THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 38, Issue of September 17, pp. 39975–39981, 2004
Printed in U.S.A.

Definition of Protein Kinase Sequence Motifs That Trigger High Affinity Binding of Hsp90 and Cdc37*

Received for publication, June 21, 2004
Published, JBC Papers in Press, July 17, 2004, DOI 10.1074/jbc.M406882200

Thomas Prince and Robert L. Matts‡

From the Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, Oklahoma 74078-3035

Hsp90 cooperates with its co-chaperone Cdc37 to provide obligatory support to numerous protein kinases involved in the regulation of cellular signal transduction pathways. In this report, the crystal structure of the Src family tyrosine kinase Lck was used to guide the creation of kinase constructs to determine features recognized by Hsp90 and its "kinase-specific" co-chaperone Cdc37. Two parameters were assayed: the ability and extent to which the constructs bound to Hsp90 and Cdc37, and the ability of the constructs to trigger salt-resistant high affinity complexes with Hsp90 and Cdc37 independent of the presence of molybdate. Although Hsp90 interacted with both the N-terminal and C-terminal lobes (NL and CL, respectively) of the catalytic domains of the kinases, the lobes themselves were not sufficient to trigger the high affinity binding of Hsp90. Only constructs containing a complete N- or C-terminal lobe and part of the adjacent lobe bound to Hsp90 and Cdc37 in salt-stable complexes independent of molybdate. The two minimum constructs that bound Hsp90 and Cdc37 contained the α-C-helix and the β4- and β5-strands of the NL through to end of the CL and the NL through to the α-E-helix and the amino acids that cap the helix. Cdc37 interacted with only the NL and minimally required the α-C-helix and β4- and β5-strands of this lobe of Lck. The results indicate that the high affinity binding activity of Hsp90 is triggered through its interaction with adjacent subdomain structures of kinase catalytic domains. Furthermore, the α-C-helix and part of its adjoining loop connection to the β4-strand appear to be the primary determinants recognized by Cdc37.

In vivo, Hsp90[1] function is essential for the biogenesis and support of numerous cellular proteins that regulate signal transduction, including transcription factors, protein kinases, and a wide variety of proteins, such as nitric-oxide synthase and telomerase (reviewed in Refs. 1–4). Despite over a decade of intense study, the basis for the recognition by Hsp90 of its diverse clients remains one of the primary mysteries in the field. Studies using site-directed and deletion mutagenesis (5–11) have identified potential sites within steroid hormone receptors and protein kinases that may be recognized by Hsp90. However, although a recent study has indicted that point mutations generated in the Cdk4 kinase (12) affect its interaction with Hsp90, no common primary, secondary, and/or tertiary structural motifs have been defined that may be responsible for Hsp90 client recognition.

Hsp90 functions in conjunction with a coterie of non-client co-chaperone partners (reviewed in Refs. 1–4). Current models suggest that co-chaperones may confer specificity to Hsp90 facilitated protein folding by also interacting with client targets (5, 13, 14). Cdc37 is one such Hsp90 co-chaperone that interacts with immature forms of Hsp90-dependent kinases (reviewed in Ref. 15). The biochemical activity of Cdc37 is essential for Hsp90-mediated support of kinase function (16–20).

In vitro, Cdc37 exhibits a "passive" chaperone activity similar to that of Hsp90. Cdc37 has been shown to prevent the aggregation of denatured protein while also being able to maintain the protein in a state that is competent to refold with no requirement for input of energy from ATP hydrolysis (16). Furthermore, the domain structure of Cdc37 has been determined, and its kinase binding and Hsp90 binding activities have been mapped to its N-terminal and middle domains, respectively (20–22), and we have demonstrated that phosphorylation of Cdc37 at Ser^{13} by casein kinase II is require for the ability of Cdc37 to bind protein kinase (23). However, although the specific basis for the recognition of protein kinases by Cdc37 is unknown, recent studies have mapped the recognition motif to the N-terminal lobe of the catalytic domain of protein kinases (12, 24, 25).

Hsp90 inhibitors have aided studies into the mechanisms underlying the function of Hsp90 and its co-chaperones (reviewed in Refs. 1–4). The N-terminal nucleotide binding domain of Hsp90 is the site of action for the Hsp90 inhibitor geldanamycin (26–28), whereas its C-terminal domain contains a binding site for novobiocin, which also inhibits Hsp90 function (29–32). Analyses with these compounds, with nucleotides and nucleotide analogs, and with site direct mutants that alter Hsp90 ATP binding and/or ATPase activity have revealed that Hsp90 function is regulated via the binding and hydrolysis of ATP, which modulates the switching of Hsp90 between at least three alternative conformations (1–4, 32). In the presence of geldanamycin, Hsp90 binds weakly to client kinases in a salt-labile fashion (20, 24, 33). These aberrant Hsp90 heterocomplexes indicate that nucleotide modulation of conformational switching is required to generate high affinity interactions of Hsp90 and Cdc37 with protein kinases (20, 24, 33). Like geldanamycin, the anion molybdate inhibits Hsp90 function. However, in contrast to geldanamycin, this inhibition reflects the ability of molybdate to "lock" or freeze Hsp90 ki-

* This work was supported by American Heart Association Award 0250556N, Oklahoma Center for the Advancement of Science and Technology Grant HR03-076, and Oklahoma Agricultural Experiment Station Project 1975. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: 246 NRC, Dept. of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078-3035. Tel.: 405-744-6200; Fax: 405-744-7799; E-mail: rmatts@biochem.okstate.edu.

[1] The abbreviations used are: Hsp90, 90-kDa heat shock protein; NL, N-terminal lobe of the Lck kinase catalytic domain; CL, C-terminal lobe of the Lck kinase catalytic domain; LBD, ligand-binding domain; Pipes, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid].

