Cdk2: A Genuine Protein Kinase Client of Hsp90 and Cdc37†

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ABSTRACT: Hsp90 and its cochaperone Cdc37 cooperate to provide requisite support to numerous protein kinases involved in cellular signal transduction. In this report, we studied the interactions of Hsp90 and Cdc37 with the cyclin-dependent kinase, Cdk2. Treatment of K562 cells with the Hsp90 inhibitor, geldanamycin, caused a 75% reduction in Cdk2 levels and reduced the levels of its activating kinase, Cdk7, by more than 60%, suggesting that both of these kinases may be Hsp90 clients. Using classical pull-down assays and the Hsp90 inhibitory agents geldanamycin and molybdate, Cdk2 is shown to be a genuine client of the Hsp90 chaperone complex. Subsequently, pull-down assays directed at helix αC of Cdk2 are shown to disrupt Hsp90 and Cdc37 binding and explain the initial difficulties in demonstrating these interactions. Mutant constructs containing deletions of secondary structural elements from the N- and C-termini of Cdk2 were prepared and assayed for their ability to coadsorb Hsp90 and Cdc37 in a salt-stable high-affinity manner with and without the addition of molybdate. Consistent with similar work done with the cyclin-dependent kinase relative Cdk4, the presence of the G-box motif of Cdk2 was shown to be critical for Cdc37 binding, whereas consistent with work done with the Src-family tyrosine kinase Lck, the presence of helix αC and the stabilization of helix αE were shown to be needed for Hsp90 binding.

Hsp90[1] is a 90 kDa ATPase that functions as a molecular chaperone for a multitude of signal transduction proteins, which play critical roles in controlling practically every facet of a cell's physiology. The ability of Hsp90 to function as a chaperone to this wide array of signal transduction proteins is due, in part, to the coterie of nonclient cochaperones that are components of the Hsp90 heteromeric superchaperone machine (*1*−*4*). In this regard, the Hsp90 cochaperone Cdc37 is a protein required for the viability of eukaryotic cells (*5*, *6*). Cdc37 carries out a function essential for Hsp90's ability to support the activity of numerous protein kinases: as such, Cdc37 is often termed the "kinase specific cochaperone" (*7*−*10*). However, Cdc37 has also been found in complexes with the androgen receptor, certain viral reverse transcriptases, and MyoD, suggesting that it likely has a broader set of clients (*11*−*13*).

As noted above, protein kinases, which are one of the largest families of signal transduction proteins (*14*), often depend on Hsp90 to carry out their function. Hsp90, together with Cdc37, interacts with protein kinases to facilitate their proper folding and/or stabilize their structure (*7*−*10*, *15*). The productive interactions of Hsp90 and Cdc37 with protein kinases are known to require the presence of specific domains or motifs within the protein kinase's bilobal structure (*15*, *16*). "Motifs" recognized by Hsp90 and Cdc37 have been defined by a number of criteria, those utilized by our laboratory being (1) the co-immunoadsorption of Hsp90 and/or Cdc37 with kinase constructs that are stabilized in the presence of molybdate, which locks Hsp90 in its high-affinity client-binding conformation; and (2) the co-immunoadsorption of Hsp90 and/or Cdc37 with kinase constructs that remain stable to washing of immune resins with buffer containing 0.5 M NaCl in the absence of molybdate, which indicates that the kinase construct has induced Hsp90 to undergo ATP-dependent conformational switching into its high-affinity binding conformation independent of molybdate.

Using these criteria, in conjunction with the deletion of defined secondary structural elements from the Lck tyrosine kinase, our laboratory has identified potential structural motifs recognized by Hsp90 and Cdc37 (*15*, *16*). Cdc37 appears to recognize only structural motifs present in the N-terminal lobe (NL) of Lck's catalytic domain, while Hsp90 appears to interact with motifs present in both the N- and C-terminal (CL) lobes (*15*, *16*). However, molybdate-independent high-affinity binding of Hsp90 and Cdc37 to protein kinase constructs required the presence of at least one complete lobe of the catalytic domain (either the NL or

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[1] Abbreviations: Hsp, heat shock protein; Hsp90, 90 kDa Hsp; Cdc37, generically used to refer to the protein product of *CDC37* gene homologues regardless of the source organism; NL, N-terminal lobe of the catalytic domain of protein kinases; CL, C-terminal lobe of the catalytic domain of protein kinases; TnT, coupled transcription and translation of a protein in nuclease-treated rabbit reticulocyte lysate; IgG, immunoglobulin G; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DMSO, dimethyl sulfoxide; PVDF, polyvinylidene difluoride; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; EGTA, ethylene glycol bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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the CL) and a portion of the adjacent lobe. The minimum constructs of the Lck catalytic domain required to induce high-affinity binding of Hsp90 and Cdc37 were found to be (a) the NL through the helix capping motif of helix αE in the CL and (b) the helix αC in the NL through the CL (*15*, *16*). However, these findings were at variance with studies done with the cyclin-dependent kinase, Cdk4 (*17*), in which the G-box (also known as the P-loop, which consists of the first two of three strands of *β*-sheet that proceed the helix αC) was identified as a motif needed for the interaction of Cdk4 with Cdc37. In our studies, the deletion of this motif had no effect on the binding of Cdc37 to Lck. Thus, the exact manner of recognition of structural motifs within protein kinases by Hsp90 and Cdc37 is still not completely understood, and consequently, we have attempted to refine our understanding of interactions of Hsp90 and Cdc37 with protein kinases by studying another kinase, the cyclin-dependent kinase Cdk2.

Cdk2, like Cdk4, is a protein kinase that regulates cell cycle progression through the coordinated association with specific regulatory proteins, known as cyclins (*18*). Once cyclin binds, Cdk2 is able to undergo a conformational change that allows its activation loop to be phosphorylated on T^{160} by Cdk-activating kinase (CAK, also known as Cdk7/cyclin H/Mat1) (*19*). Activated Cdk2 is then able to phosphorylate downstream effector proteins, which in turn help drive DNA replication and eventually S/G_2 transition. Additionally, Cdk2's kinase activity can be inhibited by phosphorylation of G-box residue Y^{15} by the bifunctional kinase Wee1 (*20*−*22*).

