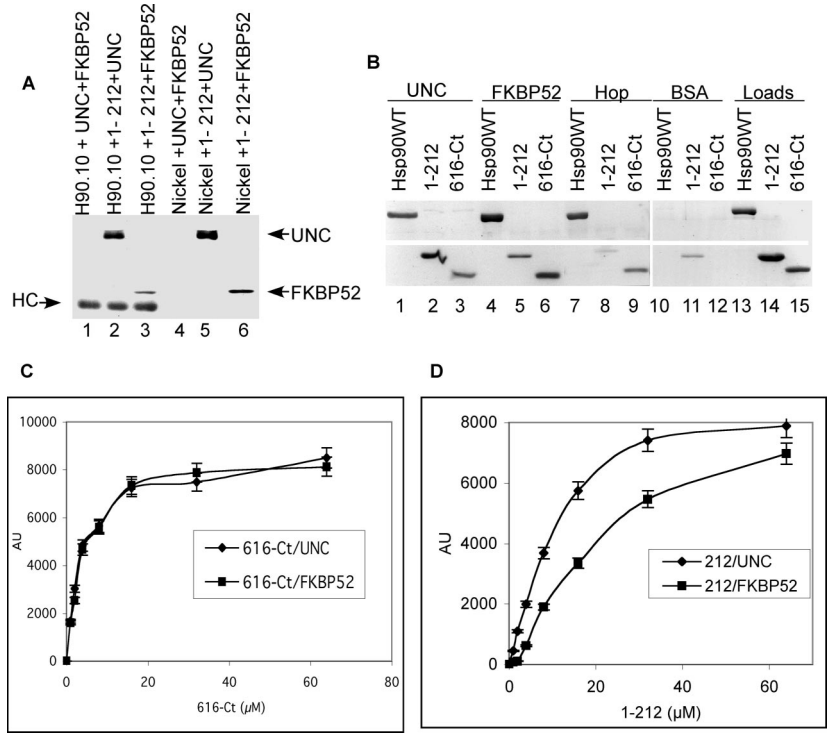

FIGURE 2: Both GCUNC45 (UNC) and FKBP52 bind to the N-terminal domain of Hsp90. (A) The His-tagged Hsp90 N-terminal fragment (1–212) (0.5 μg) was bound to the Hsp90 specific antibody H90.10−protein A-Sepharose (lanes 1–3) or to a nickel resin (lanes 4–6) and used in pull-down experiments to test for the binding of (0.5 μg) GCUNC45 (lanes 2 and 5) or FKBP52 (lanes 3 and 6). Bound proteins were detected by Western blotting using antibodies against GCUNC45 and FKBP52. (B) GCUNC45, FKBP52, Hop, or the BSA control was chemically immobilized on Sepharose-4B resin and used to pull down 0.5 μM wild-type Hsp90 (lanes 1, 4, 7, and 10), the Hsp90 N-terminal fragment (1–212) (lanes 2, 5, 8, and 11), or the Hsp90 C-terminal domain (616−Ct) (lanes 3, 6, 9, and 12). Bound complexes were separated by SDS−PAGE and stained with Coomassie Blue. Lanes 13−15 show one-tenth of the protein amounts used in the reactions. (C and D) Chemically immobilized GCUNC45 or FKBP52 was used in pull-down experiments to test the binding of increasing amounts of Hsp90 N-terminal fragment 1–212 (C) or C-terminal fragment 616−Ct (D). The protein complexes were isolated and resolved by SDS−PAGE, and Coomassie-stained bands were quantified by densitometry using NIH image 1.6. The plots of arbitrary units (AU) of bound Hsp90 fragments are shown. To estimate affinity constants for each fragment, Scatchard analysis was performed as described in Experimental Procedures.

shown). These results indicate that the same TPR domains of FKBP52 and GCUNC45 are used to bind not only the C-terminal domain but also the N-terminal domain of Hsp90.

The Binding of GCUNC45 and FKBP52 in the N-Terminal Domain Involves the ATP Binding Site. On the basis of these results, we hypothesized that a motif similar to MEEVD might exist in the N-terminal domain of Hsp90 to allow the binding of GCUNC45 and FKBP52. Analysis of the Hsp90 sequence showed that the N-terminus of Hsp90 contains an internal EEVE sequence that is conserved in Hsp90α and -β of all vertebrates but not in yeast or bacteria. However, removal of the first 24 amino acids that contain this sequence did not alter the cochaperone binding (not shown).

