Regulation of Hsp90 ATPase Activity by the Co-chaperone Cdc37p/p50<sup>cdc37</sup>*

Giuliano Siligardi‡, Barry Panaretou§, Philippe Meyer†, Shradha Singh¶, Derek N. Woolfson**, Peter W. Piper‡‡, Laurence H. Pears, and Christos Prodromou††

From the ‡Pharmaceutical Optical Spectroscopy Centre, Department of Pharmacy, the §Division of Life Sciences, King’s College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NN, ¶Section of Structural Biology, the Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6BJ, **Centre for Bimolecular Design and Drug Development, School of Biological Sciences, University of Sussex, Palmer BN1 9QG, and ††Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom

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† Present address: Protein Science, Syngenta, Jealott’s Hill International Research Centre, Bracknell, Berkshire RG42 6EY, UK.

‡ To whom correspondence should be addressed. Tel.: 44-207-970-6074; Fax: 44-207-970-6051; E-mail: prodromou@icr.ac.uk.

§ The abbreviations used are: HSP90, heat shock protein 90; AMP-PNP, adenosine 5′-(β,γ-mimino)triphosphate.

In vivo activation of client proteins by Hsp90 depends on its ATPase-coupled conformational cycle and on interaction with a variety of co-chaperone proteins. For some client proteins the co-chaperone Sti1/Hop/p60 acts as a “scaffold,” recruiting Hsp70 and the bound client to Hsp90 early in the cycle and suppressing ATP turnover by Hsp90 during the loading phase. Recruitment of protein kinase clients to the Hsp90 complex appears to involve a specialized co-chaperone, Cdc37p/P50<sup>cdc37</sup>, whose binding to Hsp90 is mutually exclusive of Sti1/Hop/p60. We now show that Cdc37p/P50<sup>cdc37</sup>, like Sti1/Hop/p60, also suppresses ATP turnover by Hsp90 supporting the idea that client protein loading to Hsp90 requires a “relaxed” ADP-bound conformation. Like Sti1/Hop/p60, Cdc37p/P50<sup>cdc37</sup> binds to Hsp90 as a dimer, and the suppressed ATPase activity of Hsp90 is restored when Cdc37p/P50<sup>cdc37</sup> is displaced by the immunophilin co-chaperone Cpr6/Cyp40. However, unlike Sti1/Hop/p60, which can displace geldanamycin upon binding to Hsp90, Cdc37p/P50<sup>cdc37</sup> forms a stable complex with geldanamycin-inhibited Hsp90 and may be sequestered in geldanamycin-inhibited Hsp90 complexes in vivo.

Although not essential, mammalian Hop/p60 increases the efficiency of steroid hormone receptor activation by Hsp90 and Hsp70 in vitro (13), and sti1<sup>−/−</sup> yeasts, although viable, are temperature-sensitive and display growth defects (14).

Whereas steroid hormone receptors have been the most studied in terms of their activation by Hsp90, protein kinases form the largest coherent class of Hsp90-dependent client proteins (1). Recruitment of many protein kinase clients to the Hsp90 system is involved in a specialized co-chaperone Cdc37p (in budding yeast) or its mammalian orthologue p50<sup>cdc37</sup> (15). Unlike Sti1/Hop/p60, which recruits clients to Hsp90 via interaction with Hsp90, Cdc37p/P50<sup>cdc37</sup> can interact directly with client protein kinases via its N-terminal region (16) and with Hsp90 via its C terminus (17). However, it is far from clear whether all protein kinases that interact with Cdc37p/P50<sup>cdc37</sup> are Hsp90-dependent if all kinases that are Hsp90-dependent are recruited by Cdc37p/P50<sup>cdc37</sup> (18). Unlike Sti1, Cdc37p is essential for yeast viability probably due to its involvement in formation of Cdc28-cyclin complexes (19).

Sti1/Hop/p60 and other TPR domain co-chaperones bind with mutual exclusivity to Hsp90 (20) via the C-terminal MEEVD sequence (10, 21), although this may not be the sole site of interaction (6, 14, 22). In contrast, Cdc37p/P50<sup>cdc37</sup> has no detectable TPR motifs and does not require the C-terminal MEEVD of Hsp90, however, its binding is mutually exclusive with Sti1/Hop/p60 (20, 23), suggesting that the binding sites overlap or are at least topologically adjacent. The common ability of Sti1/Hop/p60 and Cdc37p/P50<sup>cdc37</sup> to interact (directly or indirectly) with Hsp90 client proteins, together with their mutual exclusivity of binding to Hsp90, suggests that they may act as alternative recruitment factors for different classes of client protein. To gain further insight into this possibility, we have characterized the interaction between Cdc37p/P50<sup>cdc37</sup> and Hsp90 and investigated the effect of Cdc37p/P50<sup>cdc37</sup> co-chaperone binding on the inherent ATPase activity of Hsp90.

The results of these studies suggest a common mechanism for recruitment of client proteins into the Hsp90 complex requiring suppression of ATP turnover by Hsp90 during the loading phase.