Previous genetic and yeast two-hybrid studies have shown that both Hsp90 and Cdc37 interact with Cdk2, suggesting that Cdk2 is an Hsp90 client kinase (*23*). Furthermore, it has been shown that Cdc37 interacts with the N-terminal lobe (NL) of Cdk2, an observation supported by our findings with Lck (*15*, *16*). However, despite these and other studies, no true physical interaction between Cdk2 and Hsp90 or Cdc37 via pull-down assays has been documented (*10*, *24*, *25*), and it has been stated that Cdk2 is not an Hsp90 client protein (*10*).

In this report, we demonstrate that Cdk2 indeed physically interacts with Hsp90 and Cdc37 via pull-down assays and subsequently verify Cdk2 as a client protein kinase of the Hsp90−Cdc37 chaperone complex. A series of N-terminal and C-terminal deletions of the Cdk2 kinase were carried out to further define structural motifs that are recognized by Hsp90 and Cdc37 and the motifs required to trigger molybdate-independent high-affinity binding of Hsp90 and Cdc37 to protein kinases. Our results confirm that helix αC of Cdk2 is critical for the recognition of protein kinases by Hsp90 and Cdc37, but also reiterate that Cdc37 may recognize NL motifs in subtly different manners depending on the structure of a specific kinase species.

EXPERIMENTAL PROCEDURES

Plasmids. Cdk2 constructs were cloned via NcoI and EcoRI into a modified pSP64T plasmid (*26*) that encoded an N-terminal His_6 tag as previously described (*15*, *27*−*29*). N-Terminally His-tagged versions of each domain were constructed. Sequences represented full-length Cdk2 (FL, residues 1−298), the N-terminal lobe through the complete

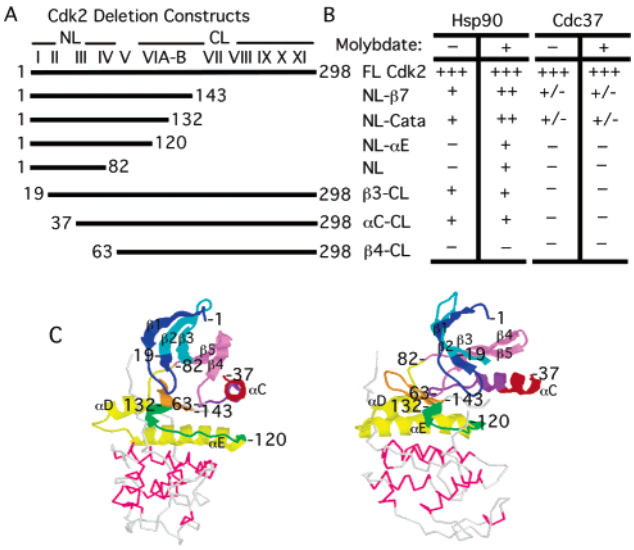

FIGURE 1: Summary of the His-tagged Cdk2 deletion constructs used for the analysis of Hsp90 and Cdc37 interactions. (A) Placement of the NL and CL constructs of Cdk2 relative to the conserved protein kinase structural motifs designated by the Roman numerals [as proposed by Hanks and Hunter (*43*)]. The numbers flanking each line indicate the first and last residue, respectively, in that construct. (B) The chart is aligned with the constructs to indicate the degree of Hsp90 and Cdc37 binding in the absence and presence of molybdate: +++, 100% of that of full-length Cdk2; ++, less than 50% of that of full-length Cdk2; +, less than 25% of that of full-length Cdk2; ±, marginal detection, or less than 5% of that of full-length Cdk2; and −, no detectable binding. (C) Structure of the Cdk2 kinase [PDB entry 1AQ1 (*51*)] looking down helix αC (left panel) or the structure rotated by approximately 90° (right panel). Amino acid residues 1−143 and 144−298 are depicted in cartoon and backbone format, respectively, and are colored blue (residues 1−19), cyan (residues 20−36), purple (residues 37−63, with the PSTAIRE epitope colored red), violet (residues 64−82), yellow (residues 83−120), green (residues 121−132), and orange (residues 133−143). Residues 144−298 are colored in structure format (magenta for helices and white for connecting strands). Positions of strands *β*1−*β*5 and helices αC−αE are indicated. This figure was rendered with RasMac2.6-UCB.

structural motif VI (NL-*β*7, residues 1−143), the N-terminal lobe through all the catalytic residues present in motif VI (NL-Cata, residues 1−132), the N-terminal lobe through structural motif VIA (NL-αE, residues 1−120; the uncapped αE helix), the NL (residues 1−82), structural motif II through the CL (*β*3-CL, residues 19−298), structural motif III through the CL (αC-CL, residues 37−298), and structural motif IV through the CL (*β*4-CL, residues 63−298) (Figure 1). The pcDNA3-CycA(wt)-AU5 plasmid which encodes T7-driven cyclin A with the C-terminal epitope tag, AU5, was a generous gift from J. Ruderman of Harvard Medical School (Boston, MA).

Generation of ^{35}S-Labeled Protein Constructs. To examine the interaction of newly synthesized Cdk2 with Hsp90 and Cdc37, ^{35}S-labeled His-tagged Cdk2 was synthesized by coupled transcription and translation in 25 *μ*L of nuclease-treated rabbit reticulocyte lysate (TnT, Promega) for 30 min at 30 °C. For samples treated with drugs, geldanamycin (GA, final concentration of 10 *μ*g/mL) or an equal volume of dimethyl sulfoxide (DMSO) was added prior to the initiation of synthesis. Lysate containing no DNA template was used as a control for nonspecific binding.