Additional clues about the binding of GCUNC45 were obtained by studying its relationship to the binding of ATP. GCUNC45 inhibits activation of the ATPase activity of Hsp90 by the cochaperone Aha1 (*19*). This suggested that GCUNC45 may interfere with the binding of ATP to Hsp90. We assessed the binding of Hsp90 to ATP in the presence or absence of GCUNC45. As shown in Figure 4A, the ability of the preformed GCUNC45−Hsp90 complex to bind an ATP affinity resin is significantly diminished compared to that of Hsp90 alone (compare lanes 1 and 3). Furthermore, occupying the Hsp90 nucleotide binding site with ADP, ATPγS, or the inhibitor geldanamycin (GA) severely weakened the interaction of Hsp90 with GCUNC45 resin (Figure 4B). Identical results were obtained with the N-terminal ATP binding domain $Hsp90_{1–212}$. FKBP52 binding to this fragment was similarly affected by geldanamycin (Figure 4C). These data strongly suggest that residues of the N-terminus of Hsp90 involved in the binding of nucleotides are also important for binding GCUNC45 and FKBP52.

Three amino acid substitutions in the ATP binding pocket of Hsp90 that cause a loss of ATP binding or hydrolysis have been reported (*36–38*). We introduced these same mutations into the $Hsp90_{1–212}$ fragment of human Hsp90β (N46A, D88A, and E42A) and tested their effects on binding to GCUNC45 and FKBP52 resins. Far-UV CD and size exclusion chromatography analysis showed no detectable alteration of secondary structure or gel filtration behavior due to these point mutations (not shown). The level of binding of Hsp90 mutant E42A or N46A to GCUNC45 and FKBP52 was reduced to 20% of that of wild-type $Hsp90_{1–212}$ (Figure 5A, lanes 2 and 3 and lanes 6 and 7, respectively), and Hsp90 D88A showed no binding activity for either cochaperone (lanes 4 and 8). Thus, cochaperone binding in this domain of Hsp90 appears to be intimately tied to residues that are also involved in ATP binding and hydrolysis.

The N-terminal domain of Hsp90 is composed of nine helices and eight antiparallel β-strands that form an $\alpha + \beta$ structure (*39–41*) (Figure 5B). The helical face contains a deep pocket for binding ATP, bordered on one side by the