EXPERIMENTAL PROCEDURES

Protein Production and Hsp90 ATPase Activity Assay—Expression and purification of His-tagged yeast Hsp90, His-tagged Sti11 (C-terminal Hsp90-binding domain of Sti1, residues 237–589), and His-tagged Cpr6 were described previously (6). DNA sequences encoding a truncated N-terminal domain of human p50<sup>cdc37</sup> (sNp50, amino acid residues 30–127) and C-terminal p50<sup>cdc37</sup> domain (Cp50, amino acid residues 128–379) were cloned in-frame with the His tag of pRSETA (pHet-Xhol). Full-length p50<sup>cdc37</sup> cloned into pET16d was a kind gift from Nick Grammatikakis. Saccharomyces cerevisiae CDC37 was
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For Cdc37p interactions by near-UV CD.

**Analytical Ultracentrifugation—**Sedimentation equilibrium experiments were conducted at 15 °C in a Beckman-Optima XL-A analytical centrifuge using an An-60 Ti rotor and 1.2-cm path length cells. p50<sup>cdc37</sup>, sNp50, and Cpd50 were dialyzed against 20 mM Tris (pH 7.4) containing 1 mM EDTA and 150 mM NaCl. The proteins were concentrated to 0.5, 1.0, 2.0, 3.0, and 4.0 M, obtained by subtracting the spectra of the free Cdc37p at 150 M using the Levenberg-Marquardt equation (dotted line).

**Circular Dichroism and K<sub>d</sub> Determinations—**CD spectra were recorded on a nitrogen-flushed Jasco J<sup>720</sup> spectropolarimeter. For an accurate determination of K<sub>d</sub> values, multisecuring was required for high data precision. At a scan speed of 20 nm min<sup>-1</sup>, nine scans were demanded in the 245–330 nm region for the CD titrations of Hsp90 with several co-chaperones. The concentration of the proteins were determined spectrophotometrically using the following molar extinction coefficients: ε<sub>560</sub> = 54,050 M<sup>-1</sup> cm<sup>-1</sup>, ε<sub>370</sub> = 21,750 M<sup>-1</sup> cm<sup>-1</sup>, ε<sub>280</sub> = 30,817 M<sup>-1</sup> cm<sup>-1</sup>, and ε<sub>260</sub> = 26,390 M<sup>-1</sup> cm<sup>-1</sup>, and ε<sub>10</sub> = 34,480 M<sup>-1</sup> cm<sup>-1</sup>. All CD spectra were reported in Δ<sub>A</sub> = (A<sub>H</sub> - A<sub>B</sub>). The spectropolarimeter was calibrated with ammonium d-champor-10-sulfonate. The dissociation constant K<sub>d</sub> was determined by analyzing the CD data using a non-linear regression analysis as described previously (6, 24).

**CD titrations were conducted in a stepwise manner adding small aliquots of ligand stock solution directly into the cuvette of 1 cm path length containing an initial volume of 520 μl of Hsp90 in 20 mM Tris-HCl. Each titration was terminated upon ligand saturation that never reached a final volume greater than 15% of the initial volume, and each CD spectrum was subsequently corrected for its dilution. To achieve the highest accuracy and precision, Finnpipette PCR with a volume range of 2–200 μl were used to add aliquots of ligand solution in a stepwise manner to reach the desired molar ratio for the solution of Hsp90 of the appropriate concentration.

**For a single binding site at equilibrium, the CD, expressed in molar equivalents, from Hsp90-Cdc37p mixtures at Hsp90:Cdc37p molar ratio of 1.13,508 Da, and Cp<sub>50</sub> were dialyzed against 20 mM Tris (pH 7.4) containing 1 mM sodium dithiothreitol were added, and the eluent was concentrated using Vivaspin molecular weight cutoff concentrations as appropriate. The protein concentrations reached are as follows: p50<sup>cdc37</sup> = 98.5 mg ml<sup>-1</sup>, sNp50 = 60 mg ml<sup>-1</sup>, Cpd50 = 50 mg ml<sup>-1</sup>, Cdc37p = 131.6 mg ml<sup>-1</sup>, ε<sub>280</sub> = 30,817 M<sup>-1</sup> cm<sup>-1</sup>, and ε<sub>260</sub> = 26,390 M<sup>-1</sup> cm<sup>-1</sup>. Further treatment of proteins destined for ATPase assays and the regenerating enzyme-linked ATPase assay itself were as described previously (3, 6). Each assay was repeated between 3 and 6 times, and average activities were calculated. Average ATPase activities were plotted as a percentage of the maximum average activity for Hsp90 at 37 °C.

For a single binding site at equilibrium, the binding of Cdc37p to Hsp90. The solid line represents the simulated curve that would be obtained if the titration were continued to obtain saturation of Cdc37p-binding sites and yields a K<sub>d</sub> of 100 μM. This is in close agreement to the estimation of 113 ± 4 μM using the Levenberg-Marquardt equation (dotted line).
The molar absorptivity coefficient \( \varepsilon \) is given by \( \varepsilon = \varepsilon_{\omega} - \varepsilon_{\ell} \) (m \( \cdot \) cm \(^{-1} \)) \( \times \) c (molarity), and l is the pathlength expressed in centimeters. For a titration carried out in a 1-cm path length cell \( (l = 1) \) with the concentration at equilibrium of the host \([H]\), ligand \([L]\), and host-ligand complex \([HL]\) (corresponding to the concentration of the bound species of the host and ligand), the CD of each species can be described as shown in Equations 2–4,

\[
\Delta A_{HL} = \Delta \varepsilon_{HL}[HL] \tag{Eq. 2}
\]

\[
\Delta A_{H} = \Delta \varepsilon_{H}[H] \tag{Eq. 3}
\]

\[
\Delta A_{L} = \Delta \varepsilon_{L}[L] \tag{Eq. 4}
\]