For reconstituting Cdk2−cyclin A complexes, ^{35}S-labeled His-tagged Cdk2 or cyclin A-AU5 (cyclin A containing a

six-amino acid TDFYLK epitope tag at its C-terminus) was synthesized in separate reactions by TnT in nuclease-treated rabbit reticulocyte lysate for 30 min at 30 °C in the presence of either 10 μg/mL geldanamycin or DMSO. Samples were then combined (20 μL each) or mixed with lysate containing no DNA template to give a total volume of 40 μL, and incubated at 37 °C for an additional 30 min.

To examine the interaction of Hsp90 and Cdc37 with mutant Cdc37 constructs, ^{35}S-labeled full-length or deletion constructs of Cdk2 were synthesized by TnT in 60 μL of nuclease-treated rabbit reticulocyte lysate for 30 min at 30 °C. Two 27 μL aliquots were supplemented with either 0.5 μL of 1 M sodium molybdate (final concentration of 17 mM) or deionized water, and then incubated for an additional 1 min. The remaining 6 μL of TnT lysate was put into 50 μL of SDS sample buffer and analyzed on a 10% SDS−PAGE gel and by autoradiography, as a control to verify that equivalent amounts of input protein were synthesized in each reaction. Lysate containing no DNA plasmid was again used as a control for nonspecific binding.

Co-Immunoadsorptions of Protein Complexes. For all immunoadsorptions, samples were immediately placed on ice, clarified by centrifugation for 5 min at maximum speed in a Fisher 235C microfuge, and immunoadsorbed with 25−30 μL of either anti-mouse IgG or anti-rabbit IgG immunoresin containing prebound mouse anti-His tag IgG (anti-His_5 Qiagen), mouse nonspecific IgG, rabbit anti-AU5 (Covance Research), rabbit sc-53 anti-PSTAIRE (Santa Cruz Biotechnology), or rabbit nonspecific IgG. Immunoresins were mixed for 1 h at 4 °C, and then washed once with buffer containing10 mM PIPES (pH 7.2), 50 mM NaCl, and 0.5% Tween 20 (P50T), two times with P500T (same as P50T except with 500 mM NaCl), and again with P50T. No molybdate was present in the wash buffers. Finally, the samples were boiled in SDS sample buffer, separated on an 8% SDS−PAGE gel, and transferred to a PVDF membrane for autoradiography and/or Western blotting to detect coadsorbed Hsp90 and Cdc37 (*15*). Band densities were quantified by scanning densitometry (Bio-Rad).

Assay of the Kinase Activity of Cdk2 and the Cdk2−Cyclin A Complex. His-tagged Cdk2 and cyclin A were synthesized, mixed (or not), and incubated in the presence or absence of geldanamycin, as described above. Samples were immunoadsorbed with anti-His tag immunoresin and washed twice with P500 (no Tween 20) and twice with P150. Subsequently, 30 μL of kinase buffer [50 mM PIPES (pH 7.2), 2 mM $MgCl_2$, 4 mM DTT, 10 mM glycerol phosphate, 5 mM NaF, and 200 μM ATP] was added along with 1 μg of histone H1 (Upstate Cell Signaling Solutions). Precomplexed and activated GST-tagged Cdk2−cyclin A fusion (0.4 μg) (Cell Signaling) was used as a positive control for kinase activity. Samples were incubated at 37 °C for 10 min followed by boiling in SDS sample buffer. Finally, samples were separated on an 8% SDS−PAGE gel and analyzed by Western blotting with anti-phosphohistone H1 antibody (Upstate Cell Signaling Solutions).

Expression of Cdk2 and Cdk7 in Geldanamycin-Treated K562 Cells. K562 cells were grown in six-well plates in RPMI 1640 medium with 10% fetal calf serum and 1% antibiotics to 50% confluency. Cells were then grown in the presence of either DMSO or 0.1 μM geldanamycin for 0, 12, or 24 h, washed once with Hank's balanced salts, and lysed in lysis buffer containing 20 mM HEPES (pH 7.4), 100 mM NaCl, 2 mM EGTA, 10% glycerol, 0.5% Igepal CA630 (Sigma), and mammalian protease inhibitor cocktail (Sigma). Lysates were clarified by centrifugation as described above, and protein concentrations were determined via the BCA assay (Pierce). Samples were then analyzed by SDS−PAGE and Western blotting with polyclonal anti-Cdk2 (rabbit sc-748, Santa Cruz Biotechnology) or anti-Cdk7 (mouse sc-7344, Santa Cruz Biotechnology) antibody.

RESULTS

Hsp90 and Cdc37 Physically Interact with Cdk2. Previous genetic studies have suggested that both Hsp90 and Cdc37 interact with Cdk2 (*23*). However, no direct physical interaction has been documented between Cdk2 and the Hsp90−Cdc37 chaperone complex. Indeed, one of the initial papers describing the interactions of Cdc37 with Cdk kinases concluded that Cdc37 did not interact with Cdk2 (*10*), yet Cdk4, a close relative of Cdk2, has been well established as a client protein kinase that physically interacts with Hsp90 and Cdc37.

Complexes between the Hsp90−Cdc37 complex and Cdk4 have been isolated from cell extracts, as well as reconstituted in vitro utilizing Cdk4 generated by coupled transcription−translation (TnT) in rabbit reticulocyte lysate (*10*, *17*). Thus, we tested whether a physical interaction between Cdk2 and the Hsp90−Cdc37 complex could similarly be reconstituted in reticulocyte lysate with newly synthesized Cdk2. Cdk2 was cloned with an N-terminal His tag, and ^{35}S-labeled His-tagged Cdk2 was synthesized by TnT in reticulocyte lysate. His-tagged Cdk2 was then immunoadsorbed with anti-His antibody, and the immune pellets were washed at high stringency (buffer containing 0.5 M NaCl) to determine whether Hsp90 and Cdc37 bound Cdk2 in a salt-stable high-affinity complex independent of the presence of molybdate. Western blot analysis indicated that endogenous Hsp90 and Cdc37 were specifically coadsorbed with Cdk2 (Figure 2A), consistent with results found for Cdk4.