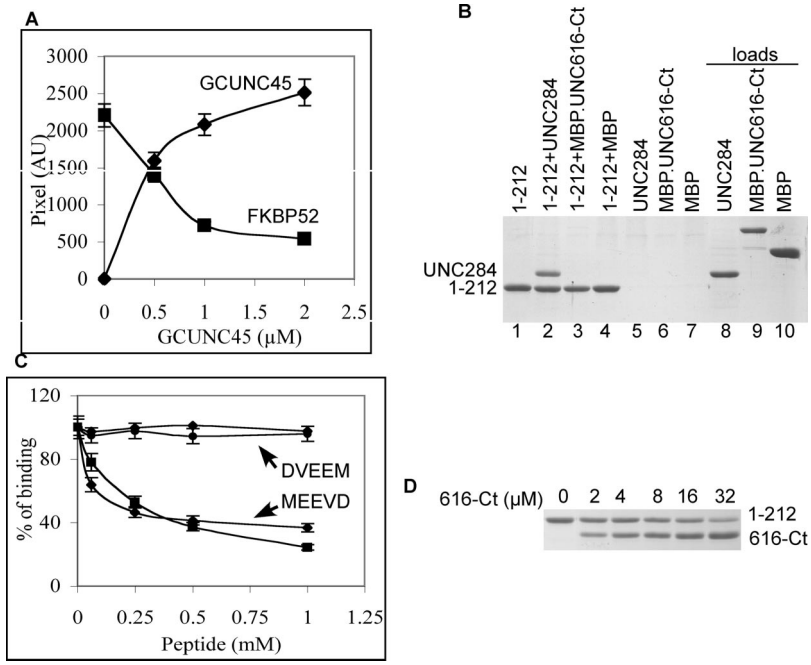

FIGURE 3: GCUNC45 and FKBP52 compete with one another for the N-terminal domain of Hsp90, and these interactions are blocked by the C-terminal domain of Hsp90. (A) The His-tagged Hsp90 N-terminal domain (1–212) was bound to nickel resin and used to pull down FKBP52 (0.5 μM) with various amounts of GCUNC45. Bound complexes were separated by SDS−PAGE and stained with Coomassie Blue. Proteins were quantified using IP LabGel software and expressed in pixels (AU). (B) Binding of GCUNC45 to the N-terminus of Hsp90 is TPR-dependent. Two micrograms of the His-tagged Hsp90 N-terminal domain (1–212) was bound to nickel resin and used to pull down the N-terminal domain (1–284) of GCUNC45 (5 μg) containing the TPR motifs (UNC284, lane 2), the C-terminal domain of GCUNC45 fused to MBP (10 μg) (UNC616-Ct, lane 3), or 10 μg of MBP alone (lane 4). Lane 1 shows Hsp90.1–212 bound to the nickel resin with no protein added. Lanes 5–7 show the nonspecific binding (resin alone) of UNC284, UNC616-Ct, and MBP, respectively. Lanes 8–10 show one-fifth of the loads. (C) His-tagged $Hsp90_{1–212}$ (2 μg) was immobilized on nickel resin and used to pull down 0.5 μM GCUNC45 (■) or FKBP52 (◆) without or with increasing concentrations of MEEVD peptide or the reversed peptide DVEEM. (D) The C-terminal domain of Hsp90 (616−Ct) competes with the N-terminal domain of Hsp90 (1–212) for binding to GCUNC45. Immobilized GCUNC45 was used to pull down $Hsp90_{1–212}$ (0.5 μM) in the presence of increasing amounts of the C-terminal domain of Hsp90 (616−Ct).

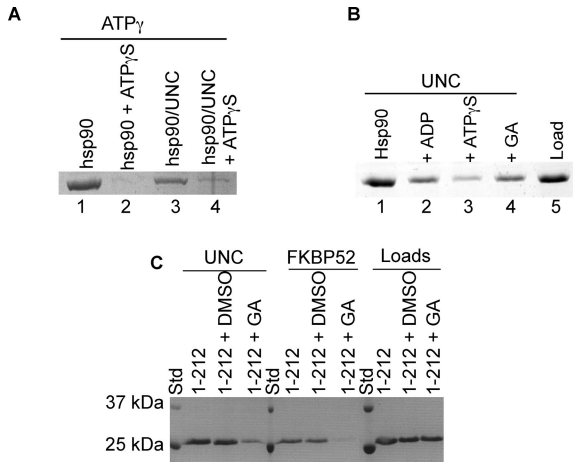

FIGURE 4: Occupying the N-terminal ATP binding pocket of Hsp90 reduces its level of binding to GCUNC45 and FKBP52. (A) Hsp90 was incubated without (lanes 1 and 2) or with GCUNC45 (UNC) (lanes 3 and 4) for 15 min at 37 °C. The mixture was then added to ATP resin for an additional 15 min without (lanes 1 and 3) or with 1 mM ATPγS (lanes 2 and 4). (B) Hsp90 was incubated alone (lane 1) or with 5 mM ADP (lane 2), 1 mM ATPγS (lane 3), or 10 μM geldanamycin (lane 4). The mixture was then added to GCUNC45 resin and further incubated for 15 min at 37 °C. Lane 5 shows one-tenth of the Hsp90 used in the reaction. (C) N-Terminal Hsp90 (1–212) alone, with DMSO (1%), or with geldanamycin (GA, 10 μM) was incubated with GCUNC45 resin or FKBP52 resin for 15 min at 37 °C. The amount of bound Hsp90 (1–212) is shown. Standards (std) are indicated.

longest helix, helix 2. This helix contains residues E42 and N46, and D88 is nearby in β-strand S3 at the base of the pocket.