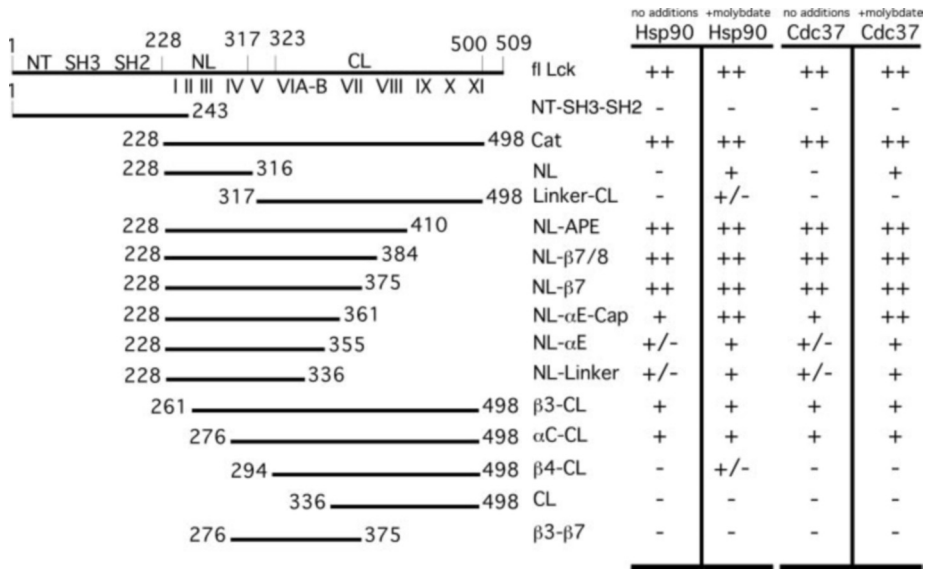

FIG. 1. **Summary of protein kinase constructs used for analysis of Hsp90 and Cdc37 binding to Lck constructs.** The domain architecture of the Lck kinase is indicate at the *top* of the diagram. The *numbers* above the *lines* indicate the amino acids residues that flank: the unique N-terminal domain of Lck (*NT*), SH2 and SH3 domains, the NL of Lck, the linker region between the NL and CL of Lck, the CL of Lck, and the C-terminal tail of Lck. The *Roman numerals* below the *line* indicate the locations of conserved kinase motifs I–XI (as proposed by Hanks and Hunter (43)). The *lower lines* indicate the amino acid residues present in each of the domain and subdomain constructs studied in this report and where the constructs begin and terminate with the abbreviation used to refer to each construct indicated to the *right* of each line. The *chart* to the *right* summarizes the binding of Hsp90 and Cdc37 to the constructs in the absence (no additions) or presence of molybdate (+*molybdate*): ++, binding equivalent or nearly equivalent to the catalytic domain; +, binding reduced by ~50% relative to the catalytic domain; +/−, binding less than 10% of that of the catalytic domain; −, no detectable binding.

nase complexes in high affinity salt-stable complexes (20, 24, 33). Thus, molybdate causes the accumulation of Hsp90 complexes containing the Hsp90 co-chaperones p23, Cdc37, and an assortment of other "late" complex components, such as tetratricopeptide repeat motif-containing immunophilins (20, 24, 33–39). However, although molybdate stabilizes the normally labile interaction of Hsp90 with steroid receptor clients (24, 33, 34), the interactions of Hsp90 with kinase clients are stable to high salt concentrations independent of molybdate freezing (20, 24, 33, 34). Thus, we have utilized the ability of kinase constructs to trigger molybdate-independent, high affinity, salt-stable interactions with Hsp90 and Cdc37 to define motifs recognized by these chaperones (24).

In this report, we have systematically deleted secondary structural elements from the catalytic domain of the Hsp90-dependent Src family protein tyrosine kinase Lck to increase the resolution of motifs recognized by Hsp90 and Cdc37. The data confirm that Cdc37 recognizes structures or sequences present in the N-terminal lobe of the catalytic domain of the kinase. Furthermore, the motifs within the N-terminal and C-terminal lobes of the catalytic domain that are required to trigger nucleotide-dependent conformational switching of Hsp90 and molybdate-independent high affinity binding of Hsp90 and Cdc37 to kinase constructs overlap at the junction of the two subdomains.

EXPERIMENTAL PROCEDURES

Plasmids—The coding sequences for protein domains and subdomains were cloned by PCR into a modified pSP64T plasmid (40) as previously described (24, 41–43). N-terminally His-tagged versions of each domain were constructed. Sequences represented: the Lck NT-terminal domains (NT-SH2-SH3; residues 1–243); catalytic domain (Cat; residues 228–498); the N-terminal lobe (NL) of the catalytic domain of Lck plus the linker region between the lobes (NL-linker; residues 228–336); the NL of the catalytic domain (NL; residues 228–316); the C-terminal lobe (CL) of the catalytic domain of Lck plus the linker between the two lobes (linker-CL; residues 317–498); the C-terminal lobe of the catalytic domain containing motifs (44) VIa-XI (CL; residues 336–498); the NL through conserved kinase motifs VIII (NL-APE; residues 228–410); the NL through conserved kinase motifs VII (NL-β7/8; residues 228–384); the NL through conserved kinase motifs VIb (NL-β7; residues 228–375); the NL through conserved kinase motifs VIa and the β6 sheet region (NL-αE-Cap; residues 228–361); the NL through conserved kinase motifs VIa (NL-αE; residues 228–355); the regions of the catalytic domain of Lck encompassing kinase motif II through the CL (β3-CL; residues 261–498); the regions of catalytic domain of Lck encompassing kinase motif III through the CL (αC-CL; residues 276–498); the regions of the catalytic domain of Lck encompassing kinase motif IV through the CL (β4-CL; residues 294–498); and the regions of the catalytic domain of Lck encompassing kinase motifs IV–VIb (αC-β7; residues 276–375). These dissections are represented graphically in Fig. 1.

Co-immunoprecipitations of Chaperones with His-tagged Kinase Constructs—Lck constructs were cloned via NcoI/EcoRI into a modified pSP64T plasmid that coded for an N-terminal His_6 tag. Using a no DNA blank as control for nonspecific binding, each construct was synthesized and radiolabeled with [^{35}S]Met by coupled transcription/translation in 60 μl of nuclease-treated rabbit reticulocyte lysate (TNT; Promega) for 24 min at 30 °C. Each sample was then split into two (30 μl) aliquots, supplemented with either 0.5 μl of 1 M sodium molybdate (final concentration, 17 mM) or deionized water, and then incubated for another 1 min. All of the samples were then immediately placed on ice, clarified, and immunoabsorbed with 25 μl of anti-His_5 antibody (Qiagen) bound to anti-mouse IgG resin for 1 h at 4 °C (24). Immunoresins were then washed once with P50T (10 mM Pipes, pH 7.2, 50 mM NaCl, and 0.5% Tween 20), three times with P500T (same as P50T except with 500 mM NaCl), and again with P50T. No $NaMoO_4$ was present in the wash buffers. Finally, the samples were boiled in SDS sample buffer, separated on SDS-PAGE, transferred to polyvinylidene difluoride membrane, and blotted for both endogenous Hsp90 and Cdc37 (24).