Hsp90 inhibitors have aided studies of the mechanisms underlying the function of Hsp90 and its cochaperones. The N-terminal nucleotide-binding domain of Hsp90 is the site of action for the Hsp90 inhibitor geldanamycin. In the presence of geldanamycin, Hsp90 is held in an "open" conformation, which binds weakly to protein clients in a salt-labile fashion (*9*, *15*, *30*). To further test whether Cdk2 interacted with Hsp90 and Cdc37, His-tagged Cdk2 was expressed in the presence or absence of the Hsp90 inhibitor geldanamycin, immunoadsorbed, and analyzed as described above. Again, consistent with the interaction of Hsp90 and Cdc37 with other client protein kinases (*15*, *30*, *31*), Hsp90 and Cdc37 did not form a complex with Cdk2 in the presence of geldanamycin that was stable to buffer containing 0.5 M NaCl (Figure 2B). Therefore, these results support the hypothesis that Cdk2 is a client of the Hsp90−Cdc37 chaperone complex.

Antibody Directed against Helix αC of Cdk2 Disrupts Its Interactions with Hsp90 and Cdc37. As noted above, in previously published work, it was concluded that Hsp90 and Cdc37 did not physically interact with Cdk2 (*10*). This work utilized the Cdk2-specific sc-53 antibody, which is directed against the amino acid sequence PSTAIRE in Cdk2's helix

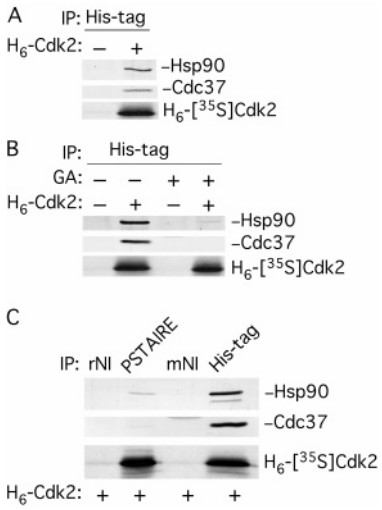

FIGURE 2: Interaction of Hsp90 and Cdc37 with newly synthesized Cdk2. (A) ^{35}S-labeled His-tagged Cdk2 was synthesized in and immunoadsorbed from reticulocyte lysate with anti-His tag antibodies, as described in Experimental Procedures. (B) ^{35}S-labeled His-tagged Cdk2 was synthesized in reticulocyte lysate in the presence or absence of geldanamycin, and immunoadsorbed with anti-His tag antibodies, as described in Experimental Procedures. Naïve reticulocyte lysate containing no template DNA was used as the control for nonspecific binding (A and B). (C) ^{35}S-labeled His-tagged Cdk2 was synthesized in and immunoadsorbed from reticulocyte lysate with mouse anti-His tag IgG or control mouse nonimmune IgG (mNI), or rabbit anti-PSTAIRE IgG or control rabbit nonimmune IgG (rNI). Samples were analyzed by SDS−PAGE, autoradiography (bottom panels), and Western blotting for co-absorption of endogenous Hsp90 (top panels) and Cdc37 (middle panels).

αC (Figure 1B, red). Since this work conflicted with the results described above, we tested whether Hsp90 and Cdc37 would coadsorb with newly synthesized His-tagged Cdk2 immunoadsorbed from reticulocyte lysate with sc-53, as opposed to anti-His tag antibody. Western blotting indicated that Hsp90 and Cd37 were coadsorbed with His-tagged Cdk2 from reticulocyte lysate with the anti-His tag antibody (Figure 2C). Again this interaction was specific, as no Hsp90 or Cdc37 was present in immune pellets containing bound mouse nonimmune IgG. Consistent with previous work, Hsp90 and Cdc37 did not coadsorb with His-tagged Cdk2 immunoadsorbed with the sc-53 antibody. These results suggest that binding of the sc-53 antibody to sequences in helix αC (Figure 1C, red) prevents Hsp90 and Cdc37 from interacting with Cdk2. The binding of the sc-53 antibody to helix αC undoubtedly distorts Cdk2's conformation, and suggests that helix αC is recognized by or is required to maintain the structure of motifs recognized by Hsp90 and/or Cdc37. This result is consistent with our previous structural studies utilizing the Lck tyrosine kinase (*15*, *16*), where Hsp90 and Cdc37 were able to bind N-terminal deletion constructs that contained helix αC in a molybdate-independent high-affinity complex. Hsp90, but not Cdc37, only formed salt-stable complexes with deletion constructs of Lck that were missing helix αC in the presence of molybdate (*16*). Together, these results suggest that helix αC may play a critical role in the recognition and interaction of protein kinases with Cdc37 and Hsp90.

Inhibition of Hsp90 Destabilizes Cdk2 and Its Activating Kinase Cdk7 in K562 Cells. Inhibition of Hsp90 function in vivo destabilizes many Hsp90-dependent proteins and accelerates their proteolytic breakdown in cells (*32*). To further test the hypothesis that Cdk2 is an Hsp90-dependent kinase, we treated K562 myeloid leukemia cells with geldanamycin or DMSO (drug vehicle control) for 0, 12, and 24 h. At the indicated times, cells were harvested, lysed in sample buffer, and then frozen. The total protein concentration was determined via the BCA assay, and equal amounts of protein from each of the cell lysates were analyzed by SDS−PAGE and Western blotting (Figure 3). Quantification of band density indicated that after 24 h the level of Cdk2 in geldanamycin-treated cells was reduced by ∼75% (Figure 3). These results are consistent with those obtained in studies with other Hsp90-dependent protein kinases (*13*, *33*), and further support the notion that Cdk2 is an Hsp90-dependent client. Furthermore, Western blot analysis indicated that geldanamycin reduced cellular levels of Cdk7, the cyclin-dependent kinase component of the activating kinase (CAK) that is responsible for the activating phosphorylation of Cdk2 and other Cdks (*34*), by more than 60% (Figure 3). Thus, geldanamycin reduces not only Cdk2 levels but also the level of the kinase required for Cdk2's activating phosphorylation, suggesting that Cdk7 may also be an Hsp90-dependent kinase.