Even though binding of GCUNC45 to $Hsp90_{1–212}$ requires the TPR motifs (*19*) and the MEEVD peptide competes very efficiently with this interaction (Figure 3A,B), the sequence of $Hsp90_{1–212}$ lacks any sequence resembling EEVD that involves residue E42, N46, or D88. Therefore, we considered the possibility that residues E42, N46, and D88 are part of a spatial positioning of amino acids with some resemblance to the EEVD motif. Neighboring residues D49, D52, and L51 of helix 2 are suitably positioned to complete a potential conformational entity recognizable by the concave surface of the TPR superhelical groove (Figure 5B).To test this hypothesis, these residues, in addition to neighboring ones, were mutated in $Hsp90_{1–212}$. As shown in Figure 5C, residues D49, L51, and D52 of helix 2 are all important for the binding of GCUNC45 and FKBP52. Mutant D49A (lane 3) shows a dramatic loss of binding, and binding is barely detected with mutants L51S and D52A (lanes 4 and 5). On the other hand, there was little change with mutant D66A (lane 6) near the end of helix 2 or E70A (lane 7), L71S, and K72A (not shown) that are part of the loop following helix 2. Also, V87S (lane 9), adjacent to the critical D88 in β-strand S3, and D75R (lane 8) of β-strand S2 showed little effect. Our findings support a spatial positioning of residues E42, N46, D49, D52, L51, and D88 in and around the ATP binding pocket of Hsp90 to generate a conformational

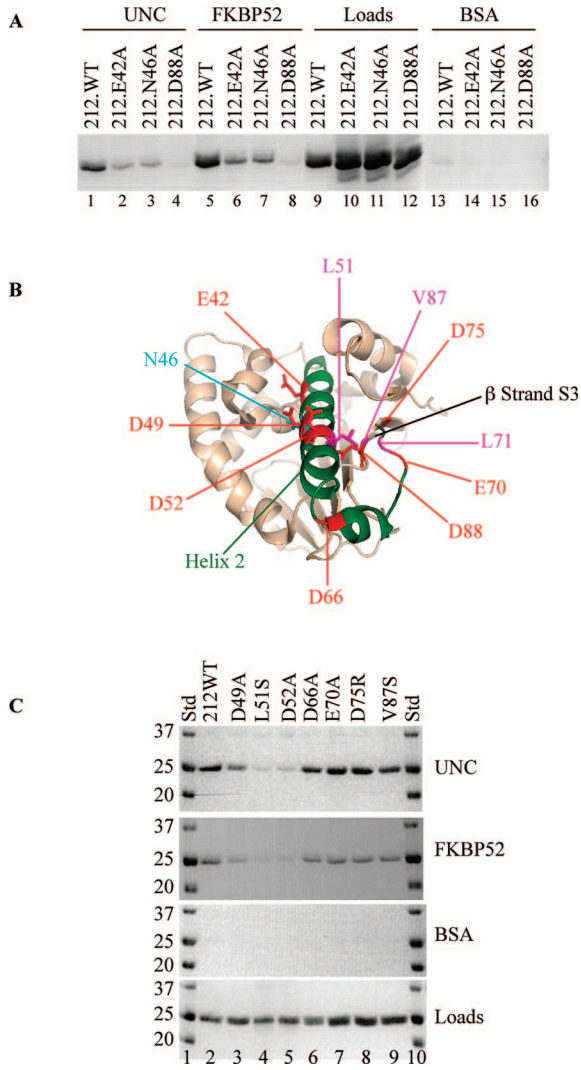

FIGURE 5: Point mutations in and around the Hsp90 N-terminal ATP binding pocket affect the binding of GCUNC45 (UNC) and FKBP52. (A) Two micromoles of purified N-terminal Hsp90 (1–212) (wild type or mutants) was incubated with GCUNC45 or FKBP52 resin, or BSA resin used as control. Bound proteins were resolved by SDS−PAGE and stained with Coomassie Blue. (B) Structure of the N-terminal domain of Hsp90β (*41*). Mutated amino acids are colored red (acidic) and magenta (hydrophobic). (C) Purified mutant proteins were tested for GCUNC45 and FKBP52 binding as in panel A. BSA resin was used as a control. Loads show one-tenth of the proteins used in the reaction.

structure required for binding TPR cochaperones to the N-terminal domain.

DISCUSSION

Hsp90 is an essential chaperone connected with a wide range of cellular functions (*1–5*). These functions depend on the interaction of Hsp90 with a number of cochaperones (*5–7*). The largest class of Hsp90 cochaperones includes those that interact with Hsp90 through their TPR domains. Members of this class differ greatly in their activities and functions (*5*). Most TPR cochaperones that have been studied have been shown to interact with the C-terminal domain of Hsp90, and the binding of GCUNC45 to a region in the Hsp90 N-terminal domain appeared to be an exception (*19*).