Substituting Equations 2–4 into 1 (Equation 5),

\[
\Delta A = \Delta \varepsilon_{HL}[HL] + \Delta \varepsilon_{H}[H] + \Delta \varepsilon_{L}[L] \tag{Eq. 5}
\]

In the titration, the total concentration of the host and the ligand is shown in Equations 6 and 7,

\[
[H_T] = [H] + [HL] \tag{Eq. 6}
\]

\[
[L_T] = [L] + [HL] \tag{Eq. 7}
\]

Rearranging Equations 6 and 7 (see Equations 8 and 9),

\[
[H] = [H_T] - [HL] \tag{Eq. 8}
\]

\[
[L] = [L_T] - [HL] \tag{Eq. 9}
\]

Substituting Equations 8 and 9 into 5 leads to Equations 10–13,

\[
\Delta A = \Delta \varepsilon_{HL}[HL] + \Delta \varepsilon_{H}[H_T] - [HL] + \Delta \varepsilon_{L}[L_T] - [HL] \tag{Eq. 10}
\]

\[
\Delta A = \Delta \varepsilon_{HL}[HL] + \Delta \varepsilon_{H}[H_T] + \Delta \varepsilon_{L}[L_T] - \Delta \varepsilon_{[HL]} \tag{Eq. 11}
\]

\[
\Delta A = (\Delta \varepsilon_{HL} - \Delta \varepsilon_{H})[HL] + \Delta \varepsilon_{L}[L_T] + \Delta \varepsilon_{[HL]} \tag{Eq. 12}
\]

\[
[H_T] = [H] + [HL] \tag{Eq. 6}
\]

\[
[L_T] = [L] + [HL] \tag{Eq. 7}
\]

\[
[H] = [H_T] - [HL] \tag{Eq. 8}
\]

\[
[L] = [L_T] - [HL] \tag{Eq. 9}
\]

Substituting Equations 8 and 9 into 5 leads to Equations 10–13,
trifuge rotor. Also shown are the simulated curves calculated assuming the concentration of the ligand was analyzed by non-linear regression to determine the dissociation constant $K_c$ in the order of $10^{-4}$ M, these conditions might not be achieved and hence saturation will not be reached, but as long as the plot $\Delta$ versus ligand concentration is of parabolic type, the $K_c$ can be still be determined by Equation 18. For $K_c$ in the region between $10^{-7}$ and $10^{-4}$ M, the plot is of Michaelis-Menten type of shape with saturation that can be achieved at 1:1 molar ratio stoichiometry. In the case of a ligand with no CD, the CD changes associated with the host plateau upon ligand saturation. In the case of a ligand with CD, the saturation is normally achieved with $[L]_0 > 5-10$-fold $[H_2]$, and $\Delta \epsilon_{\text{hl}}$ can be calculated as first approximation using the concentration of $[H_2] = [HL]$ (that is all host molecules are bound to the ligand). With the best fitting achieved by either visual inspection or by Levenberg-Marquardt method, the calculated value of $K_c$ can be used to calculate $[HL]$ from Equation 16. From Beer’s Law, a more accurate value of $\Delta \epsilon_{\text{hl}} = \Delta \epsilon_{\text{hl}}/([H]_0)$ can be calculated and used again in Equation 18 to fit again the experimental CD data in order to determine a more accurate value of $K$ and hence $K_c$.

This method has been used to calculate the $K_c$ value for 29 titrations of Hsp90 and several of its mutants with different ligands, such as ADP, ATP, AMP-PNP, Sti1, Cpr6, and geldanamycin. $K_c$ values determined from differential CD data were in very good agreement with those obtained from calorimetry. A similar approach has been used to determine the $K_c$ values of binary complexes by a non-linear regression method (27), using the difference CD obtained by subtracting the CD of the total added concentration of both host and ligand from the observed CD of the host-ligand complex.

**Simulation of CD Spectra**—To address the question of whether $X$ and $Y$ form a binary complex, three CD spectra were measured $(\chi_X), (\chi_Y)$, and $(\chi_{X+Y})$. Spectrum $(\chi_{X+Y})$ is the observed spectrum of $X$ and $Y$, whereas spectrum $(\chi_X) + (\chi_Y)$ is the simulated spectrum calculated by adding the observed spectrum of $X$ to that of $Y$. If the observed and simulated spectra are identical, one can readily infer that there is no detectable interaction between $X$ and $Y$, whereas if the spectra are different, there is unambiguously a binding interaction and hence a binary complex.

To address the question whether $X$, $Y$, and $Z$ form a ternary complex, eight CD spectra were measured $(\chi_X), (\chi_Y), (\chi_{X+Y}), (\chi_{X+Z}), (\chi_{Y+Z}), (\chi_{X+Y+Z})$, and $(\chi_{X+Y+Z})$. Spectrum $(\chi_{X+Y+Z})$ is the observed spectrum of $X$, $Y$, and $Z$; whereas spectrum $(\chi_{X+Y}) + (\chi_{X+Z}) + (\chi_{Y+Z})$ is the simulated spectrum calculated by adding the observed spectrum of the binary complex $X+Y$ to the observed spectrum of the ternary complex $(\chi_X) + (\chi_Y) + (\chi_{X+Y})$. Difference CD spectra $(\chi_{X+Y} - (\chi_X))$ and $(\chi_{X+Z} - (\chi_X))$ were calculated by subtracting the spectrum of the total concentration of $Z$ from the observed spectrum of the binary complexes $(\chi_{X+Y})$ and $(\chi_{X+Z})$, respectively.