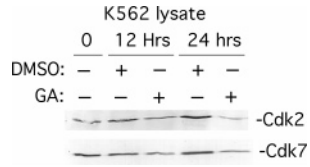

FIGURE 3: Endogenous Cdk2 protein expression is inhibited in geldanamycin-treated K562 cells. K562 cells were grown in the presence of either DMSO or 0.1 μM geldanamycin (GA) for 0, 12, or 24 h. Cells were washed and lysed as described in Experimental Procedures. The protein content in the cell extracts was determined by the BCA assay, and equal amounts or protein were separated by SDS−PAGE and analyzed by Western blotting for Cdk2 or Cdk7.

Effects of Hsp90 Inhibition on the Association of Cyclin A with Cdk2. Other Hsp90-dependent protein kinases become structurally and functionally unstable in the presence of geldanamycin (*15*, *30*, *31*). Since our results suggested that Cdk2 is a client of the Hsp90−Cdc37 chaperone complex, we hypothesized that inhibition of Hsp90's activity with geldanamycin would affect Cdk2's ability to associate with cyclin A. This was also suggested by the work of Gerber et al. (*35*) which showed that Cdc37-defective yeast are unable to properly assemble Cdc28−Cln2 complexes. We, therefore, predicted that the inability of Hsp90 to actively fold Cdk2 would prevent Cdk2 from attaining a stable conformation that could be recognized by cyclin A. To test this hypothesis, we synthesized ^{35}S-labeled His-tagged Cdk2 and cyclin A containing an AU5 epitope tag in separated TnT reactions in the presence or absence of geldanamycin. The reaction mixtures were then combined and incubated. Complexes were immunoadsorbed with anti-epitope tag or control antibodies. Samples were then analyzed by SDS−PAGE, autoradiography, and Western blotting for coadsorption of endogenous Hsp90 and Cdc37 (Figure 4).

Contrary to our prediction, the autoradiogram indicated that inhibition of Hsp90 function with geldanamycin had no significant effect on the stoichiometry of the interaction between cyclin A and Cdk2. Band intensities indicated that when Cdk2 was immunoadsorbed, cylcin A coadsorbed at

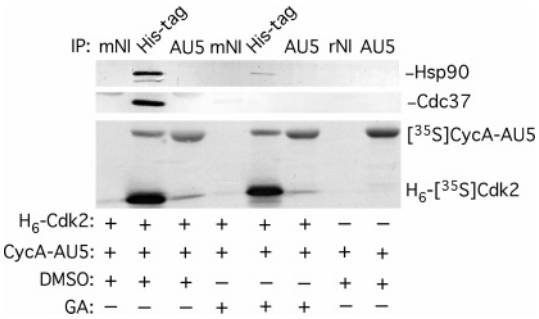

FIGURE 4: Association of Cdk2 with cyclin A in the presence and absence of geldanamycin. ^{35}S-labeled His-tagged Cdk2 and cyclin A-AU5 were synthesized in reticulocyte lysate in the presence of either DMSO or 10 μg/mL geldanamycin (GA) for 30 min at 30 °C. Samples were mixed, incubated, and immunoadsorbed with mouse anti-His tag IgG or control mouse nonimmune IgG (mNI), or rabbit anti-AU5 IgG or control rabbit nonimmune IgG (rNI), as described in Experimental Procedures. Samples were separated by SDS−PAGE and analyzed by autoradiography (bottom panel) and analyzed by Western blotting for endogenous Hsp90 (top panel) and Cdc37 (middle panel).

the same ratio in the presence or absence of geldanamycin. The reciprocal immunoadsorption of cyclin A gave a similar result, but it is noteworthy that less than 5% of the Cdk2 that was present was coadsorbed with cyclin A. Thus, at present in our hands, the inhibition of Hsp90 with geldanamycin does not seem to prevent Cdk2 from forming complexes with cyclin A. This observation is consistent with previous findings in which it was shown that Cdc37 was unable to act as an assembly factor for Cdc28−Cln2 complexes (*36*). However, the poor efficiency of Cdk2−cyclin A complex formation in reticulocyte lysate in general suggests that the question of whether molecular chaperones play a role in cyclin and cyclin-dependent kinase associations deserves more rigorous attention.

The kinase activity of Cdk2 and the reconstituted Cdk2−cyclin A complex was also assayed after being synthesized and incubated in the presence or absence of geldanamycin, as described above. Cdk2 and the reconstituted Cdk2−cyclin A complexes were immunoadsorbed with the anti-His tag antibody, and the ability of the kinase to phosphorylate histone H1 was assayed. The active recombinant GST-tagged Cdk2−cyclin A complex was used as positive control. The Cdk2 generated in reticulocyte lysate was found to be inactive regardless of the presence of cyclin A or the absence of geldanamycin (not shown). In retrospect, this result was not unexpected, as Western blotting indicated that Cdk7, the cyclin-dependent kinase whose activity is required for Cdk2 activation (*34*), is not present in reticulocyte lysate (not shown).

Interactions of Hsp90 and Cdc37 with C-Terminal Domain Deletion Constructs of Cdk2. In our previous work, we observed that Hsp90 was able to interact with deletion constructs of Lck that contained the last two strands of β-sheet (e.g., strands β4 and β5, colored violet for the Cdk2 structure shown in Figure 1C) in the N-terminal lobe (NL) and the entire C-terminal lobe (CL) of the catalytic domain of the kinase, whereas Cdc37 was found to stably interact with only Lck deletion constructs that contained helix αC (Figure 1C, colored red and purple) and strands β4 and β5 of the NL, in conjunction with the CL (*16*). These findings were at variance with results from a study utilizing Cdk4, in which the G-box/P-loop (Figure 1C, strands β1 and β2 of the β-sheet in the NL, colored blue) was found to be a critical motif required for Cdc37 binding (*17*). Deletion of this structure, which acts as a flexible flap that covers and anchors the α- and β-phosphates of ATP, has no effect on the ability of Hsp90 and Cdc37 to bind Lck (*16*).