In this study, we show that the immunophilin FKBP52 can also bind to the Hsp90 N-terminal domain. FKBP52 can

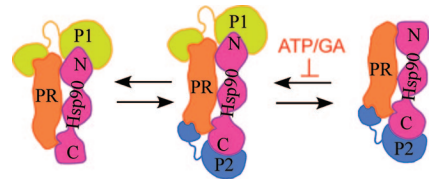

FIGURE 6: Schematic model depicting two TPR proteins (P1 and P2) interacting exclusively or simultaneously with the Hsp90−PR complex. Nucleotides regulate these interactions, and GA blocks the N-terminal TPR binding site. Other chaperones have been omitted for the sake of simplicity.

effectively compete with GCUNC45 for Hsp90 binding, and this competition is even more effective when Hsp90 is in PR complexes. Furthermore, the binding of these PR-associated cochaperones is intimately tied to structural features and regulation of Hsp90's N-terminal ATP binding pocket. Nucleotides and geldanamycin reduce the affinity of Hsp90 for GCUNC45 and FKBP52. Finally, mutation studies reveal a previously unrecognized TPR binding site involving discontinuous residues from helix 2 and β-strand S3 of the N-terminal domain of Hsp90. As discussed below, a second TPR binding site in this domain of Hsp90 helps to explain how Hsp90 efficiently utilizes its repertoire of TPR cochaperones to coordinate the chaperoning of steroid receptors and perhaps other client proteins.

The binding of TPR proteins to the Hsp90 C-terminal peptide MEEVD has been structurally characterized, and the original "two-carboxylate" clamp mechanism proposed for the TPR2a domain of Hop has been confirmed in subsequent structures with several cochaperones (*21, 34, 42*). In these interactions, basic residues of the TPR domain form electrostatic interactions with the terminal aspartate of the peptide (*21*). The affinity and specificity of interaction are enhanced by additional contacts involving charged and hydrophobic residues within and upstream of the MEEVD sequence. We now know that the TPR site in the N-terminal domain clearly differs from that at the C-terminus in that it is not anchored by a linear amino acid sequence with a terminal aspartate. Instead, the N-terminal site involves residues E42, N46, D49, D52, L51, and D88 within or near the ATP binding pocket of Hsp90. As with the C-terminal site, however, acidic and hydrophobic residues seem to be similarly important in the binding. However, the N-terminal site does not bind Hop and is likely to be selective for a subgroup of TPR cochaperones. Preliminary studies indicate that Cyp40 is also able to bind to the N-terminal site, but other TPR cochaperones have not been tested.

Initial immunoprecipitation experiments showed that PR−Hsp90 heterocomplexes contain several TPR proteins such as FKBP52, FKBP51, Cyp40, Hop, and possibly others (*15–18*). This has been interpreted to reflect an assortment of PR complexes at different stages of maturation, each complex containing only one type of TPR cochaperone. The identification of a second TPR binding site provides the possibility of two simultaneous TPR-dependent interactions. Many TPR cochaperones not only interact with Hsp90 but also have an additional domain that can interact with the client. With such a variety of client proteins and cochaperones, this interaction may occur more successfully with two binding sites on Hsp90 (Figure 6). The dynamic exchange of the TPR proteins (shown as P1 and P2) could result from a direct competition for the N- or C-terminal sites, and it is

also possible that the proteins could be exchanged through allosteric conformational modifications transmitted from one site to the other. In addition, entry of cochaperones into the Hsp90 complex would also be strongly influenced by the client or cochaperones as evidenced by the fact that FKBP52 binds much more effectively to Hsp90 in PR complexes than to Hsp90 alone (Figure 1).

The dual use of the ATP binding site for TPR cochaperone binding has additional regulatory implications. Hsp90 is known to have multiple conformational states that are determined, primarily, by a cycle of binding and hydrolysis of ATP (*1–5*). Hsp90 is thought to enter client complexes without bound nucleotide. ATP binding then induces dramatic conformational changes resulting in dimer interaction within the N-terminal domains and a reduced exposure of hydrophobic surfaces (*30, 43*). Additional conformational changes are presumed to occur upon the hydrolysis of ATP and the release of ADP. In this context, one can speculate that the binding of a cochaperone to the N-terminal site of Hsp90 would prevent ATP binding and progression of the chaperone complex to the next step, perhaps with the purpose of allowing time for additional event(s) to occur. The cochaperone interaction may be displaced by another cochaperone or weakened by conformational changes that allow ATP binding. Later, when ATP is bound, TPR cochaperones might interact with Hsp90 only at the C-terminus. Thus, cochaperone interactions at the N-terminal site would be limited to specific phases of the Hsp90 chaperoning cycle. Indeed, in the chaperoning of PR, Hsp90 enters the PR complex through the bridging protein Hop, which binds both Hsp90 and Hsp70 (bound to PR). In this intermediate complex, the C-terminal TPR site is blocked by Hop, leaving the site in the N-terminal domain available (without ATP). Progression to a mature PR conformation involves the binding of ATP, p23, and immunophilins, and the loss of Hop. In our model, the TPR cochaperone interactions would be relegated to the N-terminal site during the intermediate stages (except for Hop binding), and to the C-terminal site at later stages.