The observed spectrum $(\chi_{X+Y} + (\chi_{X+Z}) + (\chi_{Y+Z}))$ were compared with the simulated spectrum $(\chi_X + (\chi_Y) + (\chi_{X+Y})), (\chi_X + (\chi_Z) + (\chi_{X+Z})), (\chi_Y + (\chi_Z) + (\chi_{Y+Z})), (\chi_{X+Y} + (\chi_{X+Z})), (\chi_{X+Y} + (\chi_{Y+Z})), and (\chi_{X+Y} + (\chi_{X+Z})).$ The simulated spectrum $(\chi_{X+Y} + (\chi_{X+Z}))$ is the sum of the spectra of each component if no binding interactions were present among the three components $X$, $Y$, and $Z$. The simulated spectra $(\chi_X + (\chi_Y) + (\chi_{X+Y})), (\chi_X + (\chi_Z) + (\chi_{X+Z})), (\chi_Y + (\chi_Z) + (\chi_{Y+Z})), (\chi_{X+Y} + (\chi_{X+Z})), (\chi_{X+Y} + (\chi_{Y+Z})), and (\chi_{X+Y} + (\chi_{X+Z})).$ The simulated spectrum $(\chi_{X+Y} + (\chi_{X+Z}))$ can be seen as the spectrum obtained when component $Z$ does not bind to the binary complex $(\chi_{X+Y})$. The simulated spectra $(\chi_{X+Y} + (\chi_{X+Z}))$ and $(\chi_{X+Y} + (\chi_{Y+Z}))$ represent the displacement of $Y$ and $X$, respectively, from their binary $(\chi_{X+Y})$ and $(\chi_{X+Z})$ complex by ligand $Z$. Of course, if ligand $Y$ and $Z$ do not interact with each other, the simulated spectrum $(\chi_{X+Y} + (\chi_{X+Z}))$ is therefore redundant. The simulated spectrum $(\chi_{X+Y} + (\chi_{X+Z})), (\chi_{X+Y} + (\chi_{X+Z})), (\chi_{X+Y} + (\chi_{X+Z})), (\chi_{X+Y} + (\chi_{X+Z})), (\chi_{X+Y} + (\chi_{X+Z})), and (\chi_{X+Y} + (\chi_{X+Z})).$ The spectrum of $Z$ as a ternary complex was formed but without interactions between ligands $Y$ and $Z$, namely without direct contacts. The difference CD components represent the CD changes associated with each binary complex. If none of the simulated spectra superimposes the observed spectra, it excludes all the possibilities described above. There is only one possibility left, which cannot be simulated, that is all tertiary components $X$, $Y$, and $Z$ interact with each other upon forming the ternary complex.

**RESULTS**

**Stoichiometry of Hsp90-Cdc37p/p50<sup>dc37</sup> Complexes and Self-association of Cdc37p/p50<sup>dc37</sup>**—The apparently similar roles played by Cdc37p/p50<sup>dc37</sup> and by Sti1/Hop/p60 in the
recruitment of client proteins to the Hsp90 complex prompted us to characterize the binding of Cdc37p and p50\(^{cdc37}\) to Hsp90 using CD spectroscopy. As observed previously with Sti1/Hop/p60 (6), the near-UV CD spectra obtained for mixtures of Hsp90 and Cdc37p cannot be simulated by linear combination of the individual spectra of Hsp90 and Cdc37p in isolation, indicating that Hsp90 and Cdc37p interact to form a complex (Fig. 1A). Titration of Cdc37p into Hsp90 produces dose-dependent perturbations in the near-UV region (240–280 nM), and difference spectra (Fig. 1B) were obtained by subtracting the spectrum of the isolated co-chaperone (n molar equivalent) from the spectrum of Hsp90 + co-chaperone (1:n) mixtures. Although saturable changes in the near-UV region were not achieved due to the relatively weak binding of Cdc37p to Hsp90, the observed signals are consistent with changes in the environment of aromatic residues due to molecular interaction. The \(K_d\) value for the interaction of Cdc37p with Hsp90 was estimated as 113 ± 4 \(\mu M\) (Fig. 1C).

As in the case for Cdc37p, titration of human p50\(^{cdc37}\) into Hsp90 also produced dose-dependent perturbations in the near-UV region (240–280 nM). The difference spectra (Fig. 2A) showed changes that initially peaked at a molar ratio between 1:1 and 1:1.4 (Hsp90:p50\(^{cdc37}\)) and subsequently decreased at higher p50\(^{cdc37}\) concentrations. The behavior of these spectra is consistent with two different association processes occurring. The first phase results from binding of p50\(^{cdc37}\) to Hsp90 that saturates at \(\approx 1:1\), whereas the second phase results from self-association of excess p50\(^{cdc37}\). This interpretation is supported by the observation that the CD spectrum of p50\(^{cdc37}\) is concentration-dependent, consistent with self-association (Fig. 2B).