To load approximately equal molar amounts of each translation product, a portion of each TNT reaction was spotted on a piece of filter paper and precipitated with 10% trichloroacetic acid. After boiling the filters in 5% trichloroacetic acid, the filters were washed sequentially with ethanol and acetone and then dried. The filters were subsequently bleached with 15% hydrogen peroxide, dried, and counted in a scintillation counter. Equivalent molar amounts of samples were loaded from SDS elution of resins based on the amount of [^{35}S]Met incorporated into protein construct in each TNT reaction and the number of methionine residues present in each construct. The samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane for Western blotting and autoradiography. The membranes were stained lightly with Coomassie Blue to visualize protein patterns and the molecular weight markers, dried and exposed to film to determine pres-

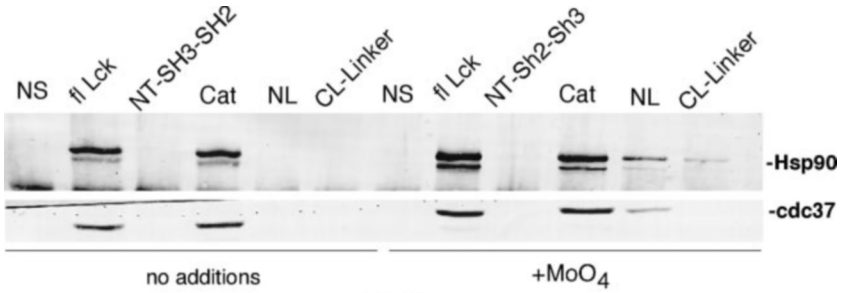

Fig. 2

FIG. 2. **Interaction of Hsp90 and Cdc37 with domain and subdomain constructs of Lck.** ^{35}S-Labeled His-tagged Lck constructs (full-length (*fl*) Lck, the N-terminal domains of Lck (*NT-SH3-SH2*), the Lck kinase catalytic domain (*Cat*), and the NL and CL-linker constructs) were synthesized in reticulocyte lysate for 25 min at 30 °C, followed by immunoadsorption to anti-His tag antibody resin in the presence (+ *MoO_4*) or absence of 17 mM molybdate. Lysate containing no template (*NS*, control) was immunoadsorbed as a control for nonspecific binding of proteins to the immunoresin. The samples were washed and analyzed by Western blotting for the co-adsorption of Hsp90 and Cdc37 as described under "Experimental Procedures." *Top panel*, Western blotted with anti-Hsp90; *bottom panel*, Western blotted with anti-Cdc37 antibodies.

ence and pattern of radioactively labeled protein bands, and then cut according to molecular weight markers for probing with the appropriate anti-chaperone antibodies. Immunoadsorption of each target kinase construct was confirmed by autoradiography (not shown in the figures).

RESULTS

Characterization of the Interactions of the Subdomains of Lck with Hsp90 and Cdc37—Although Hsp90 and Cdc37 recognize and interact with the catalytic domain of protein kinases (9, 10, 18, 20, 45–48), we have recently reported that Hsp90 also interacts with the N-terminal heme-binding domain of the heme-regulated α-subunit of eukaryotic initiation factor 2 kinase (24). Although the kinase domains of Src family kinases retain the ability to bind Hsp90, the possibility that Hsp90 may have additional interactions with the N-terminal domains of this kinase family (*i.e.* the unique N-terminal, SH3, and SH2 domains) has not been investigated. Therefore, N-terminally His-tagged full-length Lck, the N-terminal domains of Lck (NT-SH3-SH2), the Lck kinase catalytic domain (Cat), and the NL and C-terminal lobe of the catalytic domain that contained the sequences that links the two lobes (linker-CL) were cloned and expressed by coupled transcriptional/translation in nuclease-treated reticulocyte lysate. The domains were then immunoadsorbed with anti-His tag antibodies and analyzed by SDS-PAGE and Western blotting for co-adsorbing chaperones. Consistent with previous findings (24), full-length Lck and the catalytic domain of Lck formed salt-stable complexes with Hsp90 and Cdc37 independent of the presence of molybdate (Fig. 2). Hsp90 and Cdc37 also co-adsorbed with the NL construct of Lck, but molybdate was required to stabilize their interactions. Hsp90, but not Cdc37, interacted with the CL-linker construct of Lck, and molybdate was also required to stabilize this interaction to washing with high salt buffer. No Hsp90 or Cdc37 was found to co-adsorb with the NT-domains of Lck (NT-SH3-SH2) in the presence or absence of molybdate. Thus, the interaction of Hsp90 and Cdc37 with Lck was confined to its catalytic domain, and, as we have previously observed, the interaction of Cdc37 was localized to the NL of Lck (24).

Definition of the Minimal Motifs within the CL of the Catalytic Domain of Lck Required for Molybdate-independent High Affinity Binding of Hsp90 and Cdc37—To further delineate the kinase segments that interacted with Hsp90 and Cdc37, we utilized the crystal structure of the kinase domain of Lck as a guide to express constructs from which conserved kinase sequence motifs were deleted from the C-terminal lobe of the kinase (Fig. 3). The Lck constructs containing the NL and deletions that terminated the catalytic domain just after the "APE box" (motif VIII, N-APE), the conserved DFG residues (motif VII, NL-β7/8), and the conserved HRDL(K/R)*XX*N resi-

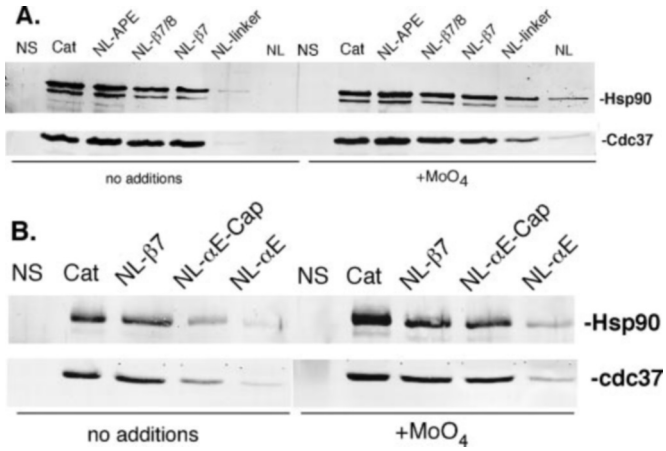

FIG. 3. **Interaction of Hsp90 and Cdc37 with domain and subdomain constructs of Lck catalytic domain containing deletions of CL kinase motifs.** *A*, ^{35}S-labeled His-tagged Lck constructs (Lck catalytic domain (*Cat*), NL-APE, NL-β7/8, NL-β7, and NL-linker) were synthesized in reticulocyte lysate for 25 min at 30 °C, followed by immunoadsorption to anti-His tag antibody resin in the presence (+ *MoO_4*) or absence of 17 mM molybdate. *B*, ^{35}S-labeled His-tagged Lck constructs (Lck catalytic domain (*Cat*), NL-β7, NL-αE-Cap, and NL-αE) were synthesized in reticulocyte lysate for 25 min at 30 °C, followed by immunoadsorption to anti-His tag antibody resin in the presence (+ *MoO_4*) or absence of 17 mM molybdate. Lysate containing no template (*NS*, control) was immunoadsorbed as a control for nonspecific binding of proteins to the immunoresin. The samples were washed and analyzed by Western blotting for the co-adsorption of Hsp90 and Cdc37 as described under "Experimental Procedures." *Top panels*, Western blotted with anti-Hsp90; *bottom panels*, Western blotted with anti-Cdc37 antibodies.

dues (motif VIb, NL-β7) formed complexes with Hsp90 and Cdc37 that were stable in high salt in the absence of molybdate, and the binding occurred at levels that were nearly equivalent to the binding of the catalytic domain (Fig. 3*A*). The Lck construct that contained the NL and sequences from the linker region terminating with the α-D-helix that connects the two kinase lobes (motif V, NL-linker) showed a very weak salt stable interaction with Hsp90 and Cdc37, which was markedly enhance in the presence of molybdate. Although again as shown in Fig. 2, Hsp90 and Cdc37 co-adsorbed with the NL but only in the presence of molybdate.