These findings may also impact our understanding of the pharmacological actions of inhibitors targeting Hsp90's N-terminal ATP binding pocket such as geldanamycin and ATP derivatives. To date, the negative effects of these pharmacological agents on Hsp90 function have been interpreted mainly through inhibition of Hsp90 ATPase activity, whereas they are likely also to alter cochaperone interactions.

In conclusion, this study defines a novel site for TPR cochaperone interaction at the N-terminal ATP binding domain of Hsp90. This site has the potential to be regulated upon structural changes in Hsp90 throughout its ATP-driven chaperoning cycle. The presence of two Hsp90 sites for TPR protein interaction provides additional flexibility in positioning this class of cochaperones to more effectively regulate client chaperoning by Hsp90.

ACKNOWLEDGMENT

We thank Dr. Sara Felts for helpful discussion about this study and Laura A. Sikkink and Dr. Marina Ramirez-Alvarado for the CD analysis. We also thank Dr. Kathryn Horwitz and Dr. Dinny Graham for their collaboration in the initial phase of this project.

REFERENCES

1. Zhao, R., Davey, M., Hsu, Y. C., Kaplanek, P., Tong, A., Parsons, A. B., Krogan, N., Cagney, G., Mai, D., Greenblatt, J., Boone, C., Emili, A., and Houry, W. A. (2005) Navigating the chaperone network: An integrative map of physical and genetic interactions mediated by the hsp90 chaperone. *Cell 120*, 715–727.
2. Young, J. C., Moarefi, I., and Hartl, F. U. (2001) Hsp90: A specialized but essential protein-folding tool. *J. Cell Biol. 154*, 267–273.
3. Pearl, L. H., and Prodromou, C. (2006) Structure and mechanism of the Hsp90 molecular chaperone machinery. *Annu. Rev. Biochem. 75*, 271–294.
4. Wegele, H., Muller, L., and Buchner, J. (2004) Hsp70 and Hsp90: A relay team for protein folding. *Rev. Physiol. Biochem. Pharmacol. 151*, 1–44.
5. Pratt, W. B., and Toft, D. O. (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp. Biol. Med. 228*, 111–133.
6. Riggs, D. L., Cox, M. B., Cheung-Flynn, J., Prapapanich, V., Carrigan, P. E., and Smith, D. F. (2004) Functional specificity of co-chaperone interactions with Hsp90 client proteins. *Crit. Rev. Biochem. Mol. Biol. 39*, 279–295.
7. Smith, D. F. (2004) Tetratricopeptide repeat cochaperones in steroid receptor complexes. *Cell Stress Chaperones 9*, 109–121.
8. Czar, M. J., Lyons, R. H., Welsh, M. J., Renoir, J. M., and Pratt, W. B. (1995) Evidence that the FK506-binding immunophilin heat shock protein 56 is required for trafficking of the glucocorticoid receptor from the cytoplasm to the nucleus. *Mol. Endocrinol. 9*, 1549–1560.
9. Riggs, D. L., Roberts, P. J., Chirillo, S. C., Cheung-Flynn, J., Prapapanich, V., Ratajczak, T., Gaber, R., Picard, D., and Smith, D. F. (2003) The Hsp90-binding peptidylprolyl isomerase FKBP52 potentiates glucocorticoid signaling in vivo. *EMBO J. 22*, 1158–1167.
10. Cheung-Flynn, J., Prapapanich, V., Cox, M. B., Riggs, D. L., Suarez-Quian, C., and Smith, D. F. (2005) Physiological role for the cochaperone FKBP52 in androgen receptor signaling. *Mol. Endocrinol. 19*, 1654–1666.
11. Tranguch, S., Cheung-Flynn, J., Daikoku, T., Prapapanich, V., Cox, M. B., Xie, H., Wang, H., Das, S. K., Smith, D. F., and Dey, S. K. (2005) Cochaperone immunophilin FKBP52 is critical to uterine receptivity for embryo implantation. *Proc. Natl. Acad. Sci. U.S.A. 102*, 14326–14331.
12. Yang, Z., Wolf, I. M., Chen, H., Periyasamy, S., Chen, Z., Yong, W., Shi, S., Zhao, W., Xu, J., Srivastava, A., Sanchez, E. R., and Shou, W. (2006) FK506-binding protein 52 is essential to uterine reproductive physiology controlled by the progesterone receptor A isoform. *Mol. Endocrinol. 20*, 2682–2694.
13. Nair, S. C., Rimerman, R. A., Toran, E. J., Chen, S., Prapapanich, V., Butts, R. N., and Smith, D. F. (1997) Molecular cloning of human FKBP51 and comparisons of immunophilin interactions with Hsp90 and progesterone receptor. *Mol. Cell. Biol. 17*, 594–603.
14. Davies, T. H., Ning, Y. M., and Sanchez, E. R. (2002) A new first step in activation of steroid receptors: Hormone-induced switching of FKBP51 and FKBP52 immunophilins. *J. Biol. Chem. 277*, 4597–4600.
15. Johnson, J. L., and Toft, D. O. (1994) A novel chaperone complex for steroid receptors involving heat shock proteins, immunophilins, and p23. *J. Biol. Chem. 269*, 24989–24993.
16. Smith, D. F., Faber, L. E., and Toft, D. O. (1990) Purification of unactivated progesterone receptor and identification of novel receptor-associated proteins. *J. Biol. Chem. 265*, 3996–4003.
17. Smith, D. F., Sullivan, W. P., Marion, T. N., Zaitsu, K., Madden, B., McCormick, D. J., and Toft, D. O. (1993) Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70. *Mol. Cell. Biol. 13*, 869–876.
18. Smith, D. F., Whitesell, L., Nair, S. C., Chen, S., Prapapanich, V., and Rimerman, R. A. (1995) Progesterone receptor structure and function altered by geldanamycin, an hsp90-binding agent. *Mol. Cell. Biol. 15*, 6804–6812.
19. Chadli, A., Graham, J. D., Abel, M. G., Jackson, T. A., Gordon, D. F., Wood, W. M., Felts, S. J., Horwitz, K. B., and Toft, D.