In the aforementioned study (*17*), Cdk4 constructs were expressed by TnT in reticulocyte as C-terminal fusions to glutathione *S*-transferase (GST fusions), the samples were placed on ice, and the ability of purified recombinant Cdc37 to bind the constructs was subsequently assessed. Unlike our studies with the catalytic domain of Lck, the Cdk4 constructs were preceded by a folded GST domain. Furthermore, GST is known to dimerize (*37*−*39*), which could potentially have additional effects on the structure and/or function of the GST−Cdk4 fusion construct. For example, the dimerization property of GST has been shown to partially reactivate Bcr-Abl lacking its oligomerization domain (*37*). In addition, Cdc37 expressed in *Escherichia coli* is not phosphorylated at Ser^{13} (*40*), a modification that has been shown to be required for high-affinity binding of Cdc37 to kinases (*40*, *41*). However, it should be noted that reticulocyte lysate has the capacity to phosphorylate Cdc37 on Ser^{13} (*40*). Thus, the differences between the experimental designs utilized in the two studies had the potential to account for the differences in the results that were obtained. Therefore, we decided to define motifs required to trigger high-affinity binding interactions between endogenous lysate Hsp90 and Cdc37, and Cdk2, with Cdk2 constructs being expressed with an N-terminal His tag (Figure 1).

Utilizing the crystal structure of Cdk2 (*42*), a series of C-terminal lobe deletion constructs of His-tagged Cdk2 were prepared (Figure 1). Each construct was expressed by TnT in reticulocyte lysate, and samples were immunoadsorbed with anti-His tag antibody in the presence or absence of molybdate to determine whether (a) the constructs bound Hsp90 and Cdc37 and (b) the constructs were capable of triggering Hsp90-conformational switching and molybdate-independent high-affinity binding, respectively. Samples were then analyzed via SDS−PAGE, Western blotting, and autoradiography. Hsp90 and Cdc37 were coadsorbed with the full-length His-tagged Cdk2, which was used as a reference and positive control (Figure 5A). Quantification of Western blot band intensities indicated that the amount of Hsp90 that was coadsorbed with either the NL-β7 construct {which contains conserved kinase motifs I (the G-box, Figure 1, blue) through VIB [the equivalent of β-sheets 6 and 7 of PKA (*43*); Figure 1, orange]} or the NL-Cata construct [which contains motifs I−VI (the loop containing the catalytic Asn residue); Figure 1, green] was approximately one-quarter of that which was coadsorbed with full-length Cdk2 in the absence of molybdate. However, in the presence of molybdate, the amount of Hsp90 that coadsorbed with the NL-β7 and NL-Cata constructs was approximately 50% of the quantity of Hsp90 that coadsorbed with the full-length protein. Furthermore, only marginally detectable levels of Cdc37 were observed to coadsorb with the NL-β7 and NL-Cata constructs, whether or not molybdate was present during the immunoadsorption. On the basis of similar experiments carried out with deletion constructs of Lck and Cdk4 (*16*, *17*), we predicted that these constructs would bind Hsp90 and Cdc37 at 50−75% of the level bound

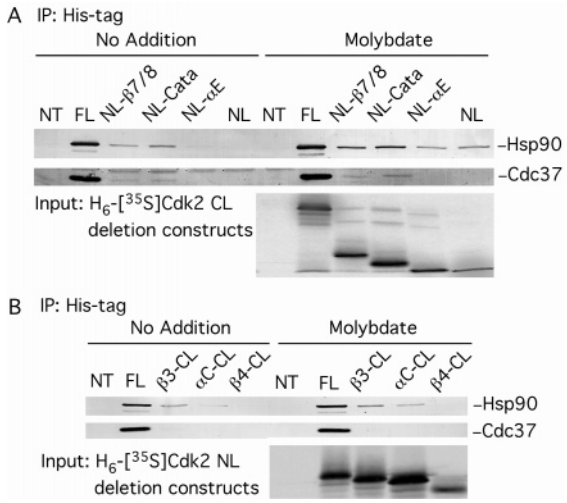

FIGURE 5: Interactions of Hsp90 and Cdc37 with His-tagged Cdk2 deletion constructs. ^{35}S-labeled His-tagged full-length (FL) Cdk2 and (A) His-tagged Cdk2 C-terminal deletion constructs or (B) His-tagged Cdk2 N-terminal deletion constructs were synthesized in and immunoadsorbed from reticulocyte lysate with anti-His tag antibodies in the presence or absence of 17 mM molybdate, as described in Experimental Procedures. Naïve reticulocyte lysate containing no template DNA was used as the control for nonspecific binding. Immunoadsorbed samples were washed, as described in Experimental Procedures, separated by SDS−PAGE on 8% gels, and analyzed by Western blotting for endogenous Hsp90 and Cdc37. Control samples for the amount of input protein that was immunoadsorbed were run on 10% SDS−PAGE and analyzed by autoradiography.

by full-length Cdk2 in the absence of molybdate, and at a level nearly equivalent to that of full-length Cdk2 in the presence of molybdate. Thus, the amount of Hsp90 and Cdc37 that coadsorbed with the NL-β7 and NL-Cata Cdk2 constructs was less than what was predicted. The interactions of Hsp90 and Cdc37 with two other Cdk2 C-terminal domain deletion mutants [the NL-αE construct, which contains helix αE of the CL (Figure 1, yellow), but not the helix capping motif, and the NL construct which lacks the entire CL and terminates after β-sheet 5 (Figure 1, violet)] were also different from those expected from our previous results. In the absence of molybdate, no Hsp90 or Cdc37 that could be detected by Western blotting was coadsorbed with these constructs (Figure 5A). In the presence of molybdate, detectable amounts of Hsp90 were coadsorbed with the constructs, whereas no coadsorbing Cdc37 was detected (Figure 5A).