To more finely characterize sequences within the C-terminal kinase lobe of Lck that were required to trigger the high affinity binding of Hsp90 and Cdc37, two more constructs containing the NL and a portion of the CL were constructed: an NL with a deletion terminating prior to the catalytic Asp residue contained in motif VIb (NL-αE-Cap) and an NL with a deletion terminating at the end of the αE-helix that lacks the three

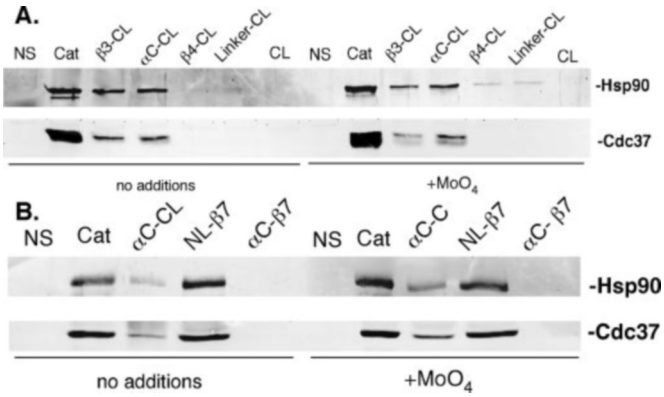

FIG. 4. **Interaction of Hsp90 and Cdc37 with domain and subdomain constructs of Lck catalytic domain containing deletions of NL and CL kinase motifs.** *A*, ^{35}S-labeled His-tagged Lck constructs (Lck catalytic domain (*Cat*), β3-CL, αC-CL, β4-CL, linker-CL, and CL) were synthesized in reticulocyte lysate for 25 min at 30 °C, followed by immunoadsorption to anti-His tag antibody resin in the presence (+ *MoO$_4$*) or absence of 20 mM molybdate. *B*, ^{35}S-labeled His-tagged Lck constructs (Lck catalytic domain (*Cat*), αC-CL, NL-β7, and αC-β7) were synthesized in reticulocyte lysate for 25 min at 30 °C, followed by immunoadsorption to anti-His tag antibody resin in the presence (+ *MoO$_4$*) or absence of 17 mM molybdate. Lysate containing no template (*NS*, control) was immunoadsorbed as a control for nonspecific binding of proteins to the immunoresin. The samples were washed and analyzed by Western blotting for the co-adsorption of Hsp90 and Cdc37 as described under "Experimental Procedures." *Top panel*, Western blotted with anti-Hsp90; *bottom panel*, Western blotted with anti-Cdc37 antibodies.

amino acids that cap the C-terminal end of this helix (equivalent to the β-6 sheet in the nomenclature for the cAMP-activated protein kinase (44)) and likely stabilizes its structure (NL-αE). The NL-αE-Cap construct showed molybdate-independent salt-stabile interactions with Hsp90 and Cdc37 that were reduced by ~50% compared with the Lck catalytic domain and the NL-β7 construct (Fig. 3*B*). However, in the presence of molybdate, the binding of Hsp90 and Cdc37 to the NL-αE-Cap construct was nearly equivalent to the binding of the proteins to the NL-β7 construct. In contrast, the interaction of Hsp90 and Cdc37 with the NL-αE construct was scarcely detectable in the absence of molybdate and was markedly decreased even in the presence of molybdate when compared with the NL-αE-Cap construct. Thus, it seems that the six amino acid residues just past the end of the α-E-helix in the C-terminal lobe of the catalytic domain of Lck appear to be required to trigger Hsp90 to adopt its high affinity binding conformation.

Definition of the Minimal Motifs within the NL of the Catalytic Domain of Lck Required for Molybdate-independent High Affinity Binding of Hsp90 and Cdc37—Similarly, to more clearly characterize sequences within the N-terminal kinase lobe of Lck that were required to trigger the high affinity binding of Hsp90 and Cdc37, we prepared constructs of the catalytic domain of Lck that contained the complete CL from which conserved kinase sequence motifs were deleted from the N-terminal lobe. Constructs of the catalytic domain were made from which the β1 and β2 sheets (motif I) were deleted (β3-CL); the β1, β2, and β3 three sheets (motifs I-II) were deleted (αC-CL); the region from the β1 sheet through the α-C-helix was deleted (motifs I-III, β4-CL); the region from the β1 sheet through the β5 sheet was deleted (motifs I- IV, linker-CL); and the region from the β1 sheet through the connecting strand including the α-D-helix was deleted (motifs I–V, CL). Of these constructs, the β3-CL and the αC-CL constructs showed molybdate-independent salt-stabile interactions with Hsp90 and Cdc37 that were reduced by ~50% compared with the Lck catalytic domain (Fig. 4*A*). The β4-CL, linker-CL, and the CL constructs showed no interaction with Hsp90 or Cdc37 in the absence of molybdate. In the presence of molybdate the β4-CL and the linker-CL constructs were found to have a very weak interaction with Hsp90 but no interaction with Cdc37. The CL construct showed no interaction with either Hsp90 or Cdc37 in the presence of molybdate. Thus, the presence of the α-C-helix within in the NL of the Lck kinase was required for the binding of Cdc37. In addition the region of Lck that connects the NL and CL of the catalytic domain is required for minimal recognition by Hsp90, and the presence of molybdate is required to stabilize this interaction.

Determination of the Effect of the Region That Links the N- and C-terminal Kinase Lobes on Hsp90/Cdc37 Binding—To test the hypothesis that the borders required for Hsp90 and Cdc37 that were identified in the experiments shown in Figs. 3 and 4*A* defined the minimal Hsp90/Cdc37-interacting motif, a construct of Lck was made that contained the region between the α-C-helix and β7 sheets (motifs III–VIb, αC-β7). Although the αC-CL and the NL-β7 constructs demonstrated molybdate-independent salt-stable binding to Hsp90 and Cdc37, albeit at a somewhat reduced level, no interaction of the αC-β7 construct was observed with Hsp90 and Cdc37, even in the presence of molybdate (Fig. 4*B*).