(2006) GCUNC-45 is a novel regulator for the progesterone receptor/hsp90 chaperoning pathway. *Mol. Cell. Biol. 26*, 1722–1730.
20. Das, A. K., Cohen, P. W., and Barford, D. (1998) The structure of the tetratricopeptide repeats of protein phosphatase 5: Implications for TPR-mediated protein-protein interactions. *EMBO J. 17*, 1192–1199.
21. Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U., and Moarefi, I. (2000) Structure of TPR domain-peptide complexes: Critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell 101*, 199–210.
22. Sinars, C. R., Cheung-Flynn, J., Rimerman, R. A., Scammell, J. G., Smith, D. F., and Clardy, J. (2003) Structure of the large FK506-binding protein FKBP51, an Hsp90-binding protein and a component of steroid receptor complexes. *Proc. Natl. Acad. Sci. U.S.A. 100*, 868–873.
23. Taylor, P., Dornan, J., Carrello, A., Minchin, R. F., Ratajczak, T., and Walkinshaw, M. D. (2001) Two structures of cyclophilin 40: Folding and fidelity in the TPR domains. *Structure 9*, 431–448.
24. Young, J. C., Obermann, W. M., and Hartl, F. U. (1998) Specific binding of tetratricopeptide repeat proteins to the C-terminal 12-kDa domain of hsp90. *J. Biol. Chem. 273*, 18007–18010.
25. Carrello, A., Ingley, E., Minchin, R. F., Tsai, S., and Ratajczak, T. (1999) The common tetratricopeptide repeat acceptor site for steroid receptor-associated immunophilins and hop is located in the dimerization domain of Hsp90. *J. Biol. Chem. 274*, 2682–2689.
26. Russell, L. C., Whitt, S. R., Chen, M. S., and Chinkers, M. (1999) Identification of conserved residues required for the binding of a tetratricopeptide repeat domain to heat shock protein 90. *J. Biol. Chem. 274*, 20060–20063.
27. Ward, B. K., Allan, R. K., Mok, D., Temple, S. E., Taylor, P., Dornan, J., Mark, P. J., Shaw, D. J., Kumar, P., Walkinshaw, M. D., and Ratajczak, T. (2002) A structure-based mutational analysis of cyclophilin 40 identifies key residues in the core tetratricopeptide repeat domain that mediate binding to Hsp90. *J. Biol. Chem. 277*, 40799–40809.
28. Ratajczak, T., and Carrello, A. (1996) Cyclophilin 40 (CyP-40), mapping of its hsp90 binding domain and evidence that FKBP52 competes with CyP-40 for hsp90 binding. *J. Biol. Chem. 271*, 2961–2965.
29. Price, M. G., Landsverk, M. L., Barral, J. M., and Epstein, H. F. (2002) Two mammalian UNC-45 isoforms are related to distinct cytoskeletal and muscle-specific functions. *J. Cell Sci. 115*, 4013–4023.
30. Prodromou, C., Panaretou, B., Chohan, S., Siligardi, G., O'Brien, R., Ladbury, J. E., Roe, S. M., Piper, P. W., and Pearl, L. H. (2000) The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains. *EMBO J. 19*, 4383–4392.