While some of the findings described above were a bit different than expected, the data indicated that Hsp90, and to a lesser extent Cdc37, was able to bind only Cdk2 constructs that contained the NL and C-terminal kinase lobe motifs through helix αE, complete with its capping motif. These results support our previous findings with Lck (*16*), and again emphasize the importance of the stability of helix αE, and most likely its interface with the hinge−loop motif (Figure 1, purple and violet) between helix αC and strand β4 in the NL. However, in the presence of molybdate, Hsp90 and Cdc37 were observed to bind the NL-αE and NL constructs of Lck (*15*, *44*), while the comparable Cdk2 constructs were devoid of stably bound Cdc37. This finding also differs from previous work on Cdk4 (*17*) which demonstrated that Cdc37 was able to bind Cdk4 constructs that contained only the NL.

Interactions of Hsp90 and Cdc37 with N-Terminal Domain Deletion Constructs of Cdk2. To similarly define the high-affinity binding interactions of Hsp90 and Cdc37 with the N-terminal lobe of Cdk2, we prepared a series of N-terminal deletion constructs (Figure 1) and tested them accordingly (Figure 5B). Hsp90 was coadsorbed, albeit at a very low level, with the β3-CL (β3, Figure 1, cyan) [which is missing kinase motif I (the G-box/P-loop motif; Figure 1, blue)] and the αC-CL [which starts at conserved motif III (helix αC; Figure 1, red and purple) and contains an invariant catalytic Glu residue] Cdk2 constructs, whether or not molybdate was present to stabilize binding. Conversely, the β4-CL construct [which initiates after the hinge−loop motif in kinase motif IV (Figure 1, violet)] did not bring down any Hsp90, even in the presence of molybdate. These results are rather consistent with those obtained with N-terminal deletion constructs of Lck, in which the molybdate-independent high-affinity interaction of Hsp90 with N-terminal deletion constructs was lost upon deletion of helix αC (*16*). However, unlike the β4-CL construct of Cdk2, the interaction of Hsp90 with the β4-CL construct of Lck was stabilized to a degree upon addition of molybdate, again underlying the significance of helix αC and the hinge−loop motif. On the other hand, no Cdc37 was detected coadsorbing with any of the N-terminal deletion constructs of Cdk2, even in the presence of molybdate. This result was consistent with results obtained with Cdk4 (*17*), where the G-box was identified as a critical motif for Cdc37 binding. In contrast, Hsp90 and Cdc37 do coadsorb with the β3-CL construct of Lck (*16*), even in the absence of molybdate.

DISCUSSION

The results presented above indicate that Cdk2, a cyclin-dependent kinase and a close relative of the Hsp90-dependent Cdk4 kinase, is a genuine client of the Hsp90−Cdc37 chaperone complex (*10*). Previous studies suggested that Cdc37 associated with and was required for the proper function of Cdc28, the yeast homologue of Cdk2 (*23*, *35*, *36*). However, in other studies, a physical interaction of Hsp90 and Cdc37 with Cdk2 was not observed, and Cdk2 was concluded not to be a client of Hsp90 and Cdc37 (*45*). Here we demonstrate that Cdk2 indeed physically interacts with both Hsp90 and Cdc37 and demonstrates classic client behavior upon inhibition of Hsp90 by geldanamycin.

The rate at which Cdk2 levels decreased in K562 cells in the presence of geldanamycin suggests that Hsp90 may not be required for maintenance of the stability of mature Cdk2 molecules. The kinetics of geldanamycin-induced reduction of Cdk2 levels was much slower than that observed for ErbB2 (*46*), Raf (*47*), and oncogenic mutants of Lck and Hck (*48*, *49*), which require Hsp90 to maintain their stability even after their maturation into an active form. Hsp90 is required both for the proper folding of these kinases when they are newly synthesized and for maintaining the stability of the kinases after they have matured to an active form. However, the kinetics of geldanamycin-induced depletion of Cdk2 from K562 cells is consistent with studies of other Hsp90-dependent protein kinases. The geldanamycin-induced turnover of wild-type Lck (*33*, *49*), Hck (*48*), Fyn (*13*), and Akt (*32*) in cells is slow because mature kinase molecules do not depend on Hsp90 for their stability. Geldanamycin-induced turnover of the kinases is primarily due to the

inability of cells to replace mature kinase molecules, as they turn over at their normal rate, with newly synthesized kinase molecules, which are Hsp90-dependent and very labile in the presence of geldanamycin. Only nascent Cdk2 may require Hsp90 and Cdc37 for its proper folding. Furthermore, the absence of a requirement for Hsp90 and Cdc37 to maintain the stability of mature Cdk2 molecules may explain why it is difficult to coadsorb Hsp90 and Cdc37 with Cdk2 from cultured cell lysates (data not shown). In cultured cells, the Hsp90−Cdc37 complex-dependent population of Cdk2 molecules may be limited to a small population of newly synthesized Cdk2 molecules, with the vast majority of Cdk2 being mature and stable. In contrast, the majority of Cdk2 molecules in TnT reticulocyte lysate are newly synthesized nascent polypeptides.

The results reported here also reaffirm the importance of helix αC in protein kinases for their proper recognition by and interaction with Hsp90 and Cdc37. The sc-53 anti-PSTAIRE antibody, which is directed against residues in helix αC of Cdk2, blocked the interaction of Hsp90 and Cdc37 with Cdk2. This observation explains results from earlier studies utilizing this antibody, which concluded that Cdk2 did not physically associate with Hsp90 and Cdc37 (*45*). This finding also supports previous work with Lck and Cdk4, which indicated that the presence of helix αC was required for the interaction of Cdc37 with kinase molecules (*16*, *17*). However, it cannot yet be concluded that helix αC is the site at which Cdc37 recognizes and binds kinase, as the binding of the antibody could block the interaction of Cdc37 by (i) sterically inhibiting the interaction of Cdk2 with Cdc37, (ii) distorting helix αC, as antibodies are known to bind peptide epitopes in an extended conformation, (iii) altering the structure of other regions of the kinase that are dependent on the helix for folding or maintaining their structure, or (iv) a combination of these effects.