The \(K_d\) value calculated for the interaction of p50\(^{cdc37}\) with Hsp90 was 2.5 \(\mu M\) (Fig. 2C) and that for p50\(^{cdc37}\) dimerization was 9.8 ± 1.9 \(\mu M\) (Fig. 2D), assuming saturation of Hsp90 by p50\(^{cdc37}\) at a 1:1 molar ratio. Because binding of the mammalian p50\(^{cdc37}\) to Hsp90 was significantly tighter than that of its yeast orthologue Cdc37p, further analysis of interactions with Hsp90 was conducted mainly with p50\(^{cdc37}\).

Analytical Ultracentrifugation—To confirm and quantitatively analyze the self-association of p50\(^{cdc37}\) observed in the CD studies, p50\(^{cdc37}\) oligomerization was analyzed by equilibrium sedimentation in an analytical ultracentrifuge. Equilibrium data sets obtained were intermediate between theoretical traces calculated assuming ideal single species with molecular weights equal to one or two p50\(^{cdc37}\) units. These data could be fitted with high confidence to an equation describing a monomer-dimer equilibrium mixture (Fig. 3) with a \(K_d\) for the mon...
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**Fig. 5. Analysis of Hsp90, geldanamycin, and cSti1 interactions by near-UV CD.** A, near-UV CD spectra for Hsp90 (10 μM), geldanamycin (10 μM), and Hsp90-geldanamycin (1:1 molar ratio), and the simulated spectrum for the mixture (SIM). The simulated spectrum is derived using the spectra of the individual components and is dissimilar to OBS, which indicates that Hsp90 and geldanamycin form a complex. Of particular interest are the changes above 310 nm that are due to geldanamycin alone. B, near-UV difference spectra for titration of cSti1 into Hsp90 (2.42 μM), obtained by subtracting the spectra of the free cSti1 at a molar ratio close to 1:1 (2.6, 3.1, and 5.0). Changes in the difference spectra are consistent with changes in the environment of aromatic residues due to molecular interaction. The saturable changes seen at a molar ratio close to 1:1 (curve 2 is observed previously (1.0.8 and curve 3 represents 1:1.4 molar ratios of Hs90:cSti1) suggests that there are two cSti1-binding sites on Hsp90. Subsequent changes (dotted line spectra), seen above a molar ratio of 1:4, indicate that the excess cSti1 can homodimerize or that a second cSti1-binding site exists. C, estimation of the Kd for the binding of cSti1 to Hsp90. The solid line represents the simulated curve that would be obtained if the titration were continued to obtain saturation of cSti1-binding sites and yields a Kd of 0.1 μM. D, near-UV spectra for Hsp90 (10 μM), cSti1 (30 μM), and spectra for mixtures of Hsp90 and geldanamycin (Hsp + GELD, 1:1 molar ratio), Hsp90 and cSti1 (Hsp + cSti1, 1:3 molar ratio), cSti1 and geldanamycin (cSti1 + GELD, 1:3 molar ratio), and Hsp90, geldanamycin, and cSti1 (OBS, 1:1:3 molar ratio). The simulated spectrum for the mixture of all three components (SIM1) is also shown and was derived from the spectra of the individual components and their spectra resulting from appropriate interactions. SIM2 represents the simulated spectrum of a cSti1 and geldanamycin mixture (3:1 molar ratio), and this spectrum exactly predicts the observed spectrum for this mixture (cSti1 + GELD). Changes above 310 nm are due to geldanamycin, and a reduction in signal above 310 nm indicates that geldanamycin is expelled from Hsp90, and this is observed to occur in the presence of cSti1.

It has been demonstrated previously (16, 17) that Cdc37p/p50cdc37 possesses two separable domains as follows: an N-terminal domain capable of binding Hsp90-dependent protein kinases and a C-terminal domain that binds Hsp90 itself. To determine which domain was responsible for the inherent dimerization of p50cdc37, the separate N- and C-terminal domains of p50cdc37 were subjected to analytical centrifugation in a similar way to the full-length protein. For both domains the data could only be fitted with high confidence to an equation describing monomer-dimer equilibrium mixtures (results not shown) indicating that homodimerization is a consequence of both N to N-terminal domain and C to C-terminal domain interactions. However, dimerization of the N-terminal kinase-binding domain was very weak with a Kd estimated as 971 μM (95% confidence limits = 812–1160 μM) and that for the C-terminal domain was 167 μM (95% confidence limits = 145–192), indicating that the main dimerization interface is between the C-terminal domains. However, it should be noted that the p50cdc37 N-terminal domain used in the current studies lacks the first 29 N-terminal amino acids which, although relatively poorly conserved, could contribute to dimerization.