31. Owen, B. A., Sullivan, W. P., Felts, S. J., and Toft, D. O. (2002) Regulation of heat shock protein 90 ATPase activity by sequences in the carboxyl terminus. *J. Biol. Chem. 277*, 7086–7091.
32. Kosano, H., Stensgard, B., Charlesworth, M. C., McMahon, N., and Toft, D. (1998) The assembly of progesterone receptor-hsp90 complexes using purified proteins. *J. Biol. Chem. 273*, 32973–32979.
33. Cintron, N. S., and Toft, D. (2006) Defining the requirements for Hsp40 and Hsp70 in the Hsp90 chaperone pathway. *J. Biol. Chem. 281*, 26235–26244.
34. Zhang, M., Windheim, M., Roe, S. M., Peggie, M., Cohen, P., Prodromou, C., and Pearl, L. H. (2005) Chaperoned ubiquitylation: Crystal structures of the CHIP U box E3 ubiquitin ligase and a CHIP-Ubc13-Uev1a complex. *Mol. Cell 20*, 525–538.
35. Barral, J. M., Hutagalung, A. H., Brinker, A., Hartl, F. U., and Epstein, H. F. (2002) Role of the myosin assembly protein UNC-45 as a molecular chaperone for myosin. *Science 295*, 669–671.
36. Grenert, J. P., Johnson, B. D., and Toft, D. O. (1999) The importance of ATP binding and hydrolysis by hsp90 in formation and function of protein heterocomplexes. *J. Biol. Chem. 274*, 17525–17533.
37. Obermann, W. M., Sondermann, H., Russo, A. A., Pavletich, N. P., and Hartl, F. U. (1998) In vivo function of Hsp90 is dependent on ATP binding and ATP hydrolysis. *J. Cell Biol. 143*, 901–910.
38. Panaretou, B., Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1998) ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *EMBO J. 17*, 4829–4836.
39. Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. (1997) Crystal structure of an Hsp90-geldanamycin complex: Targeting of a protein chaperone by an antitumor agent. *Cell 89*, 239–250.
40. Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997) Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell 90*, 65–75.
41. Wright, L., Barril, X., Dymock, B., Sheridan, L., Surgenor, A., Beswick, M., Drysdale, M., Collier, A., Massey, A., Davies, N., Fink, A., Fromont, C., Aherne, W., Boxall, K., Sharp, S., Workman, P., and Hubbard, R. E. (2004) Structure-activity relationships in purine-based inhibitor binding to HSP90 isoforms. *Chem. Biol. 11*, 775–785.
42. Cliff, M. J., Harris, R., Barford, D., Ladbury, J. E., and Williams, M. A. (2006) Conformational diversity in the TPR domain-mediated interaction of protein phosphatase 5 with Hsp90. *Structure 14*, 415–426.
43. Shiau, A. K., Harris, S. F., Southworth, D. R., and Agard, D. A. (2006) Structural analysis of *E. coli* hsp90 reveals dramatic nucleotide-dependent conformational rearrangements. *Cell 127*, 329–340.

BI7023332