Currently, we favor the notion that the binding of the sc-53 antibody to the helix αC globally distorts the NL and subsequently weakens the ability of Cdc37 to interact. This hypothesis predicts that helix αC is required to maintain a number of intramolecular interactions that stabilize the protein and that distortion of the Cdk2 NL by any number of mechanisms could lead to the loss of Hsp90 and Cdc37 interaction. We favor this notion, as it helps to explain the variant results obtained in studies on the Lck and Cdk4 kinases (*16*, *17*).

While results from the deletion analysis of Cdk2 conflicted with some aspects of previous experiments carried out on Cdk4 and Lck (*16*, *17*), the primary points of each of these studies were upheld. The requirement for the G-box/P-loop structure for the interaction of Hsp90 and Cdc37 with Cdk4, as compared to the binding of Hsp90 and Cdc37 to Lck constructs containing deletions of this motif, is unlikely to be due to differences in experimental paradigms, as the G-box/P-loop motif was also found to be required for the binding of Hsp90 and Cdc37 to Cdk2. Furthermore, as in studies with Lck, the stabilization of Cdk2's helix αE by its capping motif was found to be required to induce molybdate-independent high-affinity binding of Hsp90 and Cdc37 to Cdk2. Nevertheless, the difference in the interaction of Cdc37 with C-terminal deletion mutants of Cdk4 and Cdk2 suggests that there are subtle structural differences between the protein kinases. Sequence alignment of the N-terminus of the two catalytic kinase lobes shows that Cdk4 contains (1) extra amino acids in the loop between strand β3 and the αC-helix, which is a region that is not resolved in the crystal structure of the inactive conformation of Cdk2 [Figure 1, cyan to red (*50*−*52*)]; (2) an elongated helix αC or extra amino acids within the loop connecting helix αC (Figure 1, red and purple) and strand β4 (Figure 1, purple to violet); and (3) extra amino acids in the loop between strands β4 and β5 (Figure 1, violet). The amino acid residues within these expanded sequences may provide a larger interface for Cdc37 recognition and binding and/or added stability to the interaction of Cdc37 with Cdk4. On the other hand, the β3−αC loop of Cdk4, which is composed of seven continuous glycines, may be intrinsically unstructured due to its increased flexibility, or it may allow the NL to adopt the conformation that can more stably interact with Cdc37.

Interestingly, the importance of the G-box for the interaction of both Cdk4 and Cdk2 with Cdc37 may also be related by the fact that the kinase activity of each protein can be inhibited by the phosphorylation of a Tyr residue within this motif (*53*). The crystal structure of Cdk2 complexed to cyclin A (*54*) indicates that this Tyr residue forms a hydrogen bond with the catalytic Glu residue in helix αC, which likely helps to stabilize the orientation of the helix within the NL and the active conformation of the kinase. Inhibition of kinase activity induced by phosphorylation is most likely due to the distortion of the conformation of the G-box and/or helix αC, as slight perturbations in the structure of either of these motifs would likely have global effects upon the overall structure and function of the NL. What is more, it should be noted that the G-box of the client kinase c-Raf, which much like Cdk4 is critical for Cdc37 interaction (*17*), may also contain a possible regulatory phosphorylation site at S357 or S359. Furthermore, mutation of the third G residue in the G-box of B-Raf to Ala leads to the hyperactivation of its kinase activity, an observation that further supports the notion that slight changes in the conformation of this region can have significant effects on the structure of the NL (*55*).

The data presented here further support our previous findings that the stability of the interaction of Hsp90 with the catalytic domain of protein kinases requires structural motifs present in both lobes of the catalytic domain. As observed previously for Lck (*16*), stabilization of the C-terminal lobes's helix αE (Figure 1, yellow), by extending the sequence to provide a proper capping motif (Figure 1, green), seems to be critical for the maintenance of the interaction of Hsp90 and possibly Cdc37 with Cdk2. The deletion construct containing a capped helix αE is more likely retain its α-helical structure and possibly the orientation of its side chains. We postulate that this stabilized α-helix is then able to give the αC−β4 hinge−loop motif (Figure 1, purple to violet) a complementary surface interface with which to interact, allowing in turn the semistable kinase construct to attain a conformation that is recognized by Hp90 and Cdc37. The Cdk4 C-terminal deletion studies also showed that the αC−β4 hinge−loop motif is required for Cdc37 binding, although interestingly enough, further stabilization of the hinge−loop structure by the structural motifs following it was not required (*17*). It is possible that this result is a consequence of expressing the Cdk4 NL as a C-terminal fusion to glutathione *S*-transferase, in contrast to the expression of the isolated NL of Cdk2 described here.

Probably the most telling evidence supporting the significance of the hinge−loop structure is the work of Xu and co-workers comparing the ErbB1 and ErbB2 receptor tyrosine kinases (*56*). ErbB2 was demonstrated to require ongoing interactions with Hsp90 and Cdc37 to maintain its cellular stability, whereas ErbB1 required Hsp90 and Cdc37 only for its initial folding. The differences in the stability of each kinase were localized to amino acid residues in the hinge−loop region. However, our results support the notion that other structural motifs are also likely to be important for Hsp90 binding, such as the linker region (Figure 1, violet to yellow), which interacts with the hinge−loop region and connects the two lobes of the kinase. Thus, it appears that while protein kinases share common structural features that are recognized by Hsp90 and Cdc37, it is now apparent that there are subtle differences in how a client kinase interacts with the Hsp90 chaperone complex, just as how there are differences in how kinase activities are regulated through alterations in the interactions between the two lobes of their catalytic domains.

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