DISCUSSION

The work presented here further defines the motifs or structures that are required for recognition of kinases by Hsp90 and Cdc37, as defined by molybdate-stabilized binding of Hsp90 and Cdc37 to kinase constructs and for triggering conformational switching of Hsp90, as defined by salt-stable binding of Hsp90 and Cdc37 to kinase constructs that occurs independent of the presence of molybdate. The data presented here indicate that the Hsp90-Cdc37 heterocomplex binds to kinase molecules primarily via the specific recognition of kinase motifs or structures present in both the N-terminal and C-terminal lobes of the kinase catalytic domain of Lck. This is consistent with our previous work demonstrating that multiple kinase motifs present within both lobes of kinases are required to trigger molybdate-independent high affinity binding of Hsp90 and Cdc37 to protein kinases (24).

The linker region that connects the N- and C-terminal lobes of the Lck kinase appears to play a critical role for Hsp90 binding. The NL-linker and the linker-CL both interacted with Hsp90 albeit with reduced affinity. Stabilization of this interaction of Hsp90 with these constructs by the addition of molybdate markedly increased the level of Hsp90 binding, indicating that the minimal constructs were deficient in triggering Hsp90 conformational switching. The NL and CL constructs that lacked the linker region failed to bind Hsp90 in the absence of molybdate and only the NL exhibited any detectable interaction with Hsp90 (~5% compared with the catalytic domain control) in the presence of molybdate.

The results presented here together with previously published data (12, 24) localize the Cdc37 recognition motif to the α-C-helix and the αC-β4 loop motif in the NL of the catalytic domain of protein kinases. Cdc37 did not interact with the linker-CL Lck construct, whereas Hsp90 bound the construct. Lck constructs of the CL containing deletions of the β1, β2, and β3 sheets within the NL stably bound Cdc37 and Hsp90 in an molybdate-independent manner, although the level was reduced by ~50% compare with the intact catalytic lobe. Deletion of the α-C-helix negated the interaction of Cdc37, but not Hsp90 with Lck constructs containing the complete CL. Previous work investigating the interaction of Cdc37 with Cdk4 constructs fused to the C terminus of glutathione *S*-transferase indicated that GST-Cdk4 constructs containing the NL of Cdk4 through the α-C-helix and residues within the loop between the

α-C-helix and β4 sheet bound Hsp90 and Cdc37 (12). Deletion of residues in the loop caused a marked reduction in Hsp90/Cdc37 binding. Thus, the results of the two studies intersect at the region of the α-C-helix-β4 loop.

The presence of a β-sheet structure appears be necessary for recognition of the NL of kinase catalytic domains by Hsp90 and/or Cdc37. However, our results differ from those obtained in the studies utilizing the Cdk4 kinase (12). Single layered β-sheet structures are not stable, because the hydrophobic face of the sheet in solution would be exposed to water. Thus, β-sheet structures must interact with an additional "layer" for their structure to be stabilized. The crystal structure of Cdk2 (the protein kinase with the greatest sequence similarity to Cdk4 that has been crystallized (49)) suggests that there is no interaction between the hydrophobic face of the first three strands of β-sheet in the kinase NL and the hydrophobic face of the α-C-helix. However, the crystal structures of the inactive conformations of c-Src and Hck indicate that their β-4/5 sheets may be stabilized through interactions with conserved hydrophobic residues in the linker region that connects the SH2 domain to the catalytic domain (50–53). Thus, Hsp90 and/or Cdc37 may also act to stabilize the β-sheet structure within the NL during folding in the absence of other interactions that stabilize the domain in the mature structure.

Thus, the results obtained in this study utilizing the Lck kinase catalytic domain compared with the results obtained with Cdk4 kinase suggest that there are subtle differences between protein kinase families relative to how their five-stranded β-sheet layers are stabilized during kinase folding, in addition to how they are packed onto and influence the orientation of the α-C-helix. Subtleties in the topology of NL β-sheet layers likely account for the observation that mutation of Gly^{15} in the P-loop in the Cdk4 kinase or deletion of the first two strands of the β-sheet markedly decreases the binding of Cdc37, whereas deletion of the entire P-loop structure of the Lck catalytic domain has no effect on the binding of Hsp90 and Cdc37 to the β3-CL Lck construct. Because Cdc37 functions to foster the active conformation of protein kinases, the interactions of Hsp90/Cdc37 with components of the NL may function to stabilize the β-sheet structure in a manner that facilitates Cdc37-induced rotation of the α-C-helix. The crystal structures of the inactive conformations of Cdk2, Src, and Hck indicate that the catalytic Glu in the α-C-helix lies on the outer surface of the proteins and that the helix must be rotated to properly position of the Glu within the catalytic pocket (49–53). However, the crystal structures suggest that interactions between residues within the α-C-helix and β4-loop with residues present in the CL would sterically impede or prevent such a rotation from occurring spontaneously.

Our data indicate that the minimal kinase structure that is required to trigger molybdate-independent high affinity binding of Hsp90/Cdc37 is one complete lobe of the catalytic domain of the protein kinase, the connecting linker, and a portion of the second lobe. For the constructs containing the complete CL of Lck, the minimum NL structure consists of the α-C-helix and the β-4/5 sheets, whereas for the complete NL the minimum CL structure consists of the α-E-helix and the residues that cap its structure at its C terminus (equivalent in kinase nomenclature to the β6 strand of the cAMP-activated protein kinase (44)). Although it is noteworthy that inclusion of catalytic residues that follow the β-7 sheet in the CL restore nearly wild-type binding of Hsp90 and Cdc37 to construct of the NL of Lck. However, the combination of these minimum structures, the construct containing the α-C-helix through the α-E-helix-cap (αC-β7), does not bind Hsp90 or Cdc37. This finding is consistent with observation that substantial or partial folding of client proteins appears to occur prior to recruitment of Hsp90 to the client. Thus, we have further defined the minimum motifs within protein kinase clients that are recognized and "trigger" the Hsp90 chaperone machine to undergo its nucleotide-mediated conformational switching. Furthermore, the results indicate that the presence of the minimal Cdc37 recognition motif is not sufficient to stabilize the binding of Cdc37 to the construct in the absence of a stabile interaction of the construct with Hsp90.