Modulation of the Hsp90 ATPase Activity by Cdc37p/p50cdc37—The similar recruiting roles that Cdc37p/p50cdc37 and Sti1/Hop/p60 play in the Hsp90 system, and the fact that both are capable of dimerization and of binding to Hsp90 as dimers, led us to question whether, like Sti1/Hop/p60, Cdc37p/p50cdc37 could also inhibit the inherent ATPase activity of Hsp90. By using a regenerating enzyme-linked ATPase assay as described previously (3), we observed that both Cdc37p and p50cdc37 can inhibit the ATPase activity of Hsp90 (Fig. 4, A and B). At a given co-chaperone concentration, p50cdc37 is a more potent inhibitor of the Hsp90 ATPase than Cdc37p, consistent with the higher affinity of p50cdc37 for Hsp90 observed in the CD spectroscopy. As observed previously (6) with Sti1/Hop/p60, inhibition of ATPase activity by Cdc37p or p50cdc37 could be reversed by competition with the TPR domain-binding co-chaperone Cpr6/Cyp40 (Fig. 4, C and D). When the isolated N- or C-terminal domains of p50cdc37 were added to Hsp90 ATPase assays, the N-terminal domain showed no inhibitory effect, whereas the C-terminal domain of p50cdc37 was as potent as the full-length protein (Fig. 4, E and F). Localization of inhibitory activity in the
C-terminal domain of p50\(^{dc37}\) is consistent with its known function as the domain that interacts with Hsp90 (16).

**Co-chaperone Interaction with ATP-binding Domain—** Binding of geldanamycin to the nucleotide-binding site in the N-terminal domain of Hsp90 produces changes in the near-UV CD spectrum above 300 nm, where contributions from protein are negligible, so that the intensity of the difference CD in this region indicates the degree of saturation of the geldanamycin-binding site in the protein (6) (Fig. 5A). Geldanamycin CD thus provides a sensitive probe for measuring the accessibility of the nucleotide-binding pocket and for detecting interactions with co-chaperones or client proteins close to or in that pocket. By using this system we have shown previously that binding of Sti1/Hop/p60 displaces Hsp90-bound geldanamycin (6). As a C-terminal fragment of Sti1 (cSti1, residues 237–589) can reproduce suppression of Hsp90-ATPase by full-length Sti1/Hop/ p60, we investigated the effect of this fragment on geldanamycin binding. Titration of cSti1 into Hsp90 produced dose-dependent perturbations in the near-UV region (240–280 nm), and difference spectra (Fig. 5B) showed changes that initially peak close to a molar ratio of 1:1 (Hsp90:cSti1), as in the binding of full-length Sti1. However, unlike the full-length protein, titration with cSti1 results in further perturbations in the difference spectra above molar ratios of 1:1.4 (Hsp90:cSti1) that are consistent with cSti1 undergoing a second binding interaction (Fig. 5C) either by self-association, as seen with p50\(^{dc37}\) (Fig. 2B), or by binding to a second site on Hsp90. Because a second binding interaction was not observed previously (6) with Sti1, it is most likely that cSti1 is undergoing homodimerization at these concentrations. This situation is analogous to that observed for p50\(^{dc37}\) and suggests that the observed homodimerization of Sti1 also involves contributions from N- to N-terminal domain and C to C-terminal domain interactions. Changes in the difference CD spectra due to the dimerization of cSti1 were taken into account in subsequent experiments. Addition of cSti1 to geldanamycin-bound Hsp90 produces a decrease in the CD above 300 nm indicative of displacement of geldanamycin from the complex (Fig. 5D), as observed previously (6) with the full-length Sti1. This suggests a possible correlation between the ability of the Sti1 constructs to suppress Hsp90 ATPase activity and to interact with the nucleotide-binding pocket.

To determine whether the ATPase inhibitory properties of p50\(^{dc37}\) also involved interaction with the nucleotide-binding pocket, we examined the interaction of p50\(^{dc37}\) with geldanamycin and Hsp90. Whereas geldanamycin shows no interaction with Sti1 (Fig. 5D), initial experiments indicated that geldanamycin has a weak affinity for p50\(^{dc37}\) (Fig. 6A), and this interaction was therefore taken into account in subsequent experiments. Unlike the observation with Sti1 binding, the addition of p50\(^{dc37}\) to an Hsp90-geldenamycin complex did not diminish the difference CD signal above 300 nm, indicating that geldanamycin was not displaced (Fig. 6B). Furthermore, and in contrast to Sti1, the same spectrum was obtained whether geldanamycin was added to Hsp90 prior to or following addition of p50\(^{dc37}\). The observed spectrum for the Hsp90/p50\(^{dc37}\)/geldanamycin mixture could not be simulated by any combination of spectra for a binary complex and one unbound component, nor could it be simulated by combination of spectra for binary interactions. This implies the presence of a ternary Hsp90-p50\(^{dc37}\)-geldanamycin complex in which additional changes have occurred above those resulting from binary interactions of the components.

**DISCUSSION**

Cdc37p/p50\(^{dc37}\) and Sti1/Hop/p60 have been widely implicated in recruitment of client proteins, such as protein kinases and steroid hormone receptors, respectively, into the Hsp90 chaperone system. Cdc37p/p50\(^{dc37}\) forms a direct structural bridge between Hsp90 and protein kinases (23, 28–30), whereas Sti1/Hop/p60 couples Hsp90 to Hsp70 that is in turn bound to a steroid hormone receptor (7–9). Both co-chaperones bind at or near the C terminus of Hsp90, and their binding is mutually exclusive and exclusive of other TPR domain co-chaperones such as Cpr6/Cyp40. The precise location of the Cdc37p/p50\(^{dc37}\)-binding site on Hsp90 is not yet known, but it does not involve the C-terminal MEEVD peptide that is essential...
that there is no direct interaction between Cdc37p/p50 and the C terminus of Hsp90, suggesting that isolated Sti1 is a stable homodimer at a sub-micromolar concentration and binds to the Hsp90 dimer as such, simultaneously occupying both Hsp90 C-terminal TPR domain-binding sites. We have shown here that Cdc37p/p50 also homodimerizes, so that it too binds as a dimer to the Hsp90 dimer.