The localization of this "switching motif/conformational trigger" is also noteworthy as the minimal structures within the two domains intersect at the region that makes up the "hinge" between the two lobes of the kinase (54). With the exception of the "activation loop," comparison of crystal structures of inactive and active conformations of protein kinases indicate that the position of residues contained within the C-terminal lobe of protein kinases are nearly superimposable. Additionally, this analysis of kinase structures indicates that the αC-helix-β4 loop and the peptide strand connecting the N-terminal and C-terminal catalytic lobes act as a key region for global changes in kinase conformation that regulate the activation and activity of protein kinases (54). Our data suggest that these kinase motifs are critical for the binding of Hsp90 and Cdc37 to kinases. Furthermore, the superimposition of kinase structures indicate that the α-C-helix of the NL is rather randomly disposed but with structural elements meeting at the point of the hinge (54). This region is located where the α-E helix intersects with the loop between the α-C-helix and the β4-strand of the NL and the linker region between the two kinase lobes. At this point the β7/8 strands, which contain the catalytic kinase residues present in the CL, loop over the α-E-helix and pass between the loop connecting the α-C-helix and β4-strand and the strand that connects the two kinase lobes (Fig. 5*A*). Premature packing of the α-C-helix-β4 loop and the peptide strand connecting the two catalytic lobes onto the α-E-helix would preclude the proper positioning of the β7/8 strands. Thus, the Hsp90 chaperone complex may function to allow the independent folding of the two catalytic subdomains of protein kinase prior to their subsequent packing into an active or activable structure. This hypothesis would be consistent with the apparent requirement for at least one full kinase subdomain and additional sequences within the second subdomain and that the minimal motifs required for triggering high affinity binding of Hsp90 intersect at the point at which the two subdomains interact.

Comparisons of crystal structures indicate that twisting motions about the hinge affect the relative position of the N-terminal kinase lobe and the α-C-helix. This motion affects the position of the conserve catalytic Lys and Glu residues relative to the catalytic residues present in the C-terminal lobe (54), such that small perturbations of these structural elements have significant effects on kinase conformation and kinase activity (54). Furthermore, in the absence of bound nucleotide, the hydrophobic pocket that is occupied by the adenine ring may collapse upon exposure of the ATP-binding pocket to water. Thus, the Hsp90 chaperone machinery may function to correct structural perturbations that render the catalytic domain inactive by physically separating the two subdomains of the catalytic lobe of a kinase, allowing them to refold independently. Concurrently, Cdc37 might function to stabilize the orientation of the catalytic Glu residue in the α-C-helix relative to the position of the catalytic Lys. Subsequently, the kinase subdomains would then be released from the Hsp90·Cdc37 complex and allowed to interact (repack) into an active or activable conformation that is capable of binding ATP.

What mechanisms then govern the recognition by Hsp90 of

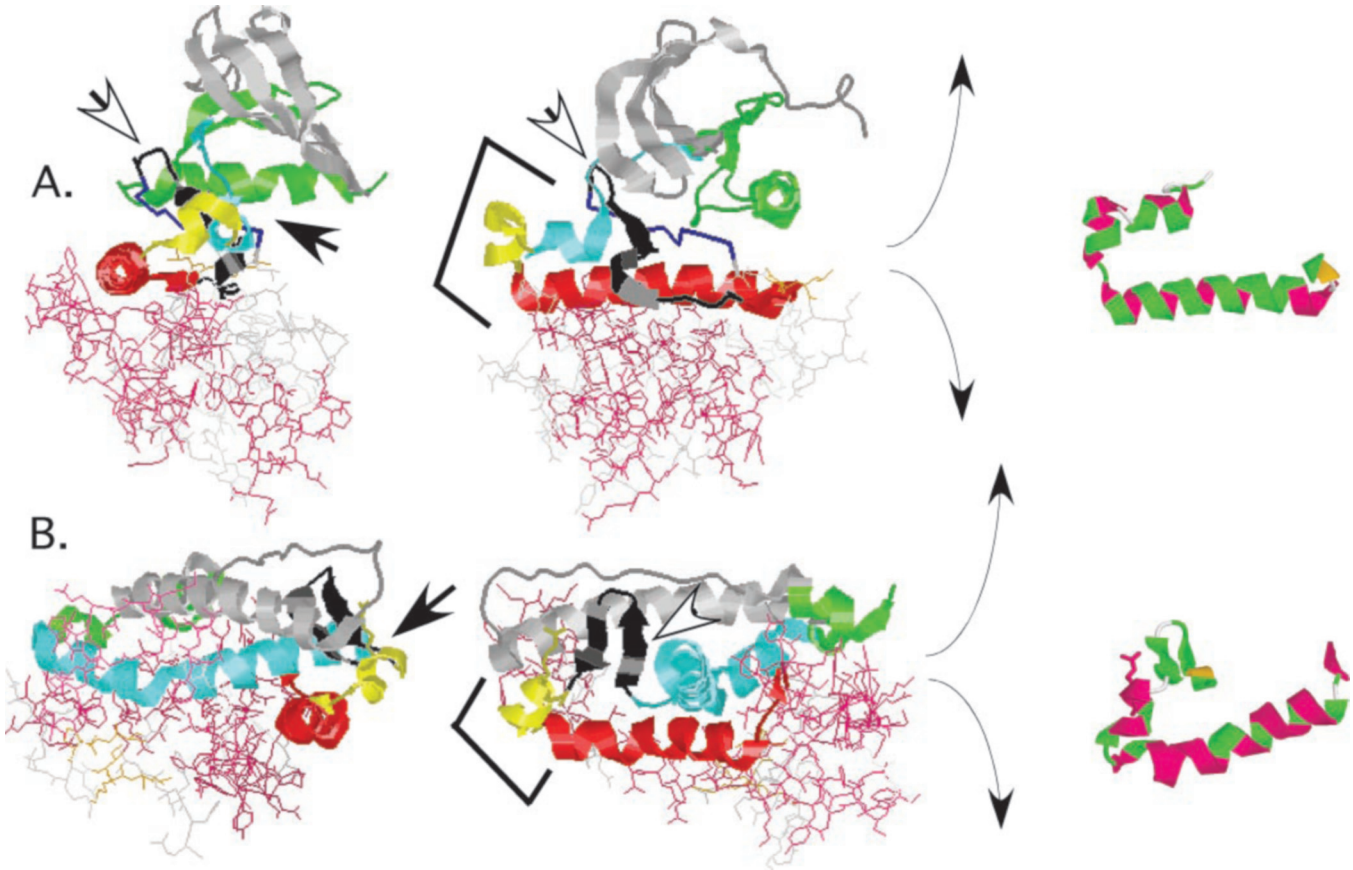

FIG. 5. **Diagrams of the Lck kinase catalytic (*A*) and the glucocorticoid receptor ligand-binding domain (*B*) highlighting structural motifs that have been implicated in their interactions with Hsp90.** The diagrams were constructed using RasMac2.6-UCB and the Protein Data Bank entries 1QPC (Lck catalytic domain) and 1NHC (glucocorticoid receptor-LBD). *A*, *left* and *center panels*, residues 231–275 (the first three β-strands of the Lck catalytic domain) are colored *gray*; residues 276–317 (the α-C-helix and β-strands 4/5) are colored *green*; residues 318–329 (the random coil and α-D-helix) are colored *cyan*; residues 330–336 (the α-helical insert between the α-D and α-E-helix) are colored *yellow*; residues 337–361 (the a-E-helix and its C-cap) are colored *red*; residues 362–375 (motif VIB containing the β-7 sheet) are colored *black*; the backbone of residues 376–384 (motif VII containing the β-8 sheet) are colored *dark blue*; and the remainder of the molecule is shown in wire frame mode and colored by structure (*magenta*, α-helix; *white*, random coil). *B*, *left* and *center panels*, residues 530–537 (the α-1-helix) and residues 582–587 (a short α-helix between helix 3 and 4 of the glucocorticoid receptor-LBD that are proposed to be part of the "hydrophobic clamp") are colored *green*; residue 538–581 (the end of the α-1helix through the α-3-helix) are colored *gray*); residues 588–619 (the α-4 and α-5 helices) are colored *cyan*; residues 620–630 (the two strands of β-sheet in the linker region) are colored *black*; residues 631–638 (the α-6 helix in the hinge region containing Arg^{633} shown in stick format) are colored *yellow*; residues 639–659 (the α7-helix) are colored *red*; the remainder of the C-terminal subdomain is shown in wire frame format and colored by structure (*magenta*, α-helix; *yellow*, β-sheet; *white*, random coil). *A* and *B*, *right panels*, cartoon diagram of the proposed hinge regions of the kinase domain of Lck and the LBD of the glucocorticoid receptor followed by the minimal part of the C-terminal subdomain of each molecule that is required for Hsp90 binding colored by structure, with hydrophobic residues colored *green*. *A*, *left* and *center panels*, *open arrows*, positions of β7 and β8 strands that must past between the junction of the N- and C-terminal kinase subdomains. *A* and *B*, *left panel*, *closed arrow*, and *center panel*, *open sided box*, positions of linker regions between the two subdomains of each protein; *curved arrows*, indicate proposed movement of the subdomains that would be required to reposition the β-sheet structures at the interface between the two subdomains of each protein. *B*, *open arrow*, position of the ligand-binding pocket behind the two strands of β-sheet.

protein structures and its switching from low affinity to high affinity binding? The data of Pratt and co-workers (6) suggest a model of the ligand-binding domain (LBD) of the glucocorticoid receptor acting as a "hinged pocket." They propose that Hsp90 recognizes α-helical structures that interact at the interface between the two subdomains of the LBD and that sequences present in a β-sheet structure function as a hinge between the two subdomains. This interaction of Hsp90 with the LBD is proposed to break the hydrophobic clasp formed by the interaction between the two subdomains, allowing hormone to access its ligand-binding site. The trypsin sensitivity of the LBD near the β-stranded region suggests that its flexibility would allow separation of the two subdomains (6). Similar to the findings reported here, deletion analysis of the LBD (14) indicates that a construct containing the complete N-terminal subdomain of the LBD and the β-stranded hinge through the first α-helix of the C-terminal portion of the ligand-binding subdomain is the minimal construct of the glucocorticoid receptor that binds Hsp90 (Fig. 5*B*).

Of further interest is the observation that the two strands of β-sheet that make up this proposed hinge are a single layer motif with the sequence ^{620}LLCFAPDLII^{629}. The side of the sheet facing the ligand-binding pocket is composed exclusively of hydrophobic amino acids, with the surface of the sheet also having considerable hydrophobic character. The "hole" representing the ligand-binding pocket results in both sides of the β-sheet being exposed to solvent. Thermodynamics might favor the collapse of the β-sheet structure into the interior of the protein in the absence of hormone occupying its binding pocket, considering that this region has been reported to be conformationally mobile (6). Thus, an additional consequence of Hsp90 separating the two subdomains of the LBD would be to reposition the β-strands and allow refolding of the hormone-binding pocket.

The findings of Pratt and co-workers, together with the data presented here suggest a common theme for Hsp90 function. Hsp90 and its associated co-chaperones may function to facilitate protein folding by breaking interactions between subdo-

mains within the structure of a protein. Inspection of the crystal structures of the kinase domain of Lck and the LBD of the glucocorticoid receptor indicate that the structures of the flexible linker regions between the N- and C-terminal subdomains of each of these proteins and the α-helical region that follows are strikingly similar (Fig. 5, *right panels*). The separation of the subdomains of steroid hormone receptors or protein kinases may function to displace protein structures that impede the ability of critical amino acid residues to be properly positioned or to reposition critical amino acids, which would subsequently allow the protein to attain its active or activable (regulatable) conformation and attain a structure capable of binding ATP or hormone, respectively. For the *de novo* folding of protein kinases, Hsp90 may be required to reorient a two-stranded β-sheet structure, subsequently allowing the structure to occupy a hole formed at the junction between the two catalytic subdomains and for the ATP-binding pocket to be properly folded. Although for steroid hormone receptors, Hsp90 would function to reorient a two-stranded β-sheet structure, removing the structure from a hole that exists because of the absence of hormone occupying its LBD, subsequently allowing the unoccupied steroid hormone binding pocket to reform. These distinct variations between protein kinases and steroid hormone receptors may then account for the differences in the characteristics of their interactions with Hsp90. Whereas the catalytic domains of protein kinases can form high affinity salt-stabile complexes with Hsp90 in the absence of molybdate (24, 33), the LBD of steroid hormone receptors require molybdate to stabilize Hsp90 binding (55). Additionally, although Cdc37 is required for Hsp90 to form high affinity complexes with protein kinases and for their productive folding, Hsp90 has not been documented to require such a cohort when restoring steroid hormone receptor function (1).

Acknowledgements—We thank the Sarkey's Biotechnology Research Laboratory (Oklahoma State University) DNA sequencing and the Oklahoma State University HYCABS Facility for antibody production.