For both co-chaperones dimerization involves extensive homomeric interactions, so that the isolated Hsp90-interacting C-terminal fragments of both are also dimeric. Binding of Sti1/Hop/p60 to Hsp90 had been shown previously (6) to inhibit the ATPase activity of Hsp90. We have shown here that Cdc37p/p50 also has this property. However, unlike Sti1/Hop/p60, inhibitory binding of Cdc37p/p50 to Hsp90 does not cause displacement of bound geldanamycin from Hsp90, suggesting that there is no direct interaction between Cdc37p/p50 and the N-terminal nucleotide-binding pocket. As with Sti1/Hop/p60, the inhibitory activity of Cdc37p/p50 is localized and can be reproduced by a C-terminal Hsp90-binding fragment of the co-chaperone, which also retains the ability to homodimerize. It is possible that the common ability of these two co-chaperones to occupy both binding sites on the Hsp90 dimer simultaneously, and to homodimerize, plays a key role in their inhibitory activity. The ATPase cycle of Hsp90 is directly coupled to changes in the juxtaposition of the monomers within the dimer (12). Effective "cross-linking" of the Hsp90 monomers due to simultaneous binding of a dimerized co-chaperone would restrict these conformational changes and inhibit the coupled ATP turnover. In other words the binding of two monomeric units to each Hsp90 site would not restrict its conformational state. Support for this model comes from the behavior of the monomeric TPR domain immunophilin Cpr6. The binding of Cpr6 monomers and Sti1/Hop/p60 or Cdc37p/p50 dimers to the C terminus of Hsp90 is competitive. However unlike the dimeric Sti1/Hop/p60 and Cdc37p/p50, Cpr6 shows no ability to dimerize and does not inhibit the ATPase activity of Hsp90 (6).

Sti1/Hop/p60 and Cdc37p/p50 show no detectable homology but have nonetheless evolved a common ability to recruit client proteins to Hsp90 and to inhibit progress through the ATP-coupled chaperone cycle. The stages in Hsp90-dependent activation of steroid hormone receptors have been well characterized (32). Sti1/Hop/p60 is associated with the early phase of this process, recruiting Hsp70 and the bound client to Hsp90, and is replaced in subsequent stages by TPR domain immunophilins (33). Whereas the activation of protein kinase clients is far less well described, Cdc37/p50 clearly has an analogous function to Sti1/Hop/p60 in the early "loading" phase of client activation. The common ability of these recruitment factors to prevent ATP turnover suggests that efficient client protein loading requires Hsp90 to be in the "relaxed" conformation in which the N-terminal domains are unassociated, rather than the N-terminally associated conformation promoted by ATP binding (12). (Fig. 7).