REFERENCES

1. Pratt, W. B., and Toft, D. O. (2003) *Exp. Biol. Med.* **228,** 111–133
2. Picard, D. (2002) *Cell Mol. Life Sci.* **59,** 1640–1648
3. Pearl, L. H., and Prodromou, C. (2002) *Adv. Prot. Chem.* **59,** 157–185
4. Richter, K., and Buchner, J. (2001) *J. Cell. Physiol.* **188,** 281–290
5. Caamano, C. A., Morano, M. I., Dalman, F. C., Pratt, W. B., and Akil, H. (1998) *J. Biol. Chem.* **273,** 20473–20480
6. Giannoukos, G., Silverstein, A. M., Pratt, W. B., and Simons, S. S., Jr. (1999) *J. Biol. Chem.* **274,** 36527–36536
7. Kaul, S., Murphy, P. J., Chen, J., Brown, L., Pratt, W. B., and Simons, S. S., Jr. (2002) *J. Biol. Chem.* **277,** 36223–36232
8. Xu, M., Dittmar, K. D., Giannoukos, G., B., P. W., and Simons, J., S. S. (1998) *J. Biol. Chem.* **273,** 13918–13924
9. Fujita, N., Sato, S., Ishida, A., and Tsuruo, T. (2002) *J. Biol. Chem.* **277,** 10346–10353
10. Sato, S., Fujita, N., and Tsuruo, T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10832–10837
11. Couette, B., Jalaguier, S., Hellal-Levy, C., Lupo, B., Fagart, J., Auzou, G., and Rafestin-Oblin, M. E. (1998) *Mol. Endocrinol.* **12,** 855–863
12. Zhao, Q., Boschelli, F., Caplan, A. J., and Arndt, K. T. (2004) *J. Biol. Chem.* **279,** 12560–12564
13. Scheibel, T., Weikl, T., and Buchner, J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1495–1499
14. Dalman, F. C., Scherrer, L. C., Taylor, L. P., Akil, H., and Pratt, W. B. (1991) *J. Biol. Chem.* **266,** 3482–3490
15. MacLean, M., and Picard, D. (2003) *Cell Stress Chaperones* **8,** 114–119
16. Kimura, Y., Rutherford, S. L., Miyata, Y., Yahara, I., Freeman, B. C., Yue, L., Morimoto, R. L., and Lindquist, S. (1997) *Genes Dev.* **11,** 1775–1785
17. Stepanova, L., Leng, X., Parker, S. B., and Harper, J. W. (1996) *Genes Dev.* **10,** 1491–1502
18. Grammatikakis, N., Lin, J.-H., Grammatikakis, A., Tsichlis, P. N., and Cochran, B. H. (1999) *Mol. Cell. Biol.* **19,** 1661–1672
19. Scholz, G., Hartson, S. D., Cartedge, K., Hall, N., Shao, J., Dunn, A. R., and Matts, R. L. (2000) *Mol. Cell. Biol.* **20,** 6984–6995
20. Shao, J., Gramatikakis, N., Scroggins, B., Uma, S., Huang, W., Chen, J.-J., Hartson, S. D., and Matts, R. L. (2001) *J. Biol. Chem.* **276,** 206–214
21. Shao, J., Irwin, A., Hartson, S. D., and Matts, R. L. (2003) *Biochemistry* **42,** 12577–12588
22. Scholz, G. M., Cartledge, K., and Hall, N. E. (2001) *J. Biol. Chem.* **276,** 30971–30979
23. Shao, J., Prince, T., Hartson, S. D., and Matts, R. L. (2003) *J. Biol. Chem.* **278,** 38117–38120
24. Scroggins, B. T., Prince, T., Shao, J., Uma, S., Huang, W., Guo, Y., Yun, B. G., Hedman, K., Matts, R. L., and Hartson, S. D. (2003) *Biochemistry* **42,** 12550–12561
25. Mort-Bontemps-Soret, M., Facca, C., and Faye, G. (2002) *Mol. Genet. Genomics* **267,** 447–458
26. Whitesell, L., Mimnaugh, E. G., De Costa, B., Myers, C. E., and Neckers, L. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 8324–8328
27. Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U., and Pavletich, N. P. (1997) *Cell* **89,** 239–250
28. Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997) *Cell* **90,** 65–75
29. Marcu, M. G., Chadli, A., Bouhouche, I., Catelli, M., and Neckers, L. M. (2000) *J. Biol. Chem.* **275,** 37181–37186
30. Marcu, M. G., Schulte, T. W., and Neckers, L. (2000) *J. Natl. Cancer Inst.* **92,** 242–248
31. Soti, C., Racz, A., and Csermely, P. (2001) *J. Biol. Chem.* **277,** 7066–7075
32. Yun, B.-G., Huang, W., Leach, N., Hartson, S. D., and Matts, R. L. (2004) *Biochemistry* **43,** 8217–8229
33. Hartson, S. D., Irwin, A. D., Shao, J., Scroggins, B. T., Volk, L., Huang, W., and Matts, R. L. (2000) *Biochemistry* **39,** 7631–7644
34. Hartson, S. D., Thulasiraman, V., Huang, W., Whitesell, L., and Matts, R. L. (1999) *Biochemistry* **38,** 3837–3849
35. Johnson, J. L., and Toft, D. O. (1995) *Mol. Endocrinol.* **9,** 670–678
36. Johnson, B. D., Schumacher, R. J., Ross, E. D., and Toft, D. O. (1998) *J. Biol. Chem.* **273,** 3679–3686
37. Sullivan, W., Stensgard, B., Caucutt, G., Bartha, B., McMahon, N., Alnemri, E. S., Litwack, G., and Toft, D. (1997) *J. Biol. Chem.* **272,** 8007–8012
38. Thulasiraman, V., and Matts, R. L. (1996) *Biochemistry* **35,** 13443–13450
39. Shao, J., Hartson, S. D., and Matts, R. L. (2002) *Biochemistry* **41,** 6770–6779
40. Kreig, P. A., and Melton, D. A. (1984) *Nucleic Acids Res.* **12,** 7057–7070
41. Hartson, S. D., and Matts, R. L. (1994) *Biochemistry* **33,** 8912–8920
42. Uma, S., Hartson, S. D., Chen, J.-J., and Matts, R. L. (1997) *J. Biol. Chem.* **272,** 11648–11656
43. Uma, S., Matts, R. L., Guo, Y., White, S., and Chen, J.-J. (2000) *Eur. J. Biochem.* **267,** 498–506
44. Hanks, S. K., and Hunter, T. (1995) *FASEB J.* **9,** 576–596
45. Chen, G., Cao, P., and Goeddel, D. V. (2002) *Mol. Cell* **9,** 401–410
46. Brugge, J. S. (1986) *Curr. Top. Microbiol. Immunol.* **123,** 1–22
47. Silverstein, A. M., Grammatikakis, G., Cochran, B. H., Chinkers, M., and Pratt, W. B. (1998) *J. Biol. Chem.* **273,** 20090–20095
48. Basso, A. D., Solit, D. B., Chiosis, G., Giri, B., Tsichlis, P., and Rosen, N. (2002) *J. Biol. Chem.* **277,** 39858–39866
49. DeBondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O., and Kim, S.-H. (1993) *Nature* **363,** 595–602
50. Xu, W., Harrison, S. C., and Eck, M. J. (1997) *Nature* **385,** 595–602
51. Xu, W., Doshi, A., Lei, M., Eck, M. J., and Harrison, S. C. (1999) *Mol. Cell* **3,** 629–638
52. Schindler, T., Sicheri, F., Pico, A., Gazit, A., Levitzki, A., and Kuriyan, J. (1999) *Mol. Cell* **3,** 639–648
53. LaFevre-Bernt, M., Sicheri, F., Pico, A., Porter, M., Kuriyan, J., and Miller, W. T. (1998) *J. Biol. Chem.* **273,** 32129–32134
54. Williams, J. C., Wierenga, R. K., and Saraste, M. (1998) *Trends Biol. Chem.* **23,** 179–184
55. Pratt, W. B., and Toft, D. O. (1997) *Endocr. Rev.* **18,** 306–360