At the present time the stoichiometry of binding between Cdc37p/p50 and Hsp90 protein kinase "client" has not been experimentally determined. However the simultaneous binding of two Cdc37p/p50 molecules to Hsp90 raises the tantalizing prospect that two different protein kinases could theoretically be bound simultaneously to the Hsp90 complex, via independent interaction with the N-terminal domain of the Hsp90-Cdc37p/p50 co-chaperones. Thus in the activation of phosphorylation cascades, the Hsp90-Cdc37p/p50 complex could simultaneously bind the client protein kinase requiring activation and the upstream kinase responsible for carrying out the phosphorylation that achieves that activation. There are in fact several examples of sequential pairs of protein kinases, which are implicated in interactions with Hsp90 and/or Cdc37p/p50. For example, in yeast cell cycle regulation the cyclin-dependent kinase Cdk2 and its activating kinase Cak1 both depend on Cdc37p for stability (34); in insulin and cell survival signaling both protein kinase B (PKB) and its activating kinase c-Src (29–39) are all dependent on interaction with Cdc37p/p50 and Hsp90 for their function. Thus, the Hsp90-Cdc37p/p50 complex could act as a "scaffold" protein, co-localizing sequential kinases and thereby improving the efficiency of the phosphorylation reaction between an upstream kinase and its specific substrate kinase in vivo. As kinases from different pathways would also be brought into proximity by simultaneous binding to the Hsp90-Cdc37p/p50 complex, such a mechanism could generate promiscuity and produce undesirable cross-talk between disparate signaling pathways. However, it is unlikely that scaffolding would overcome the inherent specificity of these phosphorylation reactions which is overwhelmingly governed by the specific amino acid sequences in the vicinity of the target serine, threonine, and tyrosine residues, and by direct specific interactions between docking sites on the sequential kinases.
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REFERENCES
1. Pearl, L. H., and Prodromou, C. (2002) Adv. Protein Chem. 59, 157–185
2. Grenert, J. P., Johnson, B. D., and Toft, D. O. (1999) J. Biol. Chem. 274, 17525–17533
3. Panaretou, B., Prodromou, C., Roe, S. M., O’Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1998) EMBO J. 17, 4829–4836
4. Obergann, W. M. J., Sondermann, H., Russo, A. A., Pavletich, N. P., and Hartl, F. U. (1988) J. Cell Biol. 143, 901–910
5. Scheibl, T., Weikl, T., and Buchner, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1495–1499
6. Prodromou, C., Siligardi, G., O’Brien, R., Woolfson, D. N., Regan, L., Panaretou, B., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1999) EMBO J. 18, 754–762
7. Smith, D. F., Sullivan, W. P., Marion, T. N., Zaitsev, K., Madden, B., McCormick, D. J., and Toft, D. O. (1993) Mol. Cell. Biol. 13, 869–876
8. Johnson, B. D., Schumacher, R. J., Ross, E. D., and Toft, D. O. (1998) J. Biol. Chem. 273, 3679–3686
9. Chen, S. Y., Prapapanich, V., Rimerman, R. A., Honore, B., and Smith, D. F. (1996) Mol. Endocrinol. 10, 682–693
10. Schreuder, C., Brinker, A., Bourkenov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U., and Mooi, R. (2000) Cell 101, 199–210
11. Russell, L. C., Whitt, S. R., Chen, M.-S., and Chinkers, M. (1999) J. Biol. Chem. 274, 20060–20063
12. 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) EMBO J. 19, 4383–4392
13. Morishima, Y., Kanelakis, K. C., Silverstein, A. M., Dittmar, K. D., Estrada, L., and Pratt, W. B. (2000) J. Biol. Chem. 275, 6894–6900
14. Chang, H. C. J., Nathan, D. F., and Lindquist, S. (1997) Mol. Cell. Biol. 17, 318–325
15. Perdew, G. H., Wiegand, H., VandenHeuvel, J. P., Mitchell, C., and Singh, S. S. (1997) Biochemistry 36, 3600–3607
16. Grammatikakis, N., Lin, J.-H., Grammatikakis, A., Tsichlis, P. N., and Cochran, B. H. (1999) Mol. Cell. Biol. 19, 1661–1672
17. Shao, J., Grammatikakis, N., Scroggins, B. T., Uma, S., Huang, W. J., Chen, J. J., Hartson, S. D., and Mattis, R. L. (2001) J. Biol. Chem. 276, 206–214
18. Hunter, T., and Poon, R. Y. C. (1997) Trends Cell Biol. 7, 157–161
19. Gerber, M. R., Farrell, A., Deshaies, R. J., Herskowitz, I., and Morgan, D. O. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4651–4655
20. Owens-Grillo, J. K., Czar, M. J., Hutchison, K. A., Hoffmann, K., Perdew, G. H., and Pratt, W. B. (1996) J. Biol. Chem. 271, 13468–13475
21. Ramsey, A. J., Russell, L. C., Whitt, S. R., and Chinkers, M. (2000) J. Biol. Chem. 275, 17587–17592
22. Chen, S. Y., Sullivan, W. P., Toft, D. O., and Smith, D. F. (1998) Cell Stress Chaperones 3, 118–129
23. Silverstein, A. M., Grammatikakis, N., Cochran, B. H., Chinkers, M., and Pratt, W. B. (1998) J. Biol. Chem. 273, 20990–20995
24. Freeman, D. J., Pattenden, G., Drake, A. F., and Siligardi, G. (1998) J. Chem. Soc. Perkin Trans. 2, 129–135
25. Renzoni, D. A., Pugh, D. J. R., Siligardi, G., Das, P., Morton, C. J., Rossi, C., Waterfield, M. D., Campbell, I. D., and Ladbury, J. E. (1996) Biochemistry 35, 15646–15653
26. Siligardi, G., and Hussein, R. (1998) Enantiomer 3, 77–87
27. Kurz, L. C., Shah, S., Crane, B. R., Donald, L. J., Duckworth, H. W., and Drysdale, G. R. (1992) Biochemistry 31, 7908–7914
28. Stepilova, L., Lang, X. H., Parker, S. B., and Harper, J. W. (1996) Genes Dev. 10, 1491–1502
29. Stancato, L. F., Chow, Y. H., Hutchinson, K. A., Perdew, G. H., Jove, R., and Pratt, W. B. (1993) J. Biol. Chem. 268, 21711–21716
30. Abbas-Toqi, T., Denze, O., and Picard, D. (2000) FEBS Lett. 467, 111–116
31. Young, J. C., Obergann, W. M. J., and Hartl, F. U. (1998) J. Biol. Chem. 273, 18007–18010
32. Smith, D. F. (2000) Semin. Cell Dev. Biol. 11, 45–52
33. Smith, D. F., Whitesel, L., Nair, S. C., Chen, S., Prapapanich, V., and Rimerman, R. A. (1995) Mol. Cell. Biol. 15, 6804–6812
34. Farrell, A., and Morgan, D. O. (2000) Mol. Cell. Biol. 20, 749–754
35. Sato, S., Fujita, N., and Tsuru, T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10832–10837
36. Fujita, N., Sato, S., Ishida, A., and Tsuru, T. (2002) J. Biol. Chem. 277, 10346–10353
37. Setalo, G. J., Singh, M., Guan, X., and Toran-Allerand, C. D. (2002) J. Neurobiol. 50, 1–12
38. Pratt, W. B. (1998) Proc. Soc. Exp. Biol. Med. 217, 420–434
39. Xu, Y., Singer, M. A., and Lindquist, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 